CHARACTERIZATION OF A TYPE III SECRETION SYSTEM AND OTHER VIRULENCE-ASSOCIATED GENES IN *AEROMONAS HYDROPHILA*

BY

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LIST OF ABBREVIATIONS

aa	amino acid
Amp^r	ampicillin-resistant
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pairs
BSA	bovine serum albumin
CFU	colony forming units
Cm	centimeter(s)
Cm^r	chloramphenicol-resistant
Col^r	colistin-resistant
ºC	degree Celsius
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
ECP	extracellular protein
EDTA	ethelyne diamine tetra acetic acid
EPC	epitelioma papillosum of carp, *Cyprinus carpio*
FBS	fetal bovine serum
g	gravitational force
HBSS	Hank's balanced salts solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	isopropyl-thiogalactoside
kb	kilobase
Kan^r	kanamycin-resistant
l	litre(s)
LB	Luria-Bertani broth
LBA	Luria-Bertani agar
M	molarity, moles/dm^3
MEM	minimal essential medium
mg	milligram(s)
min	Minute

ml	milliliter(s)
mM	milli moles/dm^3
MOI	multiplicity of infection
NBT	Nitro blue tetrazolium
orf	open reading frame
OD	optical density
ONPG	o-Nitrophenyl-beta-galactopyranoside
%	Percentage
PAGE	Poly acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PPD	Primary Prtoduction Department
ppm	parts per million
PVDF	Polyvinylidene difluoride
s	second
SDS	sodium dodocyl sulfate
Tc^r	tetracycline-resistant
TE	Tris-EDTA
TSA	tryptic soy agar
TSB	tryptic soy broth
U	unit(s)
µg	microgram(s)
µl	microlitre(s)
wt	wild type
v/v	volume per volume
w/v	weight per volume
X-gal	5- bromo-4-chloro-3-indolyl-B-D-galactopyranoside

SUMMARY

Aeromonas hydrophila, a normal inhabitant of the aquatic environment, is an opportunistic pathogen of a variety of aquatic and terrestrial animals. The pathogenesis of *A. hydrophila* is multifactorial in nature. In this study, a complete TTSS gene cluster was identified in *A. hydrophila* AH-1 by using a forward genetic approach, followed by a series of genome walking and cosmid sequencing. The genetic organization of this gene cluster was similar to those of *A. salmonicida*, *Pseudomonas aeruginosa*, and *Yersinia* species. It was present in all the 33 strains examined, irrespective of their pathogenic or non-pathogenic nature. This TTSS is located on the chromosome of *A. hydrophila*. It is required for the virulence of *A. hydrophila*. Inactivation of *aopB* or *aopD* led to decreased cytotoxicity in carp epithelial cells, increased phagocytosis and reduced virulence in blue gourami fish. Several type III secreted proteins were identified and shown to be secreted into the supernatant via this TTSS. These include type III structural proteins (AopB, AopD and AcrV), effector proteins (AopE and AopH) and a few unidentified putative effector proteins. Transfection of AopE or AopH into HeLa cells induced cell rounding, suggesting that they were cytotoxic to HeLa cells. The N-terminus of AopE was sufficient for its cytotoxicity, and mutation of the arginine residue within the arginine finger of AopE was sufficient to abolish the cytotoxicity. However, the C-terminus of AopE did not appear to be required for the cytotoxicity of AopE.

Many other virulence-associated factors were also studied in a comparative manner. These include known *A. hydrophila* virulence genes (hemolysin and aerolysin) as well as other genes showing homologies to known virulence factors, such as *bvgA*, *bvgS*, *vsdC* and *ompAI*, which have not yet been examined in *A. hydrophila* pathogenesis. Mutants were

constructed for these genes and tested for their virulence in a blue gourami fish model. The LD_{50}s of all the mutants except *ΔascN* were comparable to that of the wild type strains in blue gourami fish, indicating that disruption of more than one gene or a whole gene cluster (as in the case of TTSS) is required to increase the LD_{50}s. By comparing the virulence of a triple mutant (Δ*ahsA*Δ*serA*Δ*mepA*) and two double mutants (Δ*ahsA*Δ*mepA* and Δ*ahsA*Δ*serA*), we further demonstrated that, as is increasingly observed for other pathogens, virulence in *A. hydrophila is* complex and involves multiple virulence factors which may work in concert.

In addition, a proteomic approach and a *lacZ* transcriptional fusion study were used to characterize the major extracellular factors of *A. hydrophila* AH-1. An extracellular proteome map of *A. hydrophila* AH-1 was established and used as a reference map to compare with the extracellular proteomes of proteases, flagellar regulators and TTSS negative regulator mutants. Results suggest that a serine protease was involved in the processing of secreted enzymes such as hemolysin, GCAT and metalloprotease. We also show that temperature and other flagellar regulatory proteins (FlhA, LafK and RpoN) control the expression of polar and lateral flagellins. Mutations of *flhA* and *lafK* abolished the expression of polar and lateral flagellins, respectively. Although RpoN may play a global regulatory role in the expression of a variety of genes, the mutation of *rpoN* abolished the expression of polar and lateral flagellins but did not appear to affect the secretion of other proteins in the extracellular proteome. Of note, the TTSS appeared to have a cross-talk with the lateral flagellar secretion system via a TTSS central regulator (ExsA) as the deletion of *exsA* in a *ΔaopN* mutant background can restore the secretion of lateral flagellins. However, the TTSS did not have a cross-talk with the polar flagellar

secretion system, as the transcription levels of polar flagellins in a *ΔexsD* mutant were comparable to those in the wild type.

In conclusion, the present study attempts to characterize a TTSS and other virulence-associated factors which are involved in the process of *A. hydrophila* infection. Our results will provide great insights into the understanding of *A. hydrophila* pathogenesis and will help in developing suitable strategies to overcome diseases caused by this bacterium.

Chapter I. Introduction

I.1 Taxonomy and identification of *A. hydrophila*

I.1.1 Taxonomy

Aeromonas hydrophila, a normal inhabitant of the aquatic environment, is an opportunistic pathogen of a variety of aquatic and terrestrial animals (Thune *et al.*, 1993; Austin and Adams, 1996). It has been over 100 years since the genus *Aeromonas* was discovered. The phylogenetic classification of the genus *Aeromonas* has been a controversy for over 20 years. Members of the genus *Aeromonas* were assigned to the family Vibrionaceae, *Vibrio* and *Plessiomonas* on the basis of their biochemical characteristics (Janda and Abbott, 1998). Eventually, Colwell *et al*. (1986) proposed to include the genus *Aeromonas* in the new family Aeromonodaceae based on 16S rRNA cataloguing, 5S rRNA sequencing and RNA-DNA hybridization data (MacDonell and Colwell, 1985; MacDonell *et al*., 1986). Two other research groups also supported this proposal using phylogenetic studies based on small-subunit 16S rRNA or rDNA sequencing (Martinez-Murcia *et al*., 1992; Ruimy *et al*., 1994).

Another controversy concerns the species classification of the genus *Aeromonas*. *Aeromonas* had been divided into two species: *A. hydrophila* for motile strains and *A. salmonicida* for nonmotile strains (Janda, 2001). Unlike *A. salmonicida* species which is homogeneous at the DNA level, the *A. hydrophila* species is genetically heterogeneous and composed of many distinct taxa (Popoff and Veron, 1976; Popoff *et al*., 1981). The *A. hydrophila* species was further divided into three phenospecies: *A. hydrophila*, *A. sobria* and *A. caviae* based on biochemical characteristics (Popoff *et al*., 1981). With a number of new *Aeromonas* species having been proposed since 1987, the genus *Aeromonas* was

reclassified into 14 genomospecies: *A. hydrophila*, *A. bestiatum*, *A. popoffii*, *A. salmonicida*, *A. caviae*, *A. media*, *A. eucrenophila*, *A. sobria*, *A. jandaei*, *A. veronii*, *A. schubertii*, *A. trota*, *A. encheleia* and *A. allosaccharophila* (Janda, 2001). Thus, the extreme complexity of the classification of the genus *Aeromonas* makes the designation of *Aeromonas* strains very difficult. As a result, the assignment of appropriate species names to the genus *Aeromonas* will continue to be a great challenge.

