Virulence factor–activity relationships (VFAR) with specific emphasis on *Aeromonas* species (spp.)

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ABSTRACT

The human population most commonly inflicted with *Aeromonas* infection includes young children, the elderly and immunocompromised individuals. Importantly, the isolation rate of *Aeromonas* species from children suffering from diarrhea is similar in developing and developed countries. It is becoming clear that only a small subset of *Aeromonas* species belonging to a particular hybridization group causes disease in humans. Human infections with this pathogen occur by consuming contaminated food and water. *Aeromonas* species were isolated from wounds of patients during the tsunami in southern Thailand. Further, increased numbers of this pathogen were recovered from floodwater samples during Hurricane Katrina in New Orleans. Among various species of *Aeromonas*, *A. hydrophila*, *A. caviae* and *A. veronii* biovar sobria are mainly responsible for causing disease in humans. Our laboratory has isolated various virulence factors from a diarrheal isolate SSU of *A. hydrophila* and molecularly characterized them. In addition to various virulence factors produced by *Aeromonas* species, the status of the immune system plays an important role in inducing disease by this pathogen in the host. Taken together, we have made significant advances in better understanding the pathogenesis of *Aeromonas* infections, which will help in differentiating pathogenic from non-pathogenic aeromonads. This review covers virulence aspects of a clinical isolate of *A. hydrophila*.

Key words | *Aeromonas* species, animal models, epidemiology, gastroenteritis, septicemia, virulence factors

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SIGNIFICANCE OF *AEROMONAS* IN HUMAN DISEASES

Aeromonas spp. have emerged as an important human pathogen because of suspected food-borne outbreaks (Altwegg *et al.* 1991; Carnahan & Altwegg 1996; Hanninen *et al.* 1997) and the increased incidence of *Aeromonas* isolation from patients with gastroenteritis and traveler's diarrhea (Hanninen *et al.* 1995; Ahmed *et al.* 1997; Yamada *et al.* 1997). The ability of many *Aeromonas* strains to invade eukaryotic cells and resist complement-mediated lysis could result in bacteremia and other invasive diseases (Chopra & Houston 1999). *Aeromonas* spp., found in fresh and salt water and in virtually all foods (Schoch & Cunha 1984; Buchanan & Palumbo 1985; Palumbo *et al.* 1985; Callister & Agger 1987; Hunter & Burke 1987), cause a wide variety of human infections, including septicemia, wound infections, meningitis, pneumonia, respiratory infections, hemolytic uremic syndrome, necrotizing fasciitis and gastroenteritis (Gilardi *et al.* 1970; Davis *et al.* 1978; Fulghum *et al.* 1978; Joseph *et al.* 1979; Freij 1984; Dixon 1987; von Graevenitz 1987; Palumbo *et al.* 1989; Janda & Abbott 1998; Cheng *et al.* 2004). The ability of these microorganisms to grow well at refrigeration temperatures (Majeed *et al.* 1990) could be important in their role as food-poisoning agents. Recently,

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Aeromonas isolates (*A. simiae*) were also isolated from the feces of non-human primates (*Macaca fascicularis*), further signifying the role of this bacterium in gastroenteritis and other non-intestinal diseases (Harf-Monteil *et al.* 2004). This review specifically focuses on the mesophilic *Aeromonas* spp.

Aeromonas spp. were listed by the Environmental Protection Agency (EPA) on the "Contaminant Candidate List". In a recent 16-month study conducted in the presence of *A. hydrophila* in drinking water in Indiana, 7.7% of the biofilm samples were positive for *A. hydrophila*, indicating a potential for its regrowth and ability to contaminate distribution systems (Chauret *et al.* 2001). Some strains of *Aeromonas* isolated from food and water may be enteropathogenic, and Holmes *et al.* (1996) reported that 10% of the pipe lengths had aeromonads (specifically when present in biofilms) even after disinfection with 1 mg/L of chlorine. Further, *Aeromonas* spp. are becoming increasingly resistant to multiple antibiotics. Such resistance could lead to serious clinical sequelae associated with *Aeromonas* infections (Alavandi *et al.* 1999; Ribas *et al.* 2000). The presence of various virulence factors in *Aeromonas* spp. and their prevalence in drinking water reinforces the need to examine the health risk of this and other water-borne pathogens to better define the quality guidelines for drinking water (Pavlov *et al.* 2004). In addition, more recent studies of *Aeromonas culicicola* isolation from the midgut of mosquitos might explain *Aeromonas* infections of humans without exposure to contaminated water, soil or food (Pidiyar *et al.* 2004). In Sweden, bovine mastitis due to *A. hydrophila* was reported, which could result in human infections if milk from such animals was not properly pasteurized (Carlos *et al.* 2002).

Aeromonas spp. have been placed in a new family *Aeromonadaceae*, and, to date, more than 14 species of *Aeromonas* have been isolated (Carnahan & Joseph 1991; Carnahan 1993; Carnahan & Altwegg 1996). Misidentification of *Aeromonas* as members of the genus *Vibrio* or as an *Escherichia coli* is a continuing problem (Abbot *et al.* 1998; Holmberg 1988). Among different *Aeromonas* species, *A. caviae* is most frequently isolated from fecal specimens, followed by *A. hydrophila* and *A. veronii*. These *Aeromonas* spp. produce a variety of extracellular products that contribute to the pathogenesis of disease. The virulence factors produced by *Aeromonas* spp. include hemolysins, cytotoxins and enterotoxins (Chakraborty *et al.* 1988), proteases (Leung & Stevenson 1988), leukocidin (Scholz *et al.* 1974), phospholipases (Bernheimer *et al.* 1975), endotoxin (Rigney *et al.* 1978), fimbriae or adhesions, in addition to the capacity to form capsules (Merino *et al.* 1995). *A. hydrophila* has been shown to be invasive in HEp-2 cell monolayers (Lawson *et al.* 1985; Grey & Kirov 1993), and these bacterial cells adhere to human blood cells (Atkinson & Trust 1980). Grey & Kirov (1993) reported that adherent strains of *Aeromonas* to HEp-2 cells were virulent and enterotoxin-positive. Taken together, the ability of these bacteria to invade host cells and disseminate to virtually any organ via blood, either through the intestinal or non-intestinal route, along with their capacity to produce various virulence factors could contribute to the pathogenesis of disease mediated by *A. hydrophila*.

ROLE OF *AEROMONAS* SURFACE MOLECULES IN VIRULENCE

Many strains and species of *Aeromonas* (e.g. *A. salmonicida*) possess a regularly arrayed surface layer (S layer) tethered to the bacterial cell surface via lipopolysaccharide (LPS) that allows bacteria to resist host defenses (Kay & Trust 1991). Merino *et al.* (1996*a*) reported that *Aeromonas* spp. belonging to serogroup 0:11 with an S layer resisted complement-mediated killing by impeding complement activation. However, serum resistance of *Aeromonas* strains lacking the S layer was due to their inability to form C5b or C5b-9. In their subsequent studies, Merino *et al.* (1998) documented that a 39 kDa outer membrane protein (OMP), which bound to C1q, was not accessible in *Aeromonas hydrophila* strains possessing an O antigen, which imparted serum resistance to the organism. Recently, Esteve *et al.* (2004) reported the presence of an S layer in serogroups O:14 and O:81 of *A. hydrophila*.

The O-antigen polysaccharide, which is composed of repeating oligosaccharide units, is covalently attached to the lipid A-core complex of the LPS and extends outward from the cell surface. The genus *Aeromonas* has been classified into 96 serogroups and the O-antigen LPS of *A. hydrophila* 0:34 strains was shown to play an important role in adhesion to HEp-2 cells (Zhang *et al.* 2002). Merino *et al.* (1996*b*) showed that transposon mutants of

A. hydrophila 0:34 devoid of the O-antigen LPS were unable to colonize germfree chicken gut. This model system was used because *A. hydrophila* is a frequent inhabitant of the intestinal ecosystem of the chicken, and some strains are able to cause acute diarrhea and extraintestinal infections in this model. Further, chicken is a potential food contamination source for humans. Recently, Zhang *et al.* (2002) sequenced the O-antigen gene cluster of a virulent strain of *A. hydrophila* 0:18. The O-antigen gene cluster was 17,296 bp long and consisted of 17 genes. Seven pathway genes for the synthesis of rhamnose and mannose, six transferase genes, one O unit flippase gene and one O-antigen-chain-length determinant were identified by amino acid sequence similarity. Recently, Jimenez *et al.* (2008) characterized three different genomic regions with LPS core biosynthesis genes in *A. hydrophila* AH3 serotype O34.

Some serogroups of *A. hydrophila* (0:11 and 0:34) possess capsular polysaccharide (Zhang *et al.* 2002). Two capsule genes from an O:34 strain (*orf1* and *wcaJ*) of *A. hydrophila* conferred serum resistance on *E. coli* K-12 strains (Aguillar *et al.* 1999). The capsular gene cluster of *A. hydrophila* was 17,562 bp long and included 13 genes, which were assembled into three distinct regions. Regions I and III contained four- and two-capsule transport genes, respectively (Zhang *et al.* 2002), while region II had five genes which were highly similar to the capsule synthesis pathway genes found in other bacteria. Both the purified O antigen and capsular polysaccharides increased the ability of the avirulent *A. hydrophila* strain to survive in naive tilapia serum, suggesting that both contributed to the serum resistance of the organism. The functions of some genes in both the O antigen and capsule gene clusters are not yet known (Zhang *et al.* 2002).

Porin II was also reported to be an important surface molecule involved in serum susceptibility and C1q binding in *A. hydrophila* (Nogueras *et al.* 2000). Porin loss by different mechanisms could lead to serum resistance of *Aeromonas* spp. with a potential for the organism to develop antibiotic resistance, resulting in serious clinical problems.

