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

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,
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 (Pavlod 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 1995; 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. (1996a) 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. (1996b) 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 O54.

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-capule 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. (1995) 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 pilis differed from Bfp pilus 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 pilus 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 MgT, an Mg²⁺ transport protein, in A. hydrophila adherence and biofilm formation (Merino et al. 2004a,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,F) have been characterized from a polar flagellin locus of A. caviae (Rabaan et al. 2001).
Aeromonas to persistent or dysenteric presentation seen during also been linked to invasive ability and could be related persistent infections. Further, swarming motility has capacity to form biofilms (involved in lateral flagellar synthesis were impaired in their cultures with mutations in various genes 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 flaI, as well as the tandem 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—cytotoxic and cytotoxic (Keusch & Donta
mucosa was studied for the presence of aeromonads. Aeromonads were isolated from 125 diarrheal children 
were positive for aeromonads, 28 (22%) were infected with Aeromonas spp. from 1 to 27 months of age and suffering from diarrhea 
In our 27-month prospective study at Mercy Hospital, Chicago, we demonstrated 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 Diarrhoeal 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
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. 1985; 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* was used 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 hepatobiiliary 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 4–6h. 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 $5 \times 10^5$ 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. 1989a). The mature form of Act is 49 kDa in size (Rose et al. 1989a,b) and has various biological activities associated with it, including those that are hemolytic, cytotoxic, enterotoxic and lethality in mice (Rose et al. 1989b). 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 similar characteristics 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$_{555}$ 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 14C-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 His107,132, were crucial for aerolysin’s hemolytic activity, whereas the analogous His130,155 did not affect the hemolytic activity of Act. Likewise, Trp371 had no effect on the hemolytic titers of aerolysin; however, an analogous Trp594 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 glycosylphosphatidylinositol (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 (LD50) of these mutants in mice was 1 × 108 colony
forming units (cfu) when injected i.p., compared to 3 x 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 HGI/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. PGE2) production, which was coupled to cyclooxygenase (COX)-2 induction (Chopra et al. 2000), as specific COX-2 inhibitors NS398 and Celebrex abrogated Act-induced PGE2 production. Since AA is a substrate for PGE2 production, we showed that a newly discovered PLA2 (group V sPLA2) was involved in Act-induced PGE2 production. Further, Act evoked cAMP production in macrophages (Chopra et al. 2000). We noted that both Celebrex and sPLA2 inhibitor, which dramatically reduced Act-induced PGE2 production, also significantly decreased cAMP levels. Rat ligated intestinal loops injected with 200 ng of Act contained 4- to 5-fold-elevated PGE2 levels in the fluid. We have found that Act also induces COX-2 and initiates PGE2 production in T84 cells.

Studies conducted by Fujii et al. (2005) 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 (A2b) 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 Ca2+. 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 immunorelated, 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. 2005, 2004a). 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 Ca²⁺ 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. 1995), 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 ATTATTTT (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 fbB 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 85–87% 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 virulence of *A. hydrophila* (Sha et al. 2004).

More recently, we characterized the DNA adenine methyltransferase (dam) gene from *A. hydrophila* SSU (Erova et al. 2006a,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. 2006a).

**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. PGE₂) 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 PGE₂ 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 PGE₂ 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 PGE₂ production; however, a protein kinase A (PKA) inhibitor (H89) resulted in an approximately 77% reduction of Act-induced PGE₂ 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 PGE₂ 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 PGE₂ 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-NH₂-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).

### Cytotoxic Enterotoxins of Aeromonas

Since the first report of a cytotoxic 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. 1982a,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 cytotoxic 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 cytotoxic 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 carboxyterminus 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 CHO cells (Chopra et al. 1994). Our recent expression of the alt gene in E. coli 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\(\text{M}_3\) ganglioside based on ELISA.

We identified another cytotoxic 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 \( \text{NH}_2 \)-terminal end of Ast. We also showed that active immunization of mice with purified Ast (5 \( \mu \)g) reduced the fluid secretory response to WT \( A. \ hydrophila \) SSU by 30\%. \textit{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 \textit{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 \textit{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. \textit{Aeromonas} spp. produce either one of the siderophores, enterobactin or amonabactin (Byers \textit{et al.} 1991; Pemberton \textit{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 nonsiderophore 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 \textit{et al.} 1997). The ferric siderophore receptor gene (\( fstA \)) of \( A. \ salmonicida \) exhibited significant homology with \( fstA \) genes of \textit{Vibrio anguillarum}, \textit{Yersinia enterocolitica}, \textit{Pseudomonas aeruginosa} and \textit{Bordetella bronchiseptica}, indicating that homologs of this protein are widespread in Gram-negative bacterial pathogens (Pemberton \textit{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 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 enterotoxicogenic 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. 2004b). Our recent GeneChip data also indicated up-regulation of several genes in macrophages by Act that could be involved in apoptosis (Galindo et al. 2005, 2004a).

Recent studies mapped the opsonophagocytosis resistance to a *ftsE* and X genes in *A. hydrophila* (Merino et al. 2000a,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. 2005). 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. 2005). 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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REFERENCES


In: 4th International Symposium on Aeromonas and Plesiomonas. Atlanta, GA.


Erova, T. E., Fadl, A. A., Sha, J., Khajanchi, B. K., Pillai, L. L., Kozlova, E. V. & Chopra, A. K. 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. 74, 5763–5772.


Merino, S., Nogueras, M. M., Aguilar, A., Rubires, X., Alberti, S., Benedi, V. J. & Tomas, J. M. 1998 Activation of the complement classical pathway (C1q binding) by mesophilic...