I.1.2 Identification

Aeromonas are oxidase-positive, facultatively anerobic and Gram-negative bacilli (Millership, 1996). The accurate identification of *Aeromonas* remains very difficult, since a large number of *Aeromonas* strains or species are present and the exhibition of unusual and atypical biochemical reactions by some newly identified strains further complicates this issue (Janda and Abbott, 1998; Abbott *et al.*, 2003).

Identification of motile *Aeromonas* to the phenospecies level, such as *A. hydrophila*, *A. caviae* and *A. veronii* ("*sobria*") complexes, would result in a misidentification rate of <15% and have little impact on the treatment or diagnosis (Janda and Abbott, 1998). However, for a better characterization of *Aeromonas* strains at the molecular level, it is important to identify them to the genomospecies level (Janda, 2001).

A series of biochemical tests are frequently used to identify *Aeromonas* species found in clinical samples (Janda, 2001) (Table I.1). Many other methods have also been used as taxonomic tools for the identification of *Aeromonas* species. Multiple enzymes electrophoresis has been used to distinguish *A. hydrophila*, *A. caviae* and *A. sobria* (Picard and Goullet, 1985). As a common method, polymerase chain reaction (PCR) is used to identify *Aeromonas* genomospecies (Cascon *et al.*, 1996; Khan and Cerniglia, 1997). In

Table I.1 Biochemical features of *Aeromonas* species recovered from clinical samples

Test	*A. hydrophila*	*A. salmonicida*[a]	*A. caviae*	*A. media*	*A. veronii*		*A. jandaei*	*A. schubertii*	*A. trota*
					bv *veronii*	bv *sobria*			
Indole	+	+	+	+	+	+	+	V	+
LDC	+	V	-	-	+	+	+	V	+
ODC	-	-	-	-	+	-	-	-	-
ADH	+	V	+	+	-	+	+	+	+
VP	+	V	-	-	+	+	+	V	-
Aesculin	+	+	+	+	+	-	-	-	-
Hemolysis (β, BAP)	+	V	-[b]	V	+	+	+	V	V
L-arabinose	V	+	+	+	-	V	-	-	-
Sucrose	+	+	+	+	+	+	-	+	+
D-Mannitol	+	+	+	+	+	+	+	-	V
D-Sorbitol	-	V	-	-	-	-	-	-	-
Gas from glucose	+	V	-	-	V	+	+	-	V

[a]Human isolates of *A. salmonicida* are motile, indole-positive, and do not produce melanin-like compounds.
[b]Recently, most strains of *A. caviae* are β-hemolytic.
ADH, arginine dihydrolase; BAP, blood agar plate; LDC, lysine decarboxylase; ODC, ornithine decarboxylase; VP, Voges Proskauer.
bv: biovar; V: variable.

addition, restriction fragment length polymorphism is used to identify *Aeromonas* clinical isolates (Borrell *et al.*, 1997). More recently, macrorestriction analysis including pulsed-field gel electrophoresis (PFGE) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to type *Aeromonas* isolates (Abdullah *et al*., 2003). A matrix-assisted laser desorption/ionization mass spectrometry-based method was also developed for the protein fingerprinting and identification of *Aeromonas* species by using whole cells (Donohue *et al.*, 2005).

Although a large number of novel methods have been developed to identify *Aeromonas* to species level, their applicability to identifying other *Aeromonas* species remains unclear. The identification of *Aeromonas* to species level will continue to be a challenging issue.

I.2 *A. hydrophila* and its infection

Mesophilic *Aeromonas* spp. is a complex and ubiquitous waterborne bacterial group. The widespread presence of *Aeromonas* spp. in aquatic environments enables this bacterium to frequently come into contact with animals, such as fish, frogs and humans. It has been isolated from moribund fish, food, environment and clinical samples and is considered to be an important pathogen of fish, reptiles and humans.

I.2.1 *A. hydrophila* infections in fish

A. hydrophila and other motile aeromonads are the most common bacteria present in freshwater habitats, causing diseases in fish throughout the world (Thune *et al*., 1993). Most cultured and feral fish are susceptible to *A. hydrophila* infection, such as brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), Chinook salmon (*Oncorhynchus tshawytscha*), carp (*Cyprinus carpio*), gizzard shad (*Dorosoma cepedianum*), goldfish

(*Carassius auratus*), golden shiner (*Natemigonus crysoleucas*), snakehead (*Ophicephalus striatus*) and tilapia (*Tilapia nilotica*) (Bullock *et al.*, 1971; Egusa, 1978; Aoki, 1999).

Motile aeromonads are also present as a part of the normal intestinal microflora of healthy fish. Under stress conditions including overcrowding, high temperature, transfer of fish, physical injuries and poor nutrition, motile aeromonads can lead to the outbreak of fish disease (Trust *et al.*, 1974; Aoki, 1999). For example, Miller and Chapman (1976) reported that more than 37,000 fish died of motile aeromonad infection in a North Carolina reservoir.

Motile aeromonads cause both acute and chronic infections. Many pathologic conditions may be developed due to the infection of motile aeromonads, such as dermal ulceration, tail or fin rot, ocular ulceration and hemorrhagic septicemia (Thune *et al*., 1993). However, motile aeromonad septicemia (MAS) is the major cause of fish death (Austin and Austin, 1987). An acute motile aeromonad infection also causes exophthalmia, reddening of the skin and accumulation of fluid in the scale pockets (Faktorovich, 1969). Distended abdomens and protruding eyes may also be observed in *Aeromonas* infected fish (Ogara *et al.*, 1998). For chronic motile aeromonad infections, the dermis, epidermis and the underlying musculature can be severely injured (Huizinga *et al.*, 1979). For systematic motile aeromonad infections, the internal organs, such as livers, kidneys and spleens, are affected by acute septicemia (Bach *et al.*, 1978; Huizinga *et al.*, 1979).

I.2.2 *A. hydrophila* infections in humans

Five *Aeromonas* species have been established as human pathogens: *A. hydrophila*, *A. caviae*, *A. veronii*, *A. jandaei* and *A. schubertii* (Janda and Abbott, 1998). The major clinical syndromes attributed to *Aeromonas* infections are: gastroenteritis, wound

infections and systemic illnesses (Janda, 2001). Although a variety of serogroups of aeromonads can cause diseases in humans, serogroups O:11, O:34 and O:16 predominate clinically (Janda *et al.*, 1996).

Several outbreaks of gastroenteritis caused by *Aeromonas* infection have been reported. For example, an outbreak of diarrhea in 115 persons was attributed to the consumption of pork contaminated with *A. hydrophila* in China (Zeng *et al*., 1988). Krovacek *et al*. (1995) reported an outbreak of food poisoning caused by *A. hydrophila*. Recently, an outbreak of acute diarrhea due to *A. sobria* infection was also reported by Taher *et al*. (2000). Wound infections due to motile aeromonad infection are also quite common (Janda and Abbott, 1998). Burns, musculoskeletal or soft tissue traumas have been reported to be accompanied by aeromonad infections (Purdue and Hunt, 1988; Voss *et al*., 1992; Kohashi *et al*., 1995). Another severe symptom of aeromonad infection is motile aeromonad bacteremia. The motile aeromonad bacteremia is often present in immunocompromised adults, children less than two years old, and persons with severe wound infections (Janda and Abbott, 1996). Other than the major three symptoms discussed above, aeromonad infection also causes pneumonia, meningitis, myonecrosis, peritonitis and osteomyelitis in humans (Baltz, 1979; Reines and Cook, 1981; Siddiqui *et al*., 1992; Parras *et al*., 1993; Munoz *et al*., 1994).