Bacterial adherence and invasion

Kirov *et al.* (1993) reported that 10% of the *Aeromonas* strains isolated from food adhered to HEp-2 cells at 37°C and 5°C, produced two or more exotoxins (hemolysin, enterotoxin and cytotoxin) and expressed a flexible pilus gene at these two temperatures. Ascencio *et al.* (1998) demonstrated bacterial cell surface extracts containing mucin-binding components, varying in size from 22–95 kDa, in different species of *Aeromonas*. The presence was documented of two distinct families of type IV pili (bundle-forming pili (Bfp) and Tap (type IV *Aeromonas* pili)) in *Aeromonas* spp. associated with gastroenteritis (Barnett *et al.* 1997). Kirov *et al.* (1999) reported that removal of Bfp decreased adhesion of *Aeromonas* to intestinal cells by up to 80%. More interestingly, these investigators indicated that Bfp might also promote colonization by forming bacterium-to-bacterium linkages. Tap pili might not be as significant as Bfp pili for *Aeromonas* intestinal colonization (Kirov *et al.* 2000). The Tap biogenesis gene cluster consisted of four genes (*tapABCD*). The *tapA* gene encoded subunit protein, while *tapB* and *tapC* genes were involved in pilus biogenesis and *tapD* gene encoded a type IV prepilin peptidase/N-methyltransferase. The latter cleaved 6-amino-acid leader peptide from prepilin and catalyzed methylation of the N-terminal residue (Pepe *et al.* 1996). Tap pili differed from Bfp pili in their N-terminal sequences and molecular weights. Earlier studies of Strom *et al.* (Masada *et al.* 2002) in *A. salmonicida* indicated that the Tap pili mutant was slightly less pathogenic for rainbow trout compared to the wild-type (WT) bacterium. Likewise, recent studies of Boyd *et al.* (2008) showed that Tap pili of *A. salmonicida* made moderate contributions to bacterial virulence in Atlantic salmon.

In addition, roles have been reported for the polar flagellum in the adherence of *A. caviae* to HEp-2 cells (Rabaan *et al.* 2001) and of MgtE, an Mg^{2+} transport protein, in *A. hydrophila* adherence and biofim formation (Merino *et al.* 2001*a*,*b*). It was shown that removal of the single polar flagellum by shearing or agglutination by antiflagellin antibodies greatly reduced bacterial adhesion to HEp-2 cells (Rabaan *et al.* 2001). Further, defined polar flagella-negative mutants demonstrated a dramatic decrease in their adhesive ability to human cell lines (Rabaan *et al.* 2001). Five genes (*flaA,B,G,H,J*) have been characterized from a polar flagellin locus of *A. caviae* (Rabaan *et al.* 2001).

Although only polar flagella were seen when the organisms were grown in a liquid broth, certain *Aeromonas* strains also produced many peritrichous lateral flagella when cultured on solid agar media (Gavin *et al.* 2002). This hyperflagellation coincided with a higher adherence level of these bacteria compared to those grown in broth. Nine lateral flagellar genes (*lafA,B,C,E,F,S,T,U,X*) for *A. hydrophila* and four *A. caviae* genes, *lafA1, lafA2, lafB* and *fliU*, were characterized by Gavin *et al.* (2002). Molecular characterization revealed that these lateral flagella were distinct from the polar flagellum and were involved in swarming motility. Single mutations in *lafA1*, *lafA2* and *fliU* were reported to reduce adherence of *A. caviae* to HEp-2 cells by one-half. The adherence capability of the *lafB* mutant was only 16% of that of the WT bacterium. The *A. hydrophila* cultures with mutations in the *lafB* and *lafS* genes showed an 85% reduction in their adherence compared to that of the WT *A. hydrophila* strain, while mutation in the *lafT* gene exhibited a 50% reduction in adherence. It was suggested that the polar flagellar system controlled the synthesis of the *Aeromonas* lateral flagella, as the mutations in the polar flagellar genes *flaH* and *flaJ*, as well as the tandom *flaAB* mutants, were unable to produce both polar and lateral flagella (Kirov *et al.* 2002).

In addition to the role of lateral flagella in swarming motility, they have been linked to the formation of biofilms in a number of bacterial infections (Kirov *et al.* 2002). *A. hydrophila* cultures with mutations in various genes involved in lateral flagellar synthesis were impaired in their capacity to form biofilms (Gavin *et al.* 2002; Kirov *et al.* 2002), which are known to be a particular feature of persistent infections. Further, swarming motility has also been linked to invasive ability and could be related to persistent or dysenteric presentation seen during *Aeromonas* infections (Kirov *et al.* 2002). More recent studies of Kirov *et al.* (2004) demonstrated that both flagellar types (polar and lateral) of clinical *Aeromonas* isolates were enterocyte adhesins and needed to be fully functional for optimal biofilm formation.

AEROMONAS AS A TRUE ENTERIC PATHOGEN

Aeromonas' role as an enteric pathogen was a subject of controversy, largely because of a 1985 report indicating that oral feeding of *Aeromonas* failed to induce diarrhea in human volunteers (Morgan *et al.* 1985). It has been becoming increasingly clear now that all *Aeromonas* strains do not cause gastroenteritis. Studies of Kuhn *et al.* (1997) indicated that, based on hybridization groups (HGs) and biochemical phenotypes (Phe-nePlate (PhP) types), most human isolates were allocated to DNA HG 4 (*A. caviae*) and HG 1 (*A. hydrophila*), and were placed into BD-1 and BD-2 types, based on PhP typing. They suggested that the HG1/BD-2 type potentially represented a pathogenic *A. hydrophila* able to produce diarrhea in humans. A specific sIgA response directed against the exoproteins in patients with naturally acquired *Aeromonas* diarrhea further indicated the enteropathogenic potential of *Aeromonas* spp. (Crivelli *et al.* 2001).

Therefore, *Aeromonas* might have failed to cause diarrhea in human volunteers because strains of questionable suitability were used in the study. The tested organisms did not belong to the hybridization group that causes diarrhea (Morgan *et al.* 1985), and most of these strains were either non-enterotoxigenic in animal models or obtained from wounds and healthy individuals, and/or were sub-cultured for prolonged periods of time on a synthetic medium which, by the lack of demonstrable fecal shedding by volunteers, apparently were without the necessary attributes to survive the gastrointestinal tract. Volunteer studies with more suitable strains probably cannot be performed because of increased recognition of the ability of *Aeromonas* to cause systemic infections, which are often fatal. Since our laboratory is the first to clone and characterize three enterotoxins from a diarrheal isolate SSU of *A. hydrophila* (Chopra & Houston 1989; Chopra *et al.* 1993, 1994, 1996), we conducted case-control human studies to (1) establish an association of *Aeromonas* with diarrhea and (2) determine the role of enterotoxins in *Aeromonas*-associated gastroenteritis.

ENTEROTOXINS OF *AEROMONAS*

Aeromonas spp. possess essential virulence factors that mediate clinical infections, including acute diarrheal disease in humans (Ljungh & Wadstrom 1986). Two categories of enterotoxins–cytotonic and cytotoxic (Keusch & Donta

1975)–were discovered in culture filtrates of *Aeromonas* isolates. The cytotonic enterotoxins (heat-labile (Alt) and heat-stable (Ast)), like cholera toxin (CT), did not cause degeneration of crypts and villi of the small intestine (Ljungh & Kronevi 1982), whereas the cytotoxic enterotoxin (Act) resulted in extensive damage to epithelium (Asao *et al.* 1984; Chakraborty *et al.* 1986; Howard & Buckley 1986; Rose *et al.* 1989*a*). Act possesses hemolytic and cytotoxic activities in addition to an enterotoxic activity (Chopra *et al.* 1993).

Case-control human studies and enterotoxin-associated diarrhea

In our 27-month prospective study at Mercy Hospital, Chicago, we demonstrated *Aeromonas* spp. were the only bacterial enteropathogens isolated from children, ranging from 1 to 27 months of age and suffering from diarrhea (Challapalli *et al.* 1988). A total of 524 diarrheal children and an equal number of age- and sex-matched healthy children were examined. *Aeromonas* spp. were isolated at a rate of 7.4% from the children with diarrhea, compared to only 2.2% of the healthy children.

In our data from the International Centre for Diarroheal Diseases (ICDDR), Bangladesh, 1,735 children with diarrhea and 830 healthy children (control group) were studied for the presence of aeromonads. Aeromonads were isolated from 125 diarrheal children (7.2%) and from 27 control children (3.3%). Of the 125 diarrheal children positive for aeromonads, 28 (22%) were infected with *Aeromonas* alone (Albert *et al.* 2000). It was noted that 56% of total diarrheal isolates had both *alt* (heat-labile) and *ast* (heat-stable) genes, and this number was significantly higher compared to *alt* and *ast* gene-producing isolates from control children and environmental samples. Overall, one diarrheal isolate of *Aeromonas* produced only Act.

Aeromonas isolates, which were positive for both *alt* and *ast* genes, led to watery diarrhea, whereas patients with loose stools had only the *alt* gene. The species of *Aeromonas* which caused diarrhea in this study included *A. hydrophila* (HG 1), *A. veronii* biovar sobria (HG 8), *A. caviae* (HG 4) and *A. trota* (HG 13 now 14) (Albert *et al.* 2000; Carnahan-Martin & Joseph 2005). We examined 45 isolates of *Aeromonas* (from 2000–2002), obtained from stools of diarrheal patients at UTMB, Galveston. These cultures belonged to 2 species, *A. caviae* and *A. veronii* biovar veronii. Among 25 *A. veronii* isolates, 20 contained *act, alt* and *ast* genes and 5 isolates harbored *act* and *ast* genes, but no *alt* gene. Among *A. caviae* isolates ($n = 20$), on the other hand, none harbored the *act* gene, but contained *alt* and *ast* genes (unpublished data). We also noted a significant correlation between the presence of Act and enterotoxicity in a suckling mouse assay from environmental isolates of *Aeromonas* (Rahim *et al.* 2004).