A. hydrophila has become increasingly significant as a public health threat due to its ubiquitous presence in nature. Therefore, it is necessary to take much care when preparing food, and sterilizing drinking water in order to lower the incidence of aeromonad infection.

I.2.3 *A. hydrophila* infections in other animals

The genus *Aeromonas* was first discovered in the abdominal and peritoneal cavities of frogs (Sanarelli, 1891). *A. hydrophila* infections caused the "red-leg" disease in frogs. An increase in water temperature could even lead to an outbreak of aeromonad septicemia in frogs (Huizinga *et al.*, 1979). The *Aeromonas* infected frogs could develop several symptoms, such as sluggishness, decreased muscle tone, hemorrhages on the ventral surface of the bodies, edema of the abdomen and thighs, and hemorrhages within the eyeballs (Benirschke *et al*., 1978). It was also reported that *A. hydrophila* caused severe diseases in a group of *Xenopus laevis* within three weeks of injection (Hubbard, 1981). The primary clinical signs were marked pallor of the skin, petechiation, lethargy, anorexia, and edema in these *X. laevis*.

A. hydrophila can also cause diseases in mammals, such as dogs, horses, guinea-pigs, mice and rabbits (Gosling, 1996). Different symptoms may develop within different infection hosts. For instance, *A. hydrophila* caused pneumonia and dermatitis in dolphins, but caused septicemia in dogs (Cusick and Bullock, 1973; Andre-Fontaine *et al*., 1995).

A. hydrophila also causes diseases in cold-blooded animals and birds (Glunder and Siegman, 1989; Gosling, 1996). Birds from aquatic habitats harbor *A. hydrophila* more frequently than those from terrestrial habitats (Glunder and Siegman, 1989). Environmental stress or injury may result in *A. hydrophila* infection in birds (Shane *et al*., 1984). *A. hydrophila* causes hemorrhages, pneumonia, septicemia, and ulcers in snakes and other animals (Marcus, 1981; Gosling, 1996).

I.3 Virulence factors of *A. hydrophila*

The pathogenesis of *A. hydrophila* is multifactorial in nature. Many virulence determinants are involved sequentially for the bacterium to colonize, gain entry to, establish, replicate and cause damage in host tissues, evade the host defense systems, spread, and eventually kill the hosts (Smith, 1995). In *A. hydrophila*, a few virulence factors, such as S-layers, flagella, pili, capsules and fimbriae have been identified and characterized. However, the mechanisms of action by most of these virulence factors remain unknown. Section I.3 is a summary of literature on the pathogenicity of *A. hydrophila*.

I.3.1 *A. hydrophila* structure related virulence factors

I.3.1.1 S-layers

The two-dimensional paracrystalline surface protein or S-layer is currently regarded as an important virulence factor in aeromonads. As the outermost surface of the bacteria, the S-layers of *Aeromonas* spp. contact directly with host cells and immune defense factors. The S-layers of *Aeromonas* species may protect the bacteria against the host's innate and adaptive immune responses (Munn *et al.*, 1982). The S-layers may also protect the bacteria against proteases within phagolysomes (Kostrzynska *et al.*, 1992). The S-layer of *A. salmonicida* also allows the bacteria to bind to collagen type IV, laminin and fibonectin (Doig *et al.*, 1992; Trust *et al.*, 1993). The binding of the S-layer to host components can allow the bacteria to reside in the host persistently and to evade the host's immune response, thus contributing to the disease process.

There are significant differences between the role of the S-layer of *A. salmonicida* (*vapA*) and that of *A. hydrophila* (*ahsA*) in the pathogenesis of these bacteria (Noonan and Trust,

1997). Ishiguro *et al*. (1981) showed that loss of the S-layer at high temperature resulted in loss of virulence. Similar results were also observed with an isogenic *A. salmonicida apsE*::Tn5 mutant (Noonan and Trust, 1995). In contrast to the S-layer of *A. salmonicida*, the *A. hydrophila* S-layer may play a lesser role in virulence. Spontaneous mutants of *A. hydrophila* lacking an S-layer do not exhibit decreased virulence in animal models (Kokka *et al*., 1991, 1992).

I.3.1.2 Flagella

Aeromonas are usually motile by means of a polar, unsheathed, and monotrichous flagellum that is responsible for the swimming motility in liquid media (Thornley *et al*., 1996; Merino *et al*., 1997). The flagellum is a complex membrane-associated structure, consisting of the basal body, hook and filament (Macnab, 1996). Removal of the polar flagellum by shearing or agglutination by antiflagellin antibodies significantly reduces bacterial adhesion to Hep-2 cells, indicating that polar flagellum is required for the aeromonad colonization process (Merino *et al*., 1997). The polar flagellin locus of *A. caviae* shared the highest homology and a similar genetic organization with the *flaA* and *flaB* of *A. salmonicida* and *V. parahaemolyticus* (McCarter, 1995; Umelo and Trust, 1997; Kim and McCarter, 2000; Rabaan *et al.*, 2001).

Some *Aeromonas* strains also produce unsheathed lateral flagella when cultured on solid surfaces and 50% to 60% of mesophilic aeromonads associated with diarrhea express lateral flagella (Shimada *et al.*, 1985; Gavin *et al*., 2002). The lateral flagella, like polar flagella, also exhibit similar organization to that of *V. parahaemolyticus* although the number of flagellins is different (McCarter and Wright, 1993; McCarter, 1995). Polar flagellum is required for swimming motility, while lateral flagellum is required for

swarming motility. But both flagella are related to bacterial pathogenesis, such as adherence and invasion (Thornley *et al.*, 1996; Gryllos *et al.*, 2001; Rabaan *et al.*, 2001). In addition, they are also required for biofilm formation which is a common cause of persistent infections (Costerton *et al*., 1999; Gavin *et al*., 2002). Interestingly, another complete flagellar locus of *A. hydrophila* containing 16 genes was recently identified (Altarriba *et al*., 2003). This locus also showed high similarity to region 1 of the *V. parahaemolyticus* polar flagellum, except that no flagellin genes were found on *A. hydrophila* while *V. parahaemolyticus* showed three flagellin genes. Interestingly, FlgN within this locus is required for lateral flagella formation and swarming motility but not for polar flagellum-mediated swimming motility (Altarriba *et al*., 2003).

Both polar and lateral flagella exhibited a higher molecular weight on SDS-PAGE than that predicted based on nucleotide sequence, which may be attributed to post-translational glycosylation of flagellins (Rabaan *et al*., 2001). Recently, an *flm* operon was also reported to be widely distributed in mesophilic aeromonads and involved in flagellum assembly, possibly through glycosylation of the flagellin or other flagella components (Gryllos *et al.*, 2001).

I.3.1.3 Capsules

Capsules are present in some common *A. hydrophila* serogroups when cultured under glucose-rich conditions (Martinez *et al*., 1995; Zhang *et al*., 2002). Capsules are extracellular polysaccharides that enclose the bacteria. In *A. hydrophila*, they protect bacteria from complement-mediated serum killing by inactivating the C3b deposit (Merino *et al*., 1997; Zhang *et al*., 2002). The purified capsular polysaccharides can also increase the ability of avirulent strain PPD35/85 to survive in naive tilapia serum but have

no inhibitory effect on the adhesion of PPD134/91 to carp epithelial cells (Zhang *et al*., 2003). In addition, the capsules are also required for adherence and invasion of fish cell lines (Merino *et al*., 1996 and 1997). The genetic organization and sequences of capsule biosynthesis were recently determined in *A. hydrophila* PPD134/91 (Zhang *et al*., 2002). The capsule cluster, composed of 13 open reading frames, can be divided into three regions. Zhang *et al*. (2003) also showed that the presence of group II capsules in *A. hydrophila* strongly correlates with the serum and phagocyte survival abilities.