Taken together, the data indicated variations in the distribution of enterotoxin genes in *Aeromonas*, based on geographical location and possibly the prevalence of particular *Aeromonas* isolates at the time of study. The presence of all three enterotoxin genes in an *Aeromonas* isolate could be devastating to the patients; however, any one of the three enterotoxins (Act, Alt and Ast) could lead to severe diarrhea. It is plausible that the presence of Alt and Ast could exacerbate the pathogenic effects of Act and vice versa. Indeed, some interaction among these enterotoxins *in vivo* was noted when enterotoxin gene-deficient isogenic mutants were tested in a mouse model, which could affect the severity of diarrhea (Sha *et al.* 2002). The importance of Act is limited, not only because of its ability to evoke diarrhea but also because of its potential to cause fatal, non-intestinal diseases. In a recent report (Hofer *et al.* 2006), an acute diarrheal outbreak with 2170 cases was reported in Sao Bento do Una, Pernambuco, with isolation of enteric pathogens in 25% of the patients. *Aeromonas* spp. were most frequently isolated from the stool specimens (20% of the patients). Based on the recent literature and our data, it is evident that some strains of *Aeromonas* within certain species have true enteropathogenic potential in humans.

The environmental prevalence of these pathogenic bacteria could constitute a threat to public health, since *Aeromonas* infections are acquired through consumption of contaminated water and food (Borrell *et al.* 1998). In many non-intestinal infections, the organism gains entrance from contaminated water through wounds (Janda & Abbott 1998). The presence of enteropathogenic *Aeromonas* spp. in potable and domestic water supplies and their ability to withstand killing by chlorination in biofilms and to many antibiotics could cause serious clinical threats

(Holmes *et al.* 1996; Brandi *et al.* 1999; Sen & Rogers 2004). A widespread occurrence of Act-positive isolates (80%) of *Aeromonas* in Norwegian natural waters, including drinking water sources, suggests that these bacteria must not be ignored in drinking water and in the food industry (Ormen & Ostensvik 2001). Likewise, the incidence of *Aeromonas* in drinking water reservoirs in the Far East of Russia was reported for the first time in 2001 (Ivanova *et al.* 2001).

AEROMONAS-ASSOCIATED GASTROENTERITIS

Aeromonas-associated gastroenteritis ranges from a mild, self-limiting, watery diarrhea to a more severe, invasive type (a *Shigella*-like dysenteric form), with blood, mucus, inflammatory exudate, fever and abdominal pain (Gracey *et al.* 1982; Altwegg 1985; Agger 1986; Janda & Brenden 1987; Hichman-Brenner *et al.* 1988; Carnahan *et al.* 1989; Snower *et al.* 1989; Namdari & Bottone 1990; Borczyk *et al.* 1993). A less common manifestation of *Aeromonas* gastroenteritis is a chronic form in which the diarrhea episode exceeds 7–10 days. Chronic diarrhea exceeding 1 year's duration due to *A. caviae* or *A. hydrophila* also has been reported (Janda & Duffey 1988; Janda & Abbott 1998). Acute, self-limited diarrhea is more frequent in young children, and *Aeromonas* spp. have been detected worldwide as the only pathogen from 2–20% of the children suffering from diarrhea and from only 0–2% of children without diarrhea (Martinez-Silva *et al.* 1961; Chatterjee & Neogy 1972; Burke *et al.* 1983; Gracey 1988; Eko & Utsalo 1989; Deodhar *et al.* 1991; Hossain *et al.* 1992; Verenkar *et al.* 1995; Nojimoto *et al.* 1997). Older patients present with *Aeromonas*-mediated chronic enterocolitis (Merino *et al.* 1995). The University of Iowa Hygienic Laboratory reported 224 cases of *Aeromonas* gastroenteritis between January–June 1991, thus making this organism the most prevalent enteric pathogen (Quinn 1991). In recent years, the incidence of gastroenteritis due to *Aeromonas* spp. has increased significantly (Gromez *et al.* 1996) and, although the severity of illness is milder than that of *E. coli*, *Aeromonas* spp. have emerged as the second leading cause of traveler's diarrhea (; Hanninen *et al.* 1995; Yamada *et al.* 1997).

OTHER *AEROMONAS*-ASSOCIATED DISEASES

Aeromonas, once considered mainly an opportunistic pathogen in immunocompromised humans, is now implicated as the etiologic agent involving immunocompetent individuals of all age groups (Krovacek *et al.* 1994; Mani *et al.* 1995; Janda & Abbott 1998). Recent reports of *A. hydrophila* and *A. veronii* biovar sobria isolation from humans with sepsis, peritonitis, urinary tract infections, severe muscle degeneration and bacteremia with myonecrosis and gas–gangrene in a hemodialysis patient demonstrated the importance of this organism (Kohashi *et al.* 1995; Lin *et al.* 1996; Funada & Matsuda 1997). Reports have been published of *Aeromonas*-associated bacteremia in patients with hematologic diseases, association of *A. hydrophila* gastroenteritis with hypercalcemia, left-sided segmental colitis and severe acute diarrhea produced by *A. sobria* (*A. veronii* biovar sobria) in patients colectomized for Crohn's disease (Murakami *et al.* 1995; Riley *et al.* 1996; Deutsch & Wedzina 1997; do Sola Earle *et al.* 1997). Martino *et al.* (1997) reported that patients with acute non-lymphoblastic leukemia developed septic shock due to *Aeromonas* infection, confirming the potentially aggressive nature of these bacteria in neutropenic cancer patients (Holston *et al.* 1991). Recently, three cases of *A. veronii* biovar sobria bacteremia were reported in Denmark, with two patients having acute leukemia and HIV infection, respectively, while the third patient had colorectal cancer. The clinical presentation in all patients included chest and/or abdominal pain, with fever developing into sepsis without any known infectious focus (Thomsen & Kristiansen 2001). Diarrhea associated with *Aeromonas* spp. in AIDS patients (19%) in Thailand was also reported (Suthienkul *et al.* 2001). Leech therapy has been used successfully for medicinal purposes: however, since *A. veronii* biovar sobria constitutes a normal component of leech flora, *Aeromonas*-associated meningitis was recently reported as a result of medicinal leech therapy (Ouderkirk *et al.* 2004).

Filler *et al.* (2000) reported diarrhea-associated acute renal failure in an infant, which was caused by a hemolytic- and cytotoxic-producing strain of *A. sobria*. In general, *Aeromonas* bacteremia occurs in patients with liver cirrhosis or malignancy (Ko *et al.* 2000; Martino

et al. 2000). A fulminant fatal pneumonia due to *A. hydrophila* in a 40-year-old man with chronic renal failure and liver cirrhosis has been documented (Murata et al. 2001). A first case of necrotizing fasciitis from *A. hydrophila* that was not associated with trauma, liver disease or immunosuppression was recently reported (Minnaganti et al. 2000). Subsequent to this report, *A. hydrophila* as a causative agent of nosocomial necrotizing fasciitis was published (Cheng et al. 2004). Aeromonads, though not common pathogens in biliary sepsis, caused substantial mortality in patients with impaired hepatobiliary function (Itoh et al. 1999). *Aeromonas* has been isolated, in addition to *Helicobacter pylori*, from stomachs of squirrel monkeys with gastroenteritis (Khanolkar-Gaitondo et al. 2000).

ANIMAL MODELS OF *AEROMONAS* GASTROENTERITIS

Studies using ligated ileal loops in adult mice, rats and rabbits and the suckling mouse assay have been successfully employed by several investigators to examine enterotoxic activity in culture filtrates of *Aeromonas* isolates and purified enterotoxins (Wong et al. 1996) and, likewise, the enterotoxigenicity of *Aeromonas*. However, these methods do not use the natural oral route of infection in humans and are not suitable for determining the dose response or infectious dose of the organism. More recently, virulence of the organism was correlated successfully by oral administering organisms to suckling mice and then determining the LD_{50} (137). Sanderson et al. (1996) noted that only streptomycin-treated adult mice could colonize *Aeromonas* in the intestine when the latter was administered intragastrically; however, no diarrheal symptoms were produced in this model. Likewise, protein-malnourished mice could colonize *Aeromonas* in their intestine when the animals were given drinking water inoculated with *Aeromonas* over four days. Although the animals did not develop diarrhea, 75% of them were shedding *Aeromonas* two days after the challenge period (Sanderson et al. 1996). Therefore, an animal model is needed to demonstrate *Aeromonas*-induced gastroenteritis using a natural route of infection.

Graf (1999) suggested the possible use of medicinal leeches (*Hirudo medicinalis*) as a model for digestive tract association of *Aeromonas* spp., as *A. veronii* biovar sobria was exclusively found in the digestive tract of the leech. Interestingly, human fecal isolates of *A. hydrophila* and *A. veronii* biovar sobria colonized the digestive tract to the same extent as the symbiotic isolate. Further, human isolates proliferated to the same extent in the crop fluid as the symbiotic isolate six hours after blood feeding, indicating a potential for this digestive tract model in studying bacterial host interaction. It was reported that the proliferation of other organisms, such as *Pseudomonas aeruginosa* and *Staphylococcus aureus*, was inhibited in leeches, possibly due to continued activation of the complement inside *H. medicinalis*. These studies suggested that the symbiotic *Aeromonas* strains had mechanism(s) to prevent killing by complement (Indergand & Graf 2000). A recent study from the same group of investigators further indicated that complement resistance of *A. veronii* was essential for colonization, as serum-sensitive *A. veronii* mutants could not colonize *H. medicinalis*, while the colonization phenotype could be restored after complementation of the mutant (Braschler et al. 2003).

Fluid secretion by Act from a diarrheal isolate SSU of *A. hydrophila* in a mouse model occurred rapidly within 4–6 h. However, Alt and Ast protein enterotoxins evoked maximum fluid secretion after 12 h (Sha et al. 2002). We developed a food-restricted and streptomycin (Strp)-treated mouse model for inducing diarrhea by *Aeromonas*. Our studies indicated that Balb/c mice, which were food-restricted (20%) for three weeks and then treated with strp (5 g/L) in drinking water for 48 h, presented with an optimal fluid secretory response when challenged with 5×10^5 organisms intraluminally (Sha et al. 2002). We are aware that no animal model can replicate the conditions of the human gut, but this Balb/c model has clinical relevance, particularly in underdeveloped countries, as children are malnourished and more prone to severe infections. The immunocompromised (by injecting cyclophosphamide) mouse model has also been used in studying virulence potential of *Aeromonas* isolates when given by the intraperitoneal route (Lye et al. 2007) and studies have shown that the presence of enterotoxins and flagella was needed for bacteria to be virulent (Sen & Lye 2007).

CYTOTOXIC ENTEROTOXIN (ACT) AS A VIRULENCE FACTOR

Our laboratory purified Act from diarrheal isolate SSU of *A. hydrophila* (Asao *et al.* 1984; Chakraborty *et al.* 1986; Howard & Buckley 1986; Rose *et al.* 1989*a*). The mature form of Act is 49 kDa in size (Rose *et al.* 1989*a*,*b*) and has various biological activities associated with it, including those that are hemolytic, cytotoxic, enterotoxic and lethality in mice (Rose *et al.* 1989*b*). Subsequent cloning and sequence analysis of the *act* gene (1479 bp coding for a 493-amino acid (aa) polypeptide) indicated the predicted size of Act to be 54.5 kDa (Chopra *et al.* 1993). Two different laboratories reported the cloning of an aerolysin gene from *A. trota* and *A. bestiarum* (Chakraborty *et al.* 1986; Howard & Buckley 1986). Act and aerolysin share such similarities as (1) their production in two precursor forms (designated as pre-protoxin), and conversion to an active toxin by removal of a 23-aa-long NH_2-terminal signal peptide (protoxin) and proteolytic cleavage of the protoxins at their carboxy-terminal end (removal of 4–5 kDa peptide) to form a mature, biologically active toxin (Chopra *et al.* 1993; Ferguson *et al.* 1995, 1997) and (2) their characteristics of punching holes in the membranes. Therefore, Act and aerolysin appear to be related toxins like *E. coli* LT-1 and LT-II and/or cholera toxin (Gyles 1992; Spangler 1992). However, these toxins also exhibit significant differences structurally and functionally (Ferguson *et al.* 1995, 1997; Xu *et al.* 1998). Our laboratory was the first to pursue studies to understand the signal transduction cascade initiated in the host by Act that leads to its various biological effects.

Localization of biologically active regions within Act

By preparing deletion mutants, we determined that the Act regions (within aa residues 245–274 and 361–405) were important for the biological activity. We subsequently synthesized peptides encompassing aa residues 245–274 and 361–405 and developed anti-peptide antibodies, which significantly reduced the tested cytotoxic and hemolytic activity of the native toxin (Ferguson *et al.* 1995). We also demonstrated that pretreatment of Chinese hamster ovary (CHO) cells with a synthetic peptide (aa 245–274) resulted in dose-dependent, reduced cytotoxic activity of the native Act. These results indicated competition of the peptide with the native Act for receptors on CHO cells (Ferguson *et al.* 1995). We performed site-directed mutagenesis within aa residues 245–274 and 361–405 of the toxin molecule to identify aa residues involved in biological function(s) of Act. Among 7 aa substitutions made within region 245–274, replacing Tyr^{256} with Ser reduced Act's cytotoxic activity, with no effect on the hemolytic and enterotoxic activities. When Gly^{274} was changed to Ala, all three biological activities were reduced. Substitution of Trp^{270} with Leu, Phe and/or Gly abrogated cytotoxic activity, with some effect on the hemolytic and enterotoxic activities (Ferguson *et al.* 1995).

Among 8 substitutions made within region 361–405, replacement of Trp^{394} with Leu resulted in no biological activity. When Trp^{396} was changed to Leu, the hemolytic activity of the mutated Act was not affected; however, cytotoxic and enterotoxic activities were reduced significantly (Ferguson *et al.* 1995). A region of Act (aa residues 151–185) is moderately hydrophilic and we made 12 aa substitutions within this region. When $Asn^{177,178}$ and Asp^{179} were changed to Thr and Glu, respectively, a significant reduction in the hemolytic and cytotoxic activity of the toxin was noted, with no effect on the enterotoxicity.

With replacement of Gly^{169}, Asp^{170}, Gly^{171} and Trp^{172} by Ala, Glu, Ala and Leu, respectively, Act exhibited reduced hemolytic, cytotoxic and enterotoxic activities. There are six His residues in Act, and we mutagenized five of them, one at a time with Asn. The His^{209} residue in mutated Act did not alter hemolytic activity but reduced cytotoxic and enterotoxic activities substantially, compared to those of native toxin. The hemolytic and cytotoxic activities of Act were reduced in the His^{355} mutant, but there was no effect on the enterotoxic activity. All three biological activities were reduced when His^{144} was changed to Asn. Substitution of His^{130} and His^{155} to Asn had no effect on the biological activity of the toxin (Ferguson *et al.* 1995). All of the mutant toxins reacted with Act monoclonal antibodies to the same extent as did the native toxin, and the proper folding of all of these purified mutant toxins was confirmed by circular dichroism spectral analysis.

Detailed biochemical and structural studies were performed with aerolysin and recently reviewed (Fivaz *et al.* 2001). The crystal structure of proaerolysin revealed

that it consisted of a small, N-terminal globular domain (domain 1) and a long, elongated domain (the large lobe), which could be divided into three structural domains (domains 2–4). Based on site-directed mutagenesis studies, it was suggested that domains 1 and 2 were involved in receptor binding, although an additional role of domain 2 in the oligomerization of the toxin was also indicated. Domains 3 and 4 were found to be important in maintaining the oligomeric complex on the host cell membrane (Fivaz *et al.* 2001). Both lobes of the aerolysin seemed to play a role in toxin secretion from the bacterium, and at least 12 gene products appeared to be involved in aerolysin transport across the outer membrane of the bacterium along with ATP and electromotive forces (Wong & Buckley 1989; Howard *et al.* 1996).

Mechanism of action of Act

We demonstrated that toxin binding to erythrocytes occurred at 37°C but not at 4°C and was rapid (1–5 min). Once the toxin was bound to the erythrocytes, hemolysis proceeded at the same rate both at 4°C and 37°C. By using sugars and colloidal substances of varying diameters, we determined that Act created 1.14–2.8 nm pores in erythrocytes. The toxin's mechanism of action involves entry of water from the external milieu into erythrocytes through the pores, resulting in cell swelling and subsequent lysis. Act's ability to create pores on erythrocyte membranes was confirmed by electron microscopy (Ferguson *et al.* 1997).

We tested various phospholipids, glycolipids and cholesterol for their ability to block or reduce Act hemolytic activity. Preincubation of the toxin with only cholesterol resulted in a dose-dependent reduction in hemoglobin release from erythrocytes. Once Act interacted with cholesterol on the membranes of erythrocytes, its aggregation occurred, which was demonstrated by immunoblot analysis resulting in transmembrane pore formation and cytolysis of erythrocytes. We also showed binding of Act with ^{14}C-cholesterol using size exclusion chromatography (Ferguson *et al.* 1997).

Intriguing differences between Act and aerolysin

Four Act monoclonals, which map to different regions, neutralized Act's hemolytic activity, but failed to neutralize aerolysin. These studies were substantiated by mutagenesis of selected aa residues in Act and aerolysin (Ferguson *et al.* 1995, 1997). Some aa residues, such as $His^{107,132}$, were crucial for aerolysin's hemolytic activity, whereas the analogous $His^{130,155}$ did not affect the hemolytic activity of Act. Likewise, Trp^{371} had no effect on the hemolytic titers of aerolysin; however, an analogous Trp^{394} abrogated Act's biological activity. Act bound cholesterol but did not bind to glycophorin, which acted as the aerolysin receptor on erythrocytes. On other mammalian host cells, aerolysin was also shown to bind to glycosylphosphatidyl inositol (GPI)-anchored proteins (Fivaz *et al.* 2001). Most studies on aerolysin were targeted toward measuring hemolytic activity. Our site-directed mutagenesis data indicated possibly different loci on a single chain of mature Act, which might be associated with various biological activities (Ferguson *et al.* 1995). Therefore, abrogating hemolytic activity does not necessarily mean losing cytotoxic and enterotoxic activities. This contention is provocative because it implies the toxin has different binding sites for different target cells or that effects are not uniformly due to transmembrane pore formation.

Role of Act in *Aeromonas* pathogenesis

We performed transposon and marker exchange mutagenesis to evaluate Act's role in the pathogenesis of *A. hydrophila* SSU-induced diarrhea and septicemia. Seven transposon mutants obtained had dramatically reduced hemolytic and cytotoxic activities, and such mutants exhibited reduced virulence in mice compared to effects induced by WT *Aeromonas*. While transposition in two mutants was in the *act* structural gene, the other five appeared to be regulatory mutants. The altered virulence of the *act* transposon mutants was confirmed by developing *act* isogenic mutants of the WT *Aeromonas* via homologous recombination (Xu *et al.* 1998).