I.3.1.4 Pili

Type IV pilin is the most extensively characterized pilus in *Aeromonas* strains (Hokama and Iwanaga, 1991; Kirov and Sanderson, 1996; Nakasone *et al*., 1996). *Aeromonas* spp. possesses at least two distinct type IV pilus families (Barnett *et al*., 1997). Kirov *et al.* (1996) purified and characterized a long, flexible pilus from a gastroenteritis-associated strain of *A. veronii* biovar *sobria*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of pili preparations yielded a protein whose N-terminal amino acid sequence is homologous to those of type IV pili (Kirov *et al*., 1996). These pili can form rope-like pilus designated as "Bundle-forming pili" (BFP) and both single pilus and rope-like bundles of pili can be observed on the bacterial surface by immunogold electron microscopy with antibodies against pilin proteins (Barnett *et al*., 1997; Kirov *et al*., 1999). Bfp are intestinal adhesions and play an important role in the initial adhesion of *Aeromonas* bacteria to intestinal cells (Hokama *et al*., 1990; Kirov *et al*., 1999).

Another type IV pilus biogenesis gene cluster (TAP) was also cloned from *A. hydrophila* Ah65 (Pepe *et al.*, 1996). This cluster of genes (*tapABCD*) is homologous to

Pseudomonas aeruginosa type IV pilus biogenesis genes (*pilABCD*). TapB and TapC are functionally homologous to *P. aeruginosa* PilB and PilC, respectively. TapD is required for extracellular secretion of aerolysin, suggesting that TAP pili may play an important role in the pathogenicity of *A. hydrophila* (Pepe *et al*., 1996). However, this possibility is controversial. Kirov *et* al. (2000) showed that an insertional inactivation of *tapA* had no effect on exotoxic activities and the bacterial adherence to Hep-2 cells. There was no significant effect on the duration of colonization or incidence of diarrhea when the *A. veronii* bv. *sobria* strain was tested in the removable intestinal tie adult rabbit diarrhea model. There was also no significant effect on its ability to colonize infant mice, suggesting that TAP pili may not be as significant as Bfp pili for *Aeromonas* intestinal colonization (Kirov *et al*., 2000).

I.3.2 *A. hydrophila* extracellular enzymes and toxins

Aeromonas strains are able to produce a large number of toxins and extracellular enzymes associated with pathogenicity or the disease process.

I.3.2.1 Hemolysins and enterotoxins

Aeromonas strains are able to secrete two families of β-hemolysins: aerolysin and related β-hemolysins. The structural gene of aerolysin, *aerA*, was first cloned and sequenced from *A. hydrophila* in 1987 (Howard *et al*., 1987). Since then, different aerolysins have been cloned and sequenced from a variety of *Aeromonas* species, such as *A. hydrophila* and *A. veronii* bv. *sobria* (Husslein *et al*., 1988; Hirono *et al*., 1992; Chopra *et al*., 1993). These aerolysins show heterogeneity in size (from 49 to 65 kDa), DNA sequence, amino acid sequence and cytolytic activities (Janda, 2001). However, they show similar genetic organization and basic proteins structure (Janda, 2001). AHH1, a second family of β-

hemolysins, was first cloned and sequenced from *A. hydrophila* ATCC7966 (Hirono and Aoki, 1997). The AHH1 hemolysins showed 45~51% homology to the *Vibrio cholerae* HlyA hemolysin at the amino acid level (Wong *et al.*, 1998).

Both aerolysins and related β-hemolysins are cytotoxic for many eukaryotic cell lines, such as Hep-2, HeLa, Chinese hamster ovary, Vero and erythrocytes (Asao *et al.*, 1984; Chopra *et al*., 1993; Fujii *et al*., 1998; Wong *et al*., 1998). However, whether aerolysins are major virulence factors remains controversial. Wong *et al.* (1998) reported that the inactivation of *hlyA* or *aerA* alone showed no statistically significant attenuation in a suckling mouse model when compared to the wild type *A. hydrophila*. Chakraborty *et al*. (1987) also reported an 11-fold difference in LD_{50} values between an *aerA* mutant and the wild type *A. trota* strain when injected intraperitoneally into mice. In contrast, Xu *et al*. (1998) observed a more than 300-fold difference in LD_{50}s between an aerolysin deficient mutant and the wild type *A. hydrophila* strain.

Cytotonic enterotoxins are another family of toxins involved in the *Aeromonas*-mediated infection. These toxins are biologically and genetically different from aerolysins and aerolysin-related β-hemolysins (Janda, 2001). They cause no cytopathic effect on eukaryotic cells but induce cell elongation or rounding (Janda, 2001). Two types of cytotonic enterotoxins have been reported. The first type has a cross-reaction with cholera toxin antibodies. Potomski *et al*. (1987) revealed a toxin which induced fluid accumulation in rat ileal loops and in infant mice and caused rounding of Y1 adrenal cells. Enzyme linked immunosorbent assay (ELISA) showed that CT-cross reactivity was present in 26% of *A. sobria*, 20% of *A. hydrophila* and 24% of *A. caviae* (Potomski *et al*.,

1987). The activity of this toxin can be neutralized by CT antiserum. Preincubation with anti-CT reduced the CHO cell titer of cell lysates 10-fold (Potomski *et al*., 1987).

The second type of cytotonic enterotoxin does not cross-react with cholera toxin antibodies. Ljungh *et al.* (1981a, 1981b) described a cytotonic enterotoxin activity in *A. hydrophila*. This toxin, with a molecular weight of ~15 kDa, caused steroidgenesis in Y1 adrenal cells and appeared immunologically different from CT (Ljungh *et al*., 1981a, 1981b). Consistent with these reports, a cytotonic enterotoxin unrelated to cholera toxin antibodies was also purified and characterized from an *A. sobria* strain (Gosling, 1993). This toxin did not cross-react with components of CT or the whole toxin. It was non-hemolytic to rabbit erythrocytes but caused fluid accumulation in the infant mouse assay and an increase in cAMP activity in tissue culture cells (Gosling, 1993). Two other cytotonic enterotoxins which do not cross-react with CT have also been identified from an *A. hydrophila* strain (SSU) by Chopra *et al*. (1992, 1996). Both of them cytotonic enterotoxins caused elevated intracellular cAMP and PGE2 levels in cultured CHO cells (Chopra *et al*., 1992, 1996).

I.3.2.2 Endotoxins

The lipopolysaccharide (LPS) is present in all Gram-negative bacteria and consists of three components: lipid A, core oligosaccharide and O-antigen. The lipid A components anchor LPS in the outer membrane (Reeves *et al*., 1996). The core is composed of sugars and sugar derivatives. The O-antigen is a polysaccharide extending from the cell surface and consists of repeating oligosaccharide units.

The pathogenicity of LPS from *Aeromonas* spp. depends on several factors. Lipid A, a structure conserved among Gram-negative bacteria, acts as a T-independent mitogen

producing polyclonal B-cell activation and IgM response (Morrison, 1983). As an important recognition marker, LPS can be recognized by the innate immune system (Freudenberg *et al*., 2001). The *Aeromonas* O-antigen is an important adhesion and a colonization factor. Mutants devoid of the O-antigen lipopolysaccharide show significantly lower levels of adhesion to Hep-2 cells than the smooth strains and were unable to colonize the germfree chicken gut (Merino *et al*., 1996). The expression of LPS can be affected by both temperature and osmolarity. Mesophilic *Aeromonas* strains from serogroups O:13, O:33 and O:44 cultured at different temperatures and osmolarity showed different LPS profiles and virulence *in vivo* (Merino *et al*., 1992; Merino *et al*., 1998). Strains grown at 20°C (high or low osmolarity) or at 37°C at high osmolarity carried a smooth LPS, whereas strains grown on low osmolarity carried a rough LPS (Merino *et al*., 1992). The smooth strains were resistant to the bactericidal activity of serum and showed better adhesion to Hep-2 cells than the rough strains. The smooth strains were more virulent for fish and mice than the rough strains (Merino *et al*., 1998).