The culture filtrates from these isogenic mutants were devoid of the hemolytic, cytotoxic and enterotoxic activities (4 h observation period) associated with Act. These filtrates caused no damage to mouse small intestinal epithelium, whereas culture filtrates from WT *Aeromonas* caused complete destruction of the microvilli. The 50% lethal dose (LD_{50}) of these mutants in mice was 1×10^8 colony

forming units (cfu) when injected i.p., compared to 3×10^5 cfu for the WT *Aeromonas*. Reintegration of the native *act* gene in place of the truncated toxin gene in isogenic mutants resulted in complete restoration of Act's biological activity and virulence in mice. The animals injected with a sublethal dose of WT *Aeromonas* or revertant, but not the isogenic mutant, had circulating toxin-specific neutralizing antibodies (Xu *et al.* 1998). These studies substantiated the earlier findings of Chakraborty *et al.* (1987) in which they showed that aerolysin-deficient mutants of *A. trota* (now *A. bestiarum*) were less virulent in mice than was WT *Aeromonas*. Since the aerolysin gene from *A. trota* differed significantly from the *act* gene from *A. hydrophila* (Chopra *et al.* 1993), it was important to delete the *act* gene from an authentic strain of *A. hydrophila*, particularly as Kuhn *et al.* (1997) reported that *A. hydrophila* type HG1/BD-2 might cause diarrhea in humans. Taken together, these studies clearly established a role for Act in the pathogenesis of *Aeromonas*-mediated infections.

Act induces cytokine production

Act leads to infiltration of macrophages and mononuclear cells in the lumen of rat ligated ileal loops, indicating a host inflammatory response. Indeed Act stimulated production of tumor necrosis factor alpha (TNFα and up-regulated the expression of genes encoding interleukin (IL)-1β, IL-6 and inducible nitric oxide synthase (iNOS) in murine macrophages. The amount of LPS detected in the purified Act preparation was too negligible to contribute to these increases in pro-inflammatory cytokines. Further, treatment of Act with polymyxin B sulfate, which inactivates the biological activity of LPS, did not alter the toxin's ability to induce cytokine production. In addition, peritoneal macrophages derived from an LPS-hyporesponsive mouse strain (C3H/HeJ) responded to Act but not to LPS, to produce these cytokines (Chopra *et al.* 2000).

Role of arachidonic acid (AA) metabolism in Act-induced fluid secretion

Act evoked in macrophages prostaglandin (e.g. PGE_2) production, which was coupled to cyclooxygenase (COX)-2 induction (Chopra *et al.* 2000), as specific COX-2 inhibitors NS398 and Celebrex abrogated Act-induced PGE_2 production. Since AA is a substrate for PGE_2 production, we showed that a newly discovered PLA_2 (group V $sPLA_2$) was involved in Act-induced PGE_2 production. Further, Act evoked cAMP production in macrophages (Chopra *et al.* 2000). We noted that both Celebrex and $sPLA_2$ inhibitor, which dramatically reduced Act-induced PGE_2 production, also significantly decreased cAMP levels. Rat ligated intestinal loops injected with 200 ng of Act contained 4- to 5-fold-elevated PGE_2 levels in the fluid. We have found that Act also induces COX-2 and initiates PGE_2 production in T84 cells.

Studies conducted by Fujii *et al.* (2003) indicated that T84 cells stimulated with aerolysin-like hemolysin from *A. sobria* produced in the medium cAMP and that could lead to fluid secretion. They further delineated its mechanism of action and demonstrated that this hemolysin elevated ATP levels in T84 culture supernatant. The ATP was then converted to adenosines by ectonucleotidases. Subsequently, the adenosines stimulated the P1 adenosine receptor (A_{2B}) on T84 cells to produce cAMP. The cAMP from T84 cells was released through the channels created by the toxin. Using HT29 colonic epithelial cells, Epple *et al.* (2004) noted that β-hemolysin of *A. hydrophila* Sb induced a significant short-circuit current (I_{sc}), as measured in Ussing chambers, due to Cl^- secretion. This change in I_{sc} was inhibited by the protein kinase C inhibitor but not by a protein kinase A inhibitor or BAPTA-AM that chelates Ca^{2+}. Overall these data indicated that β-hemolysin induced Cl^- secretion in the intestinal epithelium, possibly by channel insertion into the apical membrane and by activation of protein kinase C.

Act induces activation of NF-κB (nuclear factor-kappa B) and CREB (cyclic AMP responsive element binding protein) in macrophages

Since transcription factors NF-κB and CREB have important functions in modulating the transcription of cytokine and *cox-2* genes, we showed by a gel shift assay that Act caused activation of NF-κB and CREB in nuclear extracts of macrophages (Chopra *et al.* 2000). We then used Affymetrix murine GeneChips to gain a global and molecular view of cellular transcriptional responses to Act and to identify

important genes up-regulated by this toxin. Seventy-eight genes were significantly and consistently up-regulated by Act in macrophages. Many of these genes were immune-related, and several were transcription factors, adhesion molecules and cytokines. Additionally, we identified several apoptosis-associated genes that were significantly up-regulated in Act-treated macrophages. Act-induced apoptosis of macrophages was confirmed by annexin V staining and DNA laddering. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) were used to verify any increased expression of selected inflammatory and apoptosis-associated genes identified by GeneChip analysis. Taken together, the array data provided a global view of Act-mediated signal transduction and clearly demonstrated an inflammatory response and apoptosis mediated by this toxin in host cells at the molecular level (Galindo *et al.* 2003, 2004*a*). In addition, we also provided a first description of human intestinal epithelial cell transcriptional alterations, phosphorylation or activation of signaling molecules (by using genomics and proteomics approaches), cytokine production and calcium mobilization in response to this toxin (Galindo *et al.* 2005). Our most recent studies based on protein microarray technology also indicated interaction of Act with galectin-3 and SNARE complex-scaffolding protein synaptosomal-associated protein 23 (SNAP23), which appeared crucial in Act-induced apoptosis of macrophages and epithelial cells (Galindo *et al.* 2006).

Regulation of the Act gene

We generated an *act::alkaline phosphatase (phoA)* reporter gene (including the putative promoter region and a portion of the *act* structural gene), which was subsequently integrated into the chromosome of *A. hydrophila* SSU via single crossover homologous recombination using a suicide vector. We noted that maximum PhoA activity was seen when the organism was aerated and grown at 37°C, pH 7.0. While the presence of Ca^{2+} increased *act* promoter activity, glucose and iron down-regulated the promoter activity of the *act* gene. We cloned and sequenced a ferric-uptake regulator gene (*fur*), which exhibited 69% identity at the DNA level and 79% homology at the aa level with a ferric uptake regulator (Fur) from *V. cholerae* (Sha *et al.* 2001). Complementation experiments demonstrated that the *A. hydrophila fur* gene could restore iron regulation in an *E. coli fur*-minus mutant. Using suicide vector pDMS197, we generated a *fur* isogenic mutant of *A. hydrophila*. Northern blot analysis and hemolytic activity data indicated that the repression in the transcription of the *act* gene by iron was relieved in the *fur* isogenic mutant, and iron regulation in the *fur* isogenic mutant of *A. hydrophila* could be restored by complementation (Sha *et al.* 2001). The iron-regulated genes have Fur-binding sites in their promoter region (Escolar *et al.* 1998). In the putative promoter region of the *act* structure gene of *A. hydrophila* (Chopra *et al.* 1993), we detected two Fur box-like sequences (TATTA: position -131 to -135 and position -178 to -182 nucleotides), starting from the initiation codon of the *act* structural gene. These sequences could be the potential sites within the *act* promoter region to which the Fur protein might bind and are under investigation. Interestingly, the sequence ATTATTTTT (position -173 to -181 nucleotides), starting from the starting codon of *act* structural gene and within the *act* putative promoter, has also been shown to exist within a Fur-binding sequence (19 bp) in the promoter region of the *flbB* gene (a transcriptional activator) of *E. coli* (Bartlett *et al.* 1988; Stojiljkovic *et al.* 1994).

Like Act, whose expression is increased in iron-limited environment, recent studies of Ebanks *et al.* (2004) noted that expression of genes coding for outer membrane proteins of sizes 73, 76 and 85 kDa in *A. salmonicida* was increased in iron-restricted medium *in vitro* by proteomics approaches. These three proteins represented colicin receptor, heme receptor and a ferric siderophore receptor, respectively. Similar data were obtained when bacteria were grown *in vivo* in salmon wherein iron is in limited concentration.

In search for additional regulatory genes modulating expression of the *act* gene, we obtained two mutated strains of a diarrheal isolate SSU of *A. hydrophila* by using a mini-transposon that exhibited a 50–53% reduction in hemolytic activity and 83–87% lower cytotoxic activity associated with Act. Subsequent cloning and DNA sequence analysis revealed that transposon insertion occurred at different locations in these two mutants within the same 1,890 bp, open-reading frame coding for the glucose-inhibited division gene (*gidA*). A similar reduction in hemolytic (46%)

and cytotoxic (68%) activity of Act was noted in the *gidA* isogenic mutant of *A. hydrophila* that was generated by marker-exchange mutagenesis. Northern blot analysis revealed that *act* transcription in the *gidA* transposon and isogenic mutants was not altered. However, by generating a chromosomal *act*::*phoA* reporter construct, we demonstrated significantly reduced phosphatase activity in these mutants indicating the effect of glucose-inhibited division (GidA) protein in modulating *act* gene expression at the translational level. The biological effects of Act in the *gidA* mutants were restored by complementation. The virulence of the *gidA* mutants in mice (given by the i.p. route) was dramatically reduced when compared to the WT and complemented strains of *A. hydrophila*. The histopathological examination of lungs, in particular, indicated severe congestion, alveolar hemorrhage and acute inflammatory infiltrate in the interstitial compartment and the alveolar spaces when mice were infected with the WT and complemented strains. Minimal-to-mild changes were noted in the lungs with the *gidA* mutants. Taken together, our data indicated for the first time that GidA regulated the most potent virulence factor Act of *A. hydrophila* (Sha *et al.* 2004).