I.3.2.3 Proteases

Aeromonas spp. secrete a lot of proteases which can degrade many different proteinaceous compounds such as albumin, fibrin, gelatin and native elastin molecules (Janda, 1985). Proteases contribute to pathogenicity by causing direct tissue damage, enhancing invasiveness or with the proteolytic activation of toxins (Kirov, 1997).

At least three classes of proteases have been reported in *Aeromonas* strains: a thermolabile serine protease and two thermostable metalloproteases that are EDTA-sensitive or insensitive (Rivero *et al*., 1991; Ellis, 1997). Serine proteases are inhibited by phenylmethylsulphonyl fluoride (PMSF) or diisopropyl fluorophosphates (Rivero *et al*.,

1991). Metalloproteases are typically inhibited by EDTA. Rodriguez *et al*. (1992) purified a metalloprotease from the culture supernatants of *A. hydrophila* strain. This metalloprotease (38 kDa) had no cytotoxic activity. Loewy *et al*. (1993) purified a novel zinc metalloprotease (19 kDa) which mimics the action of an isopeptidase on the gamma-chain dimers of cross-linked fibrin from *A. hydrophila*. It is inhibited by 1, 10-phenanthroline, but not by EDTA or PMSF. More recently, a novel metalloprotease AP19 was also purified from an *A. caviae* strain (Nakasone *et al*., 2004). The molecular weight of AP19 was the same as the metalloprotease reported by Loewy *et al*. (1993). A high concentration of AP19 was cytotoxic to Vero cells (Nakasone *et al*., 2004).

Proteases are potential virulence factors involved in the disease process of *Aeromonas* infection. Both serine and metalloproteases are lethal for fish at the concentration of 150 ng/g fish (Rodriguez *et al*., 1992). Recently, a gene encoding an elastolytic activity, *ahpB*, was cloned from *A. hydrophila* AG2 (Cascon *et al*., 2000). The product encoded by *ahpB* hydrolyzed casein and elastin and showed a high sequence similarity with the mature form of the *P. aeruginosa* elastase (52% identity), *Helicobacter pylori* zinc metalloprotease (61% identity), and proteases from several species of *Vibrio* (53% identity). Inactivation of *ahpB* resulted in a ~100 fold increase in LD_{50} by intraperitoneal challenge in rainbow trout, clearly suggesting this elastastic protein should be considered as a virulence factor (Cascon *et al*., 2000).

I.3.2.4 Lipases

Aeromonas spp. can secrete multiple lipases which hydrolyze esters of glycerol with preferably long-chain fatty acids (Jaeger *et al*., 1994). Anguita *et al*. (1993) purified an extracellular lipase from the culture supernatant of *A. hydrophila* H3. This lipase showed a

molecular weight of 67 kDa and contained an amino acid sequence (V-H-F-L-G-H-S-L-G-A) that is highly conserved among lipases. It exhibited both esterase and lipase activities (Anguita *et al*., 1993). Subsequently, a group of lipases with high homology has also been reported from other *A. hydrophila* strains: PLA1, LipE, Lip and Apl-1 (Ingham and Pemberton, 1995; Merino *et al*., 1999). The Apl-1 lipase exhibits non-hemolytic phospholipase C activity on lecithin and p-nitrophenylphosphorylcholine (Ingham and Pemberton, 1995). Apl-1 lipase contains a serine active lipase site (Gly-X-Ser-X-Gly) between residues 561 and 570 amino acid (aa). *Escherichia coli* strains harboring the *pla* (encoding PLA1) gene were able to degrade tributyrin but unable to exhibit any lecithinase activity on an egg yolk medium (Merino *et al.*, 1999). In the same study, Merino *et al.* (1999) also identified another phospholipase (PLC) by screening representative recombinant clones encoding *A. hydrophila* lipases on egg yolk agar plates. PLC was shown to be cytotoxic but non-hemolytic or poorly hemolytic. Inactivation of *plc* resulted in a 10-fold increase in the LD_{50}s on fish and mice, indicating that PLC is a virulence factor in the mesophilic *Aeromonas* spp. serogroup O:34 infection process (Merino *et al*., 1999).

Another well-known phospholipase is glycerophospholipid-cholesterol acyltransferase (GCAT) which has been isolated from both *A. hydrophila* and *A. salmonicida* (Thornton *et al*., 1988; Eggset *et al*., 1994). The GCAT secreted by *A. hydrophila* shares several properties in common with the mammalian enzyme lecithin-cholesterol acyltransferase (Brumlik and Buckley, 1996). Like other lipases, the GCAT contains a catalytic triad of the lipase/acyltransferase composed of Ser-16, Asp-116 and His-291. The GCAT can cause erythrocyte lysis by digesting their plasma membranes (Thornton *et al.*, 1988).

However, an *A. salmonicida* GCAT isogenic mutant revealed no major decrease in virulence in *A. salmonicida*, indicating that they may act as accessory virulence factors rather than essential virulence factors (Vipond *et al.*, 1998).

I.3.2.5 Chitinases

Chitin is a polymer of 1, 4-β-linked N-acetylglucosamine units that is abundantly present in nature. It provides carbon, nitrogen and energy for organisms capable of its degradation (Cohen and Chet, 1998). Chitin can be digested by two types of chitinases: endochitinase and exochitinase. Endochitinases cleave chitin into soluble low molecular mass multimers of N-acetylglucosamine hexasaccharide (GlcNAc) (Sahai and Manocha, 1993). Exochitinases catalyze the progressive release of di-acetylchitobiose and cleave the multimers of GlcNAc into monomers (Sahai and Manocha, 1993).

Aeromonas spp. can secrete at least three groups of extracellular chitinases: groups A, B and C (Watanabe *et al*., 1993). The ChiA of *A. caviae* shows high similarity to chitinase A of *Serratia marcescens* and belongs to group A (Sitrit *et al*., 1995). Four chitinase genes (ORFs 1-4) were clustered in *Aeromonas* spp. 10S-24 (Shiro *et al*., 1996). The amino acid sequences of ORF-1 and ORF-3 share sequence homology with chitinase D from *Bacillus circulans*, and chitinase A and B from *Streptomyces lividans*. The amino acid sequence of ORF2 showed homology with chitinase II from *Aeromonas* spp. 10S-24 and chitinase from *Saccharopolyspora erythraea*. Chitin III and the enzyme produced by ORF 3 belong to group B, while Chitin II belong to group C. Recently, the novel family 19 chitinase gene from *Aeromonas* spp. 10S-24 was cloned, sequenced, and expressed in *E. coli* (Ueda *et al.*, 2003). This enzyme contains two repeated N-terminal chitin-binding domains that are separated by two proline-threonine rich linkers.

I.3.2.6 Siderophores

Iron is essential for the survival of most organisms, including bacteria. There is little free iron available for bacteria (Wandersman and Delepelaire, 2004). Bacteria fulfill their iron needs by either direct contact between the bacterium and the exogenous iron/heme sources or by depending on molecules of siderophores and hemophores to scavenge iron or heme from various sources (Wandersman and Delepelaire, 2004). Siderophores were discovered half a century ago (Neilands, 1981). They are low-molecular-weight compounds which can chelate ferric ions with extremely high affinity and can be extracted from most mineral or organic complexes (Neilands, 1981). *Aeromonas* spp. obtain iron either from host Fe-transferrin (siderophore dependent) or from host heme-containing molecules (siderophore independent) (Byers *et al*., 1991).

Aeromonas spp. produce at least two types of siderophores: amonabactin and enterobactin (Barghouthi *et al*., 1989; Zywno *et al*., 1992). Amonabactin is predominantly synthesized by *A. hydrophila* and *A. caviae* (Barghouthi *et al*., 1989). Two forms of amonabactin are secreted by *A. hydrophila* 495A2 (Barghouthi *et al*., 1989). They consist of 2, 3-dihydroxybenzoic acid, lysine, glycine, and either tryptophan (amonabactin T) or phenylalanine (amonabactin P). Both forms are capable of stimulating growth of an amonabactin-negative mutant in an iron-deficient medium. One of the amonabactin biosynthetic genes (*amoA*) was identified from *A. hydrophila* 495A2 (Barghouthi *et al*., 1991). The product encoded by *amoA* can overcome the requirement of *E. coli* for exogenous 2, 3-DHB to support siderophore (enterobactin) synthesis. An isogenic amonabactin-negative mutant excreted neither 2, 3-DHB nor amonabactin, and was more sensitive to growth inhibition by iron restriction than the wild type. A cluster of

enterobactin biosynthesis genes was also cloned from *Aeromonas* spp. (Massad *et al*., 1994). These genes (designated *aebC*, -*E*, -*B* and –*A* for aeromonad enterobactin biosynthesis) can also complement mutations in the enterobactin genes of the *E. coli* 2, 3-DHB operon. The two types of siderophores biosynthesis clusters are functionally exchangeable with the *E. coli* 2, 3-DHB operon and thus may have diverged from an ancestral group of 2, 3-DHB genes (Massad *et al*., 1994).

There is a correlation between the genomospecies (DNA-DNA hybridization group) and the type of siderophore produced by the genus *Aeromonas* (Zywno *et al*., 1992). DNA-DNA hybridization groups 1 to 5 and group 12 predominantly produced the siderophore amonabactin, while groups 8/10 and 9 predominantly produced the siderophore enterobactin. The information about the distribution of specific siderophores in *Aeromonas* may help in the classification of the genetic species of *Aeromonas* and the evaluation of potential virulence properties.

I.4 Genomic islands and pathogenicity islands

Genomic islands (GIs) are part of a flexible bacterial gene pool with a length of 10 to 100 kilobases (kb) (Hacker and Carniel, 2001). They contain blocks of particular DNA sequences, such as transfer genes, integrases and insertion sequence (IS) elements which are derived from phages and/or plasmids. Flanking direct repeats are often found at the boundaries of GIs. They are involved in the process of integration or excision of bacterial genomes (Hacker *et al*., 1997; Buchrieser *et al*., 1998). The majorities of the clusters located within GIs are useful for the survival and transmission of the microbes and provide a selective advantage to the island-carrying organisms in a specific niche (Hacker and Carniel, 2001).

GIs carry genes encoding pathogenicity, symbiosis, antibiotics resistance or metabolic enzymes (Hacker and Carniel, 2001). Pathogenicity islands (PAIs) are a subset of GIs and they share the same general composition and organization (Hacker and Kaper, 2000). PAIs are often located on the chromosome, but they are also present as part of bacterial plasmids and phages (Hacker and Kaper, 2000).

PAI was first described in pathogenic *E. coli* (Kaper and Hacker, 1999). Many bacteria, such as *Shigella flexneri*, *Salmonella enterica*, and *Yersinia* spp., cause either intestinal or extraintestinal infections via virulence factors encoded on PAIs (Schubert *et al*., 1998; Wood *et al*., 1998; Rakin *et al*., 1999; Vokes *et al*., 1999). Extrachromosomal elements from *V. cholerae* and *P. syringae* also represent one source of PAIs (Kaper and Hacker, 1999). In general, most PAIs have most, if not all, of the characteristics described below (Hacker and Kaper, 2000):

1. Present in pathogenic organisms but absent from non-pathogenic organisms of the same or closely related species.

2. Occupy relatively large genomic regions and cover DNA regions of 10-200 kb.

3. Consist of DNA regions which differ from the average genome in G+C content.

4. Flanked by small directly repeated sequences that are possibly generated after the integration of PAI-specific DNA regions into the host genome via recombination.

5. Associated with transfer RNA (tRNA) genes which act as integration sites for foreign DNA.

6. Carry cryptic or functional genes encoding mobility elements such as integrases, transposases, and IS elements or parts of these elements.

7. Composed of mosaic-like structures which may have been acquired by a multi-step process.

8. Represent unstable DNA regions.

I.5 Type III secretion systems

I.5.1 Protein secretion systems in Gram-negative bacteria

Gram-negative bacteria can secrete a variety of proteins into extracellular media or host cells. These secreted bacterial proteins are diverse and exhibit a wide variety of functions, such as proteolysis, hemolysis, cytotoxicity, protein phosphorylation and dephosphorylation (Hueck, 1998). Five types of secretion system, types I through V, have been reported in Gram-negative bacteria. The *sec* general secretion pathway is involved in extracellular protein secretion via types II, IV, and V secretion systems. In contrast, proteins secreted via types I and III secretion systems do not require the *sec* system.

The type I secretion system was first described in the *E. coli* α-hemolysin (Salmond and Reeves, 1993). Three proteins form a channel for this secretion system: HlyB, an inner-membrane ATPase which provides energy for the system; HlyD, a membrane fusion protein which spans the periplasm (HlyD); TolC, an outer-membrane protein. The secreted proteins are not subject to proteolytic cleavage and the secretion signal is usually located within the carboxy-terminus (Hueck, 1998).

Type II secretion systems have been studied in many bacteria, such as *E. coli*, *Klebsiella oxytoca*, *V. cholerae*, *Erwinia* spp., *P. aeruginosa* and *A. hydrophila* (reviewed by Hacker and Kaper, 2000). The type II secretion system in *K. oxytoca* is the best characterized. A cluster of genes composed of at least seven cytoplasmic membrane proteins and two outer membrane proteins (PulS and PulD) are essential for the normal function of this secretion

system (Pugsley, 1993). The type II secretion system in *E. coli* consists of inner membrane proteins (SecD to F, SecY), a cytoplasmic membrane-associated ATPase (SecA), a chaperone (SecB), and a periplasmic signal peptidase (Murphy and Beckwith, 1996). The genes encoding the type II secretion apparatus are usually clustered, whereas the genes encoding proteins secreted by the apparatus are often located in a different region (Hueck, 1998). For example, the Eps type II secretion system in *V. cholerae* is located on the large chromosome, but the Hap protease secreted via this secretion system is located on the small chromosome (Trucksis *et al*., 1998).

Like the type I secretion pathway, the type III secretion system (TTSS) translocates its effector molecules across the inner and outer membranes in a *sec* independent manner (Hueck, 1998). The type III secretion apparatus, composed of about 20 proteins which are mostly located in the inner membrane, forms a needle-like structure which spans both the inner and outer membranes (Kuberi *et al*., 1998). Effector molecules are secreted without amino-terminus processing via this needle-like structure (Hueck, 1998; Kuberi *et al*., 1998). A secretin-like protein has been shown to form ring-shaped structures with large central pores that assist in the secretion of effector molecules (Gauthier *et al*., 2003). The TTSS and the bacteria flagellar secretion system share high structural and functional similarities (Blocker *et al*., 2003). Consisting of homologous component proteins with common physical and chemical properties, flagella and the TTSS may have evolved in parallel (Aizawa, 2001). The recognition and secretion of effector molecules via the TTSS may involve different mechanisms. Anderson and Schneewind (1997) proposed that a 5'mRNA signal was required for the type III secretion of Yop proteins by *Y. enterocolitica*. However, Lloyd *et al.* (2001) proposed that the N-terminus of YopE but

not the 5' end of *yopE* mRNA serves as a targeting signal for type III secretion. More interestingly, Lloyd *et al*. (2001) also demonstrated that the chaperone YerA can target YopE for type III secretion even in the absence of a functional N-terminal signal.