More recently, we characterized the DNA adenine methyltransferase *(dam)* gene from *A. hydrophila* SSU (Erova *et al.* 2006*a*,*b*). Dam exerts its function by chemically modifying DNA via methylation at adenine residues in GATC sequences. Our recent studies indicated Dam altered levels of type II secretion system (T2SS)-secreted Act, cytotoxicity associated with the T3SS, and lactone production involved in quorum sensing. Importantly, we provided evidence that Dam operated via GidA in regulating virulence of *A. hydrophila* (Erova *et al.* 2006*a*).

Cell-signaling by Act

Act rapidly mobilized calcium from intracellular stores and evoked a calcium influx from the extracellular milieu in macrophages. The release of calcium from intracellular stores was biphasic, and these results were consistent with those published by Krause *et al.* (1998) utilizing aerolysin from *A. bestiarum* in human granulocytes. Pretreatment of cells with thapsigargin indicated that, although the first phase of intracellular calcium release by Act was predominantly from endoplasmic reticulum (ER) stores, the second was from a combination of ER stores and other intracellular stores (possibly mitochondria (Ribardo *et al.* 2002)). Heat treatment of Act at 60°C for 20 min, which abolished the toxin's biological activity, did not stimulate any calcium mobilization in macrophages (Ribardo *et al.* 2002). Interestingly, however, we found that pertussis toxin (PT) reduced both the early and late phase of calcium release from intracellular stores in Act-treated macrophages, indicating that Act-associated calcium mobilization was linked to PT-sensitive G protein (Ribardo *et al.* 2002). In contrast, PT abrogated the initial rapid calcium response in human granulocytes treated with an aerolysin, with no effect on the delayed and more sustained calcium mobilization (Krause *et al.* 1998).

A direct role of calcium in Act-induced prostaglandin (e.g. PGE_2) and TNFα production was demonstrated in macrophages using a cell-permeable calcium chelator BAPTA-AM, which also down-regulated activation of transcription factor NF-κB. We showed that Act's capacity to increase PGE_2 and TNFα production could be blocked by inhibitors of tyrosine kinases and protein kinase A. The use of tyrosine kinase inhibitors genistein, herbimycin A and lavendustin A reduced Act-induced PGE_2 production by 99, 92 and 88%, respectively, after 24 h of stimulation. Chelerythrine chloride, a potent protein kinase C (PKC) specific inhibitor, did not significantly alter Act-induced PGE_2 production; however, a protein kinase A (PKA) inhibitor (H89) resulted in an approximately 77% reduction of Act-induced PGE_2 levels in macrophages. We also measured inhibition of TNFα production in Act-stimulated macrophages after treatment with tyrosine kinase, PKC and PKA inhibitors. While both tyrosine kinase and PKA inhibitors reduced Act-induced TNFα levels by 84–98%, the PKC inhibitor did not alter TNFα levels in Act-treated macrophages when compared to cells exposed to Act alone (Ribardo *et al.* 2002).

In addition, Act caused up-regulation of the DNA repair enzyme redox factor-1 (Ref-1), which potentially could promote DNA binding of the transcription factors, allowing modulation of various genes involved in the inflammatory response. Increased Ref-1 levels by Act also indicated an increase in oxidative damage within cells (Christman *et al.* 2000). We therefore examined whether Act would

cause an increase in reactive oxygen species (ROS) in macrophages, which is a primary source of oxidative damage (Xanthoudakis *et al.* 1992). Measurement by flow cytometry indicated that Act did increase ROS production within 5 min of exposure to Act. The increase in ROS and their subsequent destructive capability on tissue led us to determine if antioxidants could reduce some of the effects that Act might have on host cells. Macrophages pretreated with the antioxidant N-acetyl cysteine (NAC), followed by subsequent treatment with Act, showed a basal level of ROS production. Likewise, pretreatment of macrophages with NAC before Act treatment resulted in reduced PGE_2 and TNFα levels by approximately 54% and 83%, respectively, indicating the ability of these reactive oxygen scavengers to reduce the effect of oxidative stress in Act-treated macrophages. NAC reduced NF-κB translocation in Act-treated cells by 80% within 30 min after stimulation. Taken together, a link between Act-induced calcium release, regulation of downstream kinase cascades and Ref-1, and activation of NF-κB, leading to PGE_2 and TNFα production, was established (Ribardo *et al.* 2002). We demonstrated for the first time early cell signaling initiated in eukaryotic cells by Act, which led to various biological effects associated with this toxin. Our studies also indicated that calcium mobilization and oxidative stress pathways represented the primary mechanisms of inducing TNFα production by Act.

Act inhibited the phagocytic ability of mouse phagocytes, and interferon-gamma (IFN-γ pretreatment overcame this toxic effect (Jin *et al.* 1992). We showed that Act significantly stimulated the chemotactic activity of human leukocytes in a dose-dependent fashion. This stimulatory effect, which was inhibited by various concentrations of pertussis toxin (PT), suggested that human leukocytes possessed Act receptors, which might be coupled to PT-sensitive G-protein (Jin & Houston 1992).

We utilized five target cell types (a murine macrophage cell line (RAW 264.7), bone marrow-derived transformed macrophages, murine peritoneal macrophages and two human intestinal epithelial cell lines (T84 and HT29)) to investigate the effect of Act on mitogen-activated protein kinase (MAPK) pathways and mechanisms leading to apoptosis. As demonstrated by immunoprecipitation/kinase assays or Western blot analysis, Act activated stress-associated p38, c-Jun-NH_2-terminal kinase (JNK) and extracellular signal-regulated kinase 1/2 (ERK1/2) in these cells. Act also induced phosphorylation of upstream MAPK factors (MAPK kinase 3/6 (MKK3/6), MKK4 and MAP/ERK kinase 1 (MEK1)) and downstream effectors (MAPK-activated protein kinase-2 (MAPKAPk-2), activating transcription factor-2 (ATF-2) and c-Jun). Act evoked cell membrane blebbing, caspase 3-cleavage and activation of caspases 8 and 9 in these cells. In macrophages that did not express functional tumor necrosis factor receptors (TNFRs), apoptosis and caspase activities were significantly decreased. Immunoblotting of host whole-cell lysates revealed Act-induced up-regulation of apoptosis-related proteins, including the mitochondrial proteins cytochrome c and apoptosis-inducing factor (AIF). However, mitochondrial membrane depolarization was not detected in response to Act. Taken together, the data demonstrated, for the first time, Act-induced activation of MAPK signaling and classical caspase-associated apoptosis in macrophages and intestinal epithelial cells. Given the importance of MAPK pathways and apoptosis in inflammation-associated diseases, this study provided new insights into the mechanism of action of Act on host cells (Galindo *et al.* 2004*b*).

CYTOTONIC ENTEROTOXINS OF *AEROMONAS*

Since the first report of a cytotonic enterotoxin of *Aeromonas* (Annapurna & Sanyal 1977), several investigators identified an enterotoxic factor in culture filtrates of *Aeromonas* spp. (Sanyal *et al.* 1975; Ljungh *et al.* 1982*a*,*b*; Chakraborty *et al.* 1984; Potomski *et al.* 1987; Schulz & McCardell 1988; Singh & Sanyal 1992). However, not much effort was made to purify and characterize this enterotoxin at the molecular level. In 1984, Chakraborty *et al.* (1984) first reported cloning of a non-cholera toxin reactive cytotonic enterotoxin gene from *A. hydrophila* (later designated as *A. trota* and then *A. bestarium*). The cell lysate (heated at 56°C/20 min) from this clone caused fluid secretion in rabbit ligated ileal loops and suckling mice. However, this gene or its product was not further characterized.

We purified the heat-labile cytotonic enterotoxin (referred to as Alt) from a diarrheal isolate, SSU, of

A. hydrophila. The purified native Alt exhibited a molecular mass of 44 kDa and consisted of a single polypeptide chain having 368 amino-acid residues (Chopra *et al.* 1996). The toxin was biologically active in *in vivo* and *in vitro* models. Alt was not related to CT; however, in Chinese hamster ovary (CHO) cells, Alt elevated cAMP and PGE_2 levels (Chopra *et al.* 1986, 1992; Chopra & Houston 1989).

The gene encoding Alt was cloned (Chopra *et al.* 1994) and expressed, using various *E. coli*-based expression vectors, and subsequently purified (Chopra *et al.* 1996). The recombinant-Alt (r-Alt) in *E. coli* was slightly smaller (35–38 kDa), compared to native Alt (44 kDa) purified from *A. hydrophila* (Chopra *et al.* 1996). Alt exhibited 45–51% identity in a 175-aa overlap with the carboxy terminus of lipase and PLC of *A. hydrophila* (Chopra *et al.* 1996); however Alt did not exhibit any lipase or PLC activity with the substrates tested. An aa sequence VHFLGHSLGA (aa residues 218–227) of Alt was highly homologous to the putative substrate-binding domain found within bacterial, fungal, porcine and human lipases. Do structural homologies exist between Alt, lipase and PLC which are unrelated to their mechanisms of action? These interesting questions are being addressed in our laboratory. Interestingly, the CHO cell elongation and the fluid secretory ability of r-Alt in rat ligated ileal loop was much lower (10–15-fold) compared to those in the native Alt purified from *Aeromonas* (Chopra *et al.* 1996), indicating differential processing of the toxin in *E. coli* and *Aeromonas* which affected the toxin's enterotoxicity. Cyclic AMP levels of the intestinal mucosa in r-Alt-treated rat loops were 3- to 4-fold higher, and the PGE_2 levels in the loop fluid elevated by at least 5-fold compared to phosphate-buffered saline (PBS)-treated control loops.