The type IV secretion pathway exports proteins from the cytoplasm via the *sec* pathway with cleavage of an amino-terminal signal peptide (Hueck, 1998). The type IV secretion system is the least understood Gram-negative protein secretion pathway. It was first described in *Agrobacterium tumefaciens* (Kaper and Hacker, 1999). The type IV secretion system of *A. tumefaciens* is located on a 200-kb Ti plasmid (Kaper and Hacker, 1999). T-DNA, part of the Ti plasmid is transferred into plant cells via this type IV secretion system and integrated into the host genome. Several other systems which are quite close to the type IV secretion system have also been described in many pathogenic bacteria, such as the *Bordetella pertussis* Ptl (pertussis toxin) system (Farizo *et al*., 2000), the Dot/Icm system of *Legionella pneumophila* (Zink *et al*., 2002), and the cytotoxin-associated genes cagPAI of *H. pylori* (Backert *et al*., 2002). All these type IV like secretion systems are required for the virulence of pathogens.

Type V secretion is possibly the simplest protein secretion mechanism. It can be subdivided into three groups: an autotransporter system (type Va or AT-1), a two-partner secretion pathway (type Vb), and a type Vc system (also termed AT-2) (Henderson *et al*., 2004). Type V secretion (type Va) was first described in *Neisseria gonorrhoeae* by Pohlner *et al*. (1987) who proposed that a helper serves as a pore for the secretion of the IgA1 protease domain through an outer membrane. The IgA1 protease is released by autoproteolysis and further matured into a functional IgA1 protease. The type Vb secretion pathway is a bacterial two-partner secretion (TPS) pathway (Henderson *et al*., 2004). In

the type Vb secretion pathway, the passenger domain (also called the exoprotein) possesses a signal sequence that directs translocation across the inner membrane (Henderson *et al*., 2000). The passenger domain is exported into the periplasm and inserted into an outer membrane pore formed by a ß-barrel, and possibly followed by proteolytic processing to achieve its physiological function (Jacob-Dubuisson *et al*., 2001). The passenger domain and the pore-forming ß-domain (also called the transporter domain) are translated as TpsA and TpsB family members, respectively (Henderson *et al*., 2004). The type Vc or AT-2 pathway was recently designated, based on an alternative model for an autotransporter secretion system (Henderson *et al*., 2004). In this model, a *Y. pestis* YadA protein, the prototype of a novel class of bacterial adhesions (a subfamily of surface-attached oligomeric autotransporters), forms an oligomeric lollipop-like structure and is anchored in the outer membrane by the C-terminus (Roggenkamp *et al*., 2003).

I.5.2 Type III secretion systems and pathogenicity islands

TTSSs have been closely associated with PAIs. In *enteropathogenic E. coli*, a chromosomally encoded TTSS called the locus of enterocyte effacement (LEE) is required for the attaching and effacing (A/E) phenotype (Jarvis *et al*., 1995). The equivalent locus of LEE has also been identified in *enterohemorrhagic E. coli*, and *Citrobacter rodentium* (Jarvis and Kaper, 1996; Newman *et al*., 1999). All the equivalent LEE loci are required for the virulence of the pathogens and referred to as PAIs. More interestingly, a second pathogenicity island ETT2 was found to be present among various intestinal serovars and pathovars of *E. coli* but absent among extra-intestinal, non-pathogenic *E. coli* strains and other enteric bacteria (Hartleib *et al*., 2003). Like in *E. coli*, two PAI-related TTSSs are also present in *S. enterica*. A chromosomally encoded 40 kb fragment (called SPI-1) is

absent in *E. coli* K-12 chromosome. It consists of 31 genes encoding components of the Inv/Spa TTSS that are required for invasion of non-phagocytic cells (Mills *et al*., 1995). A second chromosomally encoded virulence gene cluster is also conserved among most *Salmonella* species but is not present in a variety of other pathogenic bacteria or in *E. coli* K-12 (Shea *et al*., 1996). Unlike the TTSS encoded by the inv/spa invasion locus, this new cluster is designated the Spi/ssa TTSS (SPI-2) and is required for systemic infections and NADPH oxidase-dependent killing by macrophages (Shea *et al*., 1996; Vazquez-Torres *et al*., 2000). The SPI-2 has a lower G+C content than that of the *Salmonella* genome and may have been acquired by insertion into a region corresponding to that between the *ydhE* and *pykF* genes of *E. coli* (Shea *et al*., 1996). In *Yersinia* spp., two PAI-related TTSSs have also been reported. One is plasmid encoded (discussed in detail in section I.5.3.1), while the other is chromosomally encoded (Ysa system) (Michiels *et al*., 1990; Haller *et al*., 2000; Foultier *et al*., 2002). The *ysa* system is closely related to the Mxi-Spa TTSS of *Shigella* (Foultier *et al*., 2002).

PAIs-related TTSSs are also present in plant pathogens. In *Xanthomonas axonopodis* pv. glycines, an approximately 29 kb region composed of *hrp* (hypersensitive response and pathogenicity) and *hrc* (*hrp* and conserved) gene clusters were identified (Kim *et al*., 2003). This region encodes a TTSS and has many characteristics of PAI, such as the presence of many virulence genes, low G+C content, and tRNA genes at the boundary. *P. syringae* also contains an *hrp/hrc* gene cluster encoding a TTSS (Alfano *et al*., 2000). This *hrp/hrc* gene cluster is conserved in three *P. syringae* strains (*Psy* 61, *Psy* B728a, and *Pto* DC3000) and ecodes an Hrp PAI composed of a tripartite mosaic structure (Alfano *et al*., 2000). It is flanked by a unique exchangeable effector locus (EEL) which contains

diversified putative effectors and a conserved effector locus (CEL) which is conserved between Psy B728a and Pto DC3000 (Alfano *et al*., 2000).

I.5.3 Type III secretion systems in animal pathogens

TTSSs are present in a variety of Gram-negative bacteria (Hueck, 1998). Pathogenicity proteins are secreted via TTSSs and injected into the cytosol of eukaryotic cells, leading to the activation of a series of host signaling pathways. Here, we describe the TTSSs of three animal pathogens.

I.5.3.1 *Yersinia* species TTSS

I.5.3.1.1 Genetic organization and regulation

Three *Yersinia* species are pathogenic to humans and rodents: *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* (Ramamurthi and Schneewind, 2002). All these pathogenic *Yersinia* species are cytotoxic to epithelial cells and able to resist the host primary immune response possibly via the inhibition of their uptake by phagocytes (Hueck, 1998). They share a similar Yop (*Yersinia* outer protein) virulon which is located on a large plasmid. The Yop virulon, the archetype of TTSSs, is required for the secretion of Yop proteins (Michiels *et al*., 1990).

An approximately 20 kb of gene cluster encoding the core type III secretion apparatus is conserved in all the three species (Hueck, 1998). This gene cluster consists of 31 genes, including genes encoding components of the type III secretion apparatus (*yscA to yscL*, *yscN* to *yscU*, and *lcrD*), genes encoding regulators (*virF*, *lcrR*, *lcrGVH*, *yopN*, and *lcrQ*), and translocators (*yopB* and *yopD*). However, the effector molecules secreted via this TTSS are usually scattered around the plasmid (Cornelis *et al*., 1989; Persson *et al*., 1995).

In *Yersinia*, the secretion of Yops is triggered by growth at 37°C under low concentration of calcium condition but is blocked at low temperature or in the presence of 2.5 mM calcium (Michiels *et al*., 1990). Contact with the host cells also leads to the activation of the TTSS and subsequent translocation of effector molecules (Yops) into the cytosol of the targeted host cells (Rosqvist *et al*., 1994; Cornelis and Wolf-Watz, 1997). The transcription and secretion of Yops are under the control of multiple factors. Both temperature and virF are required for the induction of the *yop* regulon (Lambert *et al*., 1992). The VirF protein, the key activator of the TTSS, belongs to the AraC family of transcriptional regulators (Gallegos *et al*., 1993). It binds to the promoter regions of *yopE*, *yopH*, *yscA to yscL*, and *lcrGVH* to *yopBD* (Lambert *et al*., 1992; Wattiau and Cornelis, 1994). The deletion of any one of YopN, SycN, TyeA, LcrV and LcrG also results in the derepression and/or secretion of Yops (Forsberg *et al*., 1991; Day and Plano, 1998; Iriarte *et al*., 1998; Cheng and Schneewind, 2000; DeBord *et al*., 2001). Interestingly, two hypothetical secreted negative regulators, LcrQ and YscM, are involved in the down-regulation of the expression of Yops (Hueck, 1998). The deletion of *lcrQ* or *yscM1 yscM2* leads to the reduction of feedback inhibition, whereas overproduction of any one of these proteins shuts off Yop synthesis.