Our recent expression of the *alt* gene back in *Aeromonas* using a *tac* promoter-based, multi-host-range expression vector pMMB66 indicated the r-Alt was 44 kDa when produced from plasmid pMMB66 in *Aeromonas* and secreted into the medium, like native Alt. Further, recombinant and native Alt produced from *Aeromonas* had identical biological activity. These data clearly indicated differential processing of Alt in *E. coli* compared to *Aeromonas* or the possible posttranslational modification of Alt in *Aeromonas*, which affected Alt's enterotoxic activity.

Active immunization of mice with purified r-Alt resulted in a significant decrease (39%) in the fluid secretory response when the mice were challenged with WT *A. hydrophila* (Chopra *et al.* 1996). In a study by Granum *et al.* (1998), 75% of the *Aeromonas* isolates from food and water in Norway contained the *alt* gene, based on PCR amplification, and one of the *A. hydrophila* strains was probably involved in an outbreak of food poisoning caused by ingestion of raw, fermented fish. Our data indicated elevated Ca^{2+} levels in CHO cells after treatment with purified r-Alt. Our studies also showed that pretreatment of CHO cells with a phospholipase C (PLC) inhibitor (U73122) prior to addition of Alt blocked Ca^{2+} release by 65–70%, indicating activation of a G-protein-coupled PLC. In addition, we demonstrated that PGE_2 levels in Alt-treated CHO cells were regulated by a G-like protein (phospholipase A_2-activating protein (PLAA)) (Ribardo *et al.* 2001). We noted that pretreatment of cells with an antisense *plaa* oligonucleotide generated to the first 25 bases of the human *plaa* cDNA reduced release of ^{3}H-AA (substrate for eicosanoids (e.g., PGE_2)) from Alt-induced CHO cells by 40–50% (unpublished data). Our data also indicated binding of Alt to G_{M3} ganglioside based on ELISA.

We identified another cytotonic enterotoxin gene (designated as *ast*) in the genomic library of *A. hydrophila* SSU. The *ast* gene encoded a product (Ast) which caused CHO cells to elongate, an enterotoxic activity stable at 56°C for 20 min (Morgan *et al.* 1985). The crude Ast preparations evoked fluid secretion in the rat small intestine and the cAMP levels in the mucosal cells were elevated, compared to appropriate controls (Morgan *et al.* 1985). Expression of the DNA fragment containing the *ast* gene in *E. coli* exhibited prominent bands of 32 and 67 kDa. Our DNA sequence analysis of the *ast* gene revealed it was encoded by a 1,911 bp open-reading frame (ORF), contained 636 amino-acid residues, and had a predicted molecular mass of 71 kDa with an isoelectric point of 6.9. Both Alt and Ast represent novel molecules with no significant homology to known bacterial enterotoxins (Sha *et al.* 2002).

The highly purified Ast exhibited a size of 69 kDa after SDS-PAGE and the NH_2-terminal sequence (five amino-acid residues sequenced) of the purified Ast matched with the DNA-derived amino acid sequence. A potential

hydrophobic leader sequence seemed to be present at the NH_2-terminal end of Ast. We also showed that active immunization of mice with purified Ast (5 μg) reduced the fluid secretory response to WT *A. hydrophila* SSU by 30%. McCardell *et al.* (1995) purified from *A. hydrophila* a heat-stable enterotoxin (56°C/20 min) which exhibited a size of 70 kDa. This toxin was non-CT cross-reactive and did not increase cAMP, cGMP and PGE_2 levels in CHO cells, but evoked intestinal fluid accumulation in infant mice. Whether this enterotoxin has any similarity to Ast needs to be determined.

ROLE OF VARIOUS *A. HYDROPHILA* ENTEROTOXINS IN DIARRHEA

We evaluated the role of three *A. hydrophila* enterotoxins (Act, Alt, Ast) in evoking diarrhea in a diet-restricted and streptomycin-treated murine model by developing various combinations of enterotoxin gene-deficient mutants by marker exchange mutagenesis. A total of six isogenic mutants were prepared in an *act*-positive or -negative background strain of *A. hydrophila*. We developed two single knockouts with truncation in either the *alt* or the *ast* gene; three double knockouts with truncations of genes encoding (i) *alt* and *ast*, (ii) *act* and *alt*, and (iii) *act* and *ast* genes; and a triple-knockout mutant with truncation in all three genes, *act*, *alt* and *ast*. The identity of these isogenic mutants was confirmed by Southern blot analysis. Northern and Western blot analyses revealed that the expression of different enterotoxin genes in mutants was correspondingly abrogated. Our data also indicated that all of these mutants had a significantly reduced capacity to evoke fluid secretion compared to that of WT *A. hydrophila*; the triple-knockout mutant failed to induce any detectable level of fluid secretion. The biological activity of selected *A. hydrophila* mutants was restored by complementation. We demonstrated that Act contributed greatest in evoking fluid secretion (64%), followed by Alt (38%) and Ast (27%). Our data also suggested some interaction among these various enterotoxins (Sha *et al.* 2002). All of these mutants colonized the small intestine of mice to the same extent as that noted for the WT *A. hydrophila* after 2 h and their number increased by approximately 2 logs after 12–16 h of incubation.

Interestingly, we were unable to detect a transcript for the *ast* gene, even in the WT *A. hydrophila*, although the Ast protein could be seen by Western blot analysis in WT *A. hydrophila* and its isogenic mutants with intact *ast* gene (Sha *et al.* 2002). Our data might be suggestive of a short half-life for the *ast* mRNA or its rapid degradation. This possibility was based on our observation that we could detect the *ast* gene transcript in significant amounts from *E. coli* when the toxin gene was hyperexpressed using a pET30a vector system. However, we cannot rule out the possibility that the *ast* gene expression requires interaction of *A. hydrophila* with the host cell.

IRON ACQUISITION MECHANISM

During an infection, a microbial pathogen must acquire all of its iron from the host. Because of the array of host iron-withholding defenses, an efficient mechanism to divert some of the metal ions to microbial metabolism is essential for bacterial virulence. To obtain their supply of iron, organisms synthesize and excrete iron-specific ligands of low molecular mass, collectively known as siderophores. *Aeromonas* spp. produce either one of the siderophores, enterobactin or amonabactin (Byers *et al.* 1991; Pemberton *et al.* 1997). While the amonabactin producers have evolved both siderophore-dependent and -independent means for iron acquisition from a vertebrate host, enterobactin producers have to rely exclusively on non-siderophore heme utilization because enterobactin is inactive in vertebrate serum. Both siderophores contain 2,3-dihydroxybenzoic acid (DHB), with amonabactin produced in two biologically active forms. Amonabactin T contained 2,3-DHB, lysine, glycine and tryptophan, while amonabactin P contained phenylalanine instead of tryptophan (Pemberton *et al.* 1997). The ferric siderophore receptor gene (*fstA*) of *A. salmonicida* exhibited significant homology with *fstA* genes of *Vibrio anguillarum*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa* and *Bordetella bronchiseptica*, indicating that homologs of this protein are widespread in Gram-negative bacterial pathogens (Pemberton *et al.* 1997).

QUORUM SENSING

Quorum sensing, which is a mechanism for controlling gene expression in response to an expanding bacterial population, has been reported in *Aeromonas* spp. and is a subject of intensive investigation in many Gram-negative bacteria (Swift *et al.* 1997). The quorum-sensing signal molecule belongs to the *N*-acylhomoserine lactone (AHL) family and the signal generator proteins responsible for the synthesis of AHLs belong to the LuxI family. Accumulation of this molecule above a threshold concentration provides an indication that the minimum bacterial population size has been reached and that the appropriate target gene(s) should be activated via the LuxR family of transcriptional activators. The LuxR protein consists of two domains with an AHL binding site within the N-terminal end and a helix-turn-helix DNA binding motif within the C-terminal domain. It is plausible that the expression of various virulence factors of *Aeromonas* could be controlled by quorum sensing. The role of an AHL-dependent, quorum-sensing system, based on the LuxRI homolog AhyRI in *A. hydrophila*, has been reported.

The major signal molecule synthesized by the *ahyI* locus in *A. hydrophila* was N-(butanoyl)-L-homoserine lactone (BHL) and also referred to as C4-HSL, with AHL synthesized in relatively smaller amounts (Swift *et al.* 1999). Downstream of the *ahyI* locus was a gene with homology to *iciA* gene, an inhibitor of chromosome replication *in E. coli*, suggesting that in *Aeromonas* cell division could be linked to quorum sensing. Further, it was noted that both AhyRI and BHL were required for the transcription of *ahyI*. Indeed, in other bacteria such as *P. aeruginosa*, BHL was involved in the regulation of the secretion of multiple exoproducts, including elastase, hemolysin, chitinase, alkaline protease, cyanide, lectins, staphylolytic activity, pyocyanin and the alternative stationary-phase sigma factor RpoS (Swift *et al.* 1999).

A. hydrophila produces both a serine protease and a metalloprotease, and in the *ahyI*-negative strain, both of these proteases were produced in reduced amounts. However, their production was restored after exogenous addition of C4-HSL (Swift *et al.* 1999). On the other hand, mutation in the *ahyR* gene resulted in the loss of protease activity which could not be restored by the addition of C4-HSL. However, studies of Vivas *et al.* (2004) reported no correlation between production of AHLs and protease *in vitro* in an *aroA* live vaccine strain of *A. hydrophila*. The presence of the C4-HSL in *A. hydrophila* biofilm development has also been reported (Swift *et al.* 1999). The *ahyI* mutant that could not produce C4-HSL failed to form mature biofilms. A mutation in the *ahyR* locus increased the coverage of the available surface to around 80%, with no obvious effect upon biofilm microcolony formation (Lynch *et al.* 2002). Bacteria in biofilms are more resistant to host defenses and antimicrobial agents and could express more virulent phenotypes as a result of gene activation through bacterial communication (quorum sensing) or gene transfer (Swift *et al.* 1997). Our recent studies indicated a correlation between the T3SS and Act of *A. hydrophila* and the production of lactones (Sha *et al.* 2005). More in-depth studies are needed to definitively establish the role of iron acquisition and quorum sensing in *Aeromonas*-associated infections.

OTHER VIRULENCE FACTORS

Aeromonas spp. produce a wide range of proteases which may cause tissue damage and aid in establishing an infection by overcoming host defenses, and possibly by providing nutrients for cell proliferation (Pemberton *et al.* 1997). At least three types of proteases have been identified, which include heat-labile serine protease and heat-stable and EDTA-sensitive or insensitive metalloproteases. Recently, additional proteases were described in *A. veronii* biovar sobria and *A. caviae* that could directly and indirectly play a role in bacterial virulence (Nakasone *et al.* 2004; Song *et al.* 2004). In addition, some aminopeptidases may have a specific function such as activation of Act/aerolysin. *Aeromonas* spp. produce glycerophospholipid:cholesterol acyltransferase (GCAT), which functions as a lipase or phospholipase and could cause erythrocyte lysis by digesting their plasma membranes (Pemberton *et al.* 1997). Although the role of GCAT in fish disease furunculosis has been suggested, its role as well as of proteases as a virulence factor in humans is presently undefined. We recently identified a ToxR-regulated lipoprotein (TagA) from *A. hydrophila* that provided serum

resistance to the bacterium. It also prevented erythrocyte lysis by controlling classical pathway of complement activation (Pillai *et al.* 2006).

Strains of *A. hydrophila* lacking enterotoxigenic activity and presenting minimal hemolytic activity showed a strong vacuolating activity in Caco-2 cells (Falcon *et al.* 2001). Other studies using polarized and non-polarized epithelial cells indicated that aerolysin could trigger vacuolation of the endoplasmic reticulum. Whether this vacuolation is linked to intracellular calcium release by aerolysin is not known (Fivaz *et al.* 2001). Although ER vacuolation could be observed in some forms of apoptosis, no degradation of genomic DNA was noted in cells. However, studies with Act from *A. hydrophila* indicated that it led to apoptosis in T84 and HT29 cells, as determined by cytoplasmic blebbing and nuclear condensation (Guimaraes *et al.* 2002; Galindo *et al.* 2004*b*). Our recent GeneChip data also indicated up-regulation of several genes in macrophages by Act that could be involved in apoptosis (Galindo *et al.* 2003, 2004*a*).

Recent studies mapped the opsonophagocytosis resistance to a *ftsE* and *X* genes in *A. hydrophila* (Merino *et al.* 2001*a*,*b*). The *fts* gene cluster (*ftsY*, *E* and *X*) is located at 76 min on the *E. coli* genetic map. It has been shown that *ftsE* and *X* form a complex in the inner membrane that bears the characteristics of an ABC-type transporter involved in cell division. It is believed that *A. hydrophila ftsE* mutation renders a filamentous phenotype at 37°C, which could interfere with opsonophagocytosis.

NEW VIRULENCE FACTORS IN *AEROMONAS*

By using a murine peritoneal culture (MPC) model, we identified via restriction fragment differential display PCR (RFDDPCR) five genes of *A. hydrophila* SSU that were differentially expressed under *in vivo* versus *in vitro* growth conditions. The gene encoding enolase was among those five genes that were differentially up regulated. Enolase is a glycolytic enzyme, and its surface expression was recently shown to be important in the pathogenesis of a Gram-positive bacterium *Streptococcus pyogenes*. By Western blot analysis and Immunogold staining, we demonstrated secretion and surface expression of enolase in *A. hydrophila*. We also showed that the whole cells of *A. hydrophila* had strong enolase activity. Using an ELISA assay and sandwich Western blot analysis, we demonstrated binding of enolase to human plasminogen, which is involved in the fibrinolytic system of the host. We cloned the *A. hydrophila* enolase gene, which exhibited 62% identity at the DNA level and 57% homology at the amino-acid level when compared to *S. pyogenes* enolase. This is a first report describing the increased expression of enolase gene *in vivo* that could potentially contribute to the pathogenesis of *A. hydrophila* infections (Sha *et al.* 2003). Currently, additional studies are in progress to demonstrate role of enolase in bacterial virulence.

Superoxide dismutases in *Aeromonas* spp

Recently, genes encoding SodA and B were sequenced from *A. salmonicida*. The SodA, which is 23 kDa in size, exhibited high homology with Mn-SODs (periplasmic) from other prokaryotes. The SodB was 22.3 kDa in size and showed similarity to other prokaryotic Fe-SODs (cytoplasmic) (Dacanay *et al.* 2003). SODs are responsible in detoxifying superoxide anions generated inside phagocytic cells, thus allowing bacteria to survive inside the hostile environment of the host. The SOD levels were higher in bacteria grown under *in vivo* conditions than when the organisms were cultivated *in vitro*. Further, SOD levels were lower in avirulent versus virulent cultures of *A. salmonicida*. Studies with the SODs of *A. hydrophila* showed that Fe-SOD was crucial for bacterial viability, as mutation in gene coding for SodB was lethal (Leclere *et al.* 2004). The susceptibility of Mn-SOD mutant to hydrogen peroxide was similar to that of the WT bacterium, indicating that this SOD was not involved in protection against intracellular superoxide. However, the survival of the Mn-SOD mutant was reduced compared to WT *A. hydrophila* when exposed to hypoxanthine/xanthine oxidase, indicating the role of this enzyme against external superoxide (Leclere *et al.* 2004).

Type III secretion system in *Aeromonas*

Recent studies of Burr *et al.* (2002) noted that, in *A. salmonicida*, AexT, a homolog of exoenzyme S from

Pseudomonas aeruginosa, was a crucial virulence factor in fish. This toxin led to cytotoxic effects on gonad cells of rainbow trout. Since ExoS is secreted by a type III secretion system (T3SS), and that secretion of AexT occurred only after contact with fish cells, led to the discovery of TTSS in *Aeromonas*. A total of 19 open-reading frames were identified in *A. salmonicida* that coded for the T3SS and exhibited homology with T3SS of the *Yersinia* species (Burr *et al.* 2003). Mutation in the *ascV* gene of *A. salmonicida*, a homolog of the *yscV* gene in yersiniae, and a highly conserved inner membrane protein found in every known T3SS, resulted in no toxic effect on gonad cells. Likewise, mutation in the *aopB* and *acrV* genes of *A. salmonicida*, which are homologs of *yopB* and *lcrV* of yersiniae, prevented translocation of AexT in the host cells and hence no cytotoxicity to these cells (Burr *et al.* 2003). In another fish isolate of *A. hydrophila*, 25 open-reading frames coding for T3SS were identified (Yu *et al.* 2004). Mutation in the *aopB* and *aopD* (*yopD* homolog in yersiniae) led to decreased cytotoxicity in carp epithelial cells. Such mutants also prevented mortality in fish, possibly due to increase in phagocytosis (Yu *et al.* 2004).

Simultaneously, our laboratory identified T3SS in a clinical isolate SSU of *A. hydrophila* SSU (Sha *et al.* 2005). Although the T3SS was plasmid-encoded in *A. salmonicida*, it was present on the chromosome in *A. hydrophila* (Sha *et al.* 2005). The T3SS of *A. hydrophila* SSU contained 35 genes, and the deletion of *act/aopB* genes led to a mutant that had minimal lethality in a mouse model (Sha *et al.* 2005). We provided evidence that alteration in the expression of 209 macrophage and 32 epithelial cell genes was reduced in a cell culture system based on GeneChip analysis when the *act/aopB* mutant was used, compared to when cells were infected with the WT bacterium (Fadl *et al.* 2006). We obtained similar data when mice were infected with such a mutant and their spleens subjected to GeneChip analysis (Fadl *et al.* 2007). Finally, we provided evidence that the T3SS is important not only for bacterial virulence in a mouse model but also for their colonization in medical leeches (Silver *et al.* 2007). Our laboratory recently characterized further the T3SS and identified a new effector AexU from a diarrheal isolate SSU of *A. hydrophila* (Sha *et al.* 2007; Sierra *et al.* 2007).

Using specific probes to *ascF* and *ascG* genes of T3SS, 84 clinical isolates of *Aeromonas* were examined for the presence of T3SS. It was noted that 50% of the isolates possessed the hybridizing sequences, with a higher prevalence in *A. hydrophila* and *A. veronii* than in *A. caviae* (Chacon *et al.* 2004).

Recently, we characterized the new type 6 secretion system (T6SS) from isolate SSU of *A. hydrophila* and demonstrated its role in bacterial virulence (Suarez *et al.* 2008). Likewise, a new homolysin (Erova *et al.* 2007) and VacB (virulence associated protein) (Erova *et al.* 2008) were identified in *A. hydrophila* SSU that contributed to bacterial virulence. VacB also plays an important role in bacterial growth at 4°C. Finally, we have sequenced the genome of an environmental isolate ATCC7966 of *A. hydrophila* which has provided new information on its virulence and insights as to its ability to flourish in both aquatic and host environments (Seshadri *et al.* 2006). Finally, the clinical relevance of *Aeromonas* in human diseases was recently reviewed by Figueras (2005).

In conclusion, *Aeromonas* spp. produce many virulence factors, some of which have been characterized in detail for their contribution to causing disease in the host. Identification of new virulence factors from *Aeromonas* indicates complex mechanism(s) by which this organism causes disease in the host. Further studies are needed to define the set of virulence factors that could differentiate pathogenic from non-pathogenic strains of *Aeromonas*. Our initiative in collaboration with the American Water Works Association Research Foundation and the EPA are aimed specifically at correlating *Aeromonas* virulence factors that should be targeted in isolates from drinking water.

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