I.5.3.1.2 Secreted proteins

The proteins secreted by TTSSs of *Yersinia* include: YopB, YopD, LcrG, YopE, YopH, YopJ, YopM, YopN, YopP, YopQ, YopR, YopT, YscM1, YscM2, and LcrV (Hueck, 1998). Six Yop effector proteins are delivered into the host cytosol: YopE, YopH, YopM, YopT, YopO (called YpkA in *Y. pseudotuberculosis* and *Y. pestis*), and YopP (called YopJ in *Y. pseudotuberculosis* and *Y. pestis*) (Navarro *et al*., 2005).

YopE localizes to a perinuclear region in mammalian cells (Rosqvist *et al*., 1990). The translocation of YopE into host cells elicits a cytotoxic effect, causing infected cells to round up (Rosqvist *et al*., 1990; Rosqvist *et al*., 1991). YopE exhibits GAP (GTPase activating protein) activity toward RhoA, Rac1 and Cdc42 (Black and Bliska, 2000; Pawel-Rammingen *et al*., 2000). An "arginine finger" which is conserved among mammalian Rho GAPs is also present in the C-terminus of YopE (Fu *et al*., 1999). A mutation of the arginine in the arginine finger motif to alanine (R144A) eliminated the GAP activity of YopE (Black and Bliska, 2000). *Y. pseudotuberculosis* carrying YopER144A was unable to disrupt actin filaments, induce cell rounding or inhibit phagocytosis by HeLa cells (Black and Bliska, 2000). A mutation of *yopE* led to decreased virulence in mice after oral infection, intraperitoneal infection, and intravenous injection (Rosqvist *et al*., 1990).

YopH exhibits antiphagocytic ability and is essential for the virulence of *Yersinia* (Bliska *et al*., 1991). It belongs to a protein-tyrosine phosphatase (PTPase) superfamily (Guan and Dixon, 1990). Translocation of YopH into host cells leads to the disruption of the link between focal adhesions and the actin cytoskeleton, thus inhibiting phagocytosis (Bliska *et al*., 1991). YopH can down-regulate the activation of Fc-mediated respiratory bursts in macrophages and neutrophils (Bliska and Black, 1995; Ruckdeschel *et al*., 1996).

YopM is necessary for the virulence of *Y. pestis* (Leung *et al*., 1990). It can interact with thrombin and inhibit thrombin-induced platelet aggregation (Reisner and Straley, 1992). YopM is not only translocated into the cytoplasm but also trafficks to the cell's nucleus by means of a vesicle-associated pathway (Skrzypek *et al*., 1998). YopM contains multiple leucine-rich repeats (LRR) which are involved in protein-protein interactions and required

for the localization of YopM to the nucleus in HeLa cells (Boland *et al*., 1996; McDonald *et al*., 2003; Skrzypek *et al*., 2003). YopM interferes with innate immunity by causing depletion of NK cells, possibly by affecting the expression of IL-15 receptor alpha and IL-15 (Kerschen *et al*., 2004).

YopT is a member of a large family of bacterial virulence factors found in both animal and plant pathogens (Shao *et al*., 2002). YopT acts as a cysteine protease and its proteolytic activity is dependent upon the invariant C/H/D residues conserved in the entire YopT family (Shao *et al*., 2000). Cleavage of the post-translationally modified Rho GTPases near their carboxyl termini by YopT leads to the releases of Rho GTPases from the membrane (Shao *et al*., 2000). It was recently shown that translocated YopT acts on membrane-bound and GDI-complexed RhoA but not Rac or CDC42 (Aepfelbacher *et al*., 2003).

YpkA/YopO, showing homology to serine and threonine protein kinases, is also necessary for the virulence of *Yersinia* (Galyov *et al*., 1993). YpkA/YopO not only phosphorylates actin but may also be cytotoxic to host cells (Hakansson *et al*., 1996; Juris *et al*., 2000). Overexpression of either a wild type or a kinase-inactive form of YpkA has a cytotoxic effect on yeast (Nejedlik *et al*., 2004).

Yop J functions as a ubiquitin-like cysteine protease (Orth *et al*., 2000; Carter *et al*., 2003). It is essential for the induction of apoptosis in macrophages (Monack *et al*., 1997). YopJ binds to multiple members of the MAP kinase superfamily, including MKKs and IκB kinase β, and blocks their ability to transduce signals from cell surface receptors to the nucleus by a proteolytic cleavage of ubiquitin or a ubiquitin-like protein from these

signaling molecules (Orth *et al*., 2000). More recently, YopJ was shown to act as a deubiquitinase to inhibit NF-kappa B activation (Zhou *et al*., 2005).

Taken together, these effector proteins enable *Yersinia* to obstruct the primary immune response by interfering with host cell signal transduction pathways. The functional study of the effector proteins has revealed multiple pathogenesis mechanisms which may be also used by the corresponding homologous proteins in other pathogens.

I.5.3.2 *Pseudomonas aeruginosa* TTSS

I.5.3.2.1 Genetic organization and regulation

P. aeruginosa is an opportunistic pathogen infecting immunocompromised individuals (Torres *et al.*, 1990). It also utilizes a chromosomally encoded TTSS to deliver effector molecules into a host cytosol to interfere with the physiological signaling pathway (Yahr *et al.*, 1996). The *P. aeruginosa* TTSS contains 38 genes encoding the components required for biosynthesis, secretion, and translocation of effector molecules into host cells (Frank, 1997). The genetic organization of *P. aeruginosa* TTSS is quite similar to that of *Yersinia* TTSS, except for the inversion of the *pscO-pscD* genes with respect to *Yersinia* homologues (Yahr *et al.*, 1997). The *exsD* encoded protein is unique to *P. aeruginosa* (Yahr and Frank, 1994).

As in *Yersinia* spp., contact with eukaryotic host cells, calcium chelation or the presence of serum is able to trigger the expression and activation of *P. aeruginosa* TTSS (Frank, 1997; Vallis *et al.*, 1999). The transcription of the *P. aeruginosa* TTSS is controlled by a four-tier regulatory cascade involving ExsE, ExsC, ExsD, and ExsA (McCaw *et al.*, 2002; Dasgupta *et al.*, 2004; Rietsch *et al.*, 2005; Urbanowski *et al.*, 2005). ExsA, like VirF in *Yersinia*, belongs to a family of AraC transcriptional activators and positively regulates

the expression of all TTSS genes (Yahr and Frank, 1994; Frank, 1997). ExsD functions as an anti-activator by binding to ExsA (McCaw *et al.*, 2002). ExsC, in turn, functions as an anti-anti-activator by binding to ExsD (Dasgupta *et al.*, 2004). ExsE, an inhibitor of ExsC, is a secreted regulator of the *P. aeruginosa* TTSS (Rietsch *et al.*, 2005; Urbanowski *et al.*, 2005). The reduced intracellular pool of ExsE increases the levels of ExsC available to bind ExsD and frees ExsA to activate transcription of the TTSS (Urbanowski *et al.*, 2005).

I.5.3.2.2 Secreted proteins

Nine proteins of *P. aeruginosa*, PopB, PopD, PcrG, PcrV, PopN, ExoS, ExoT, ExoU and ExoY, have been reported to be secreted into the culture supernatant and/or translocated into host cells (Finck-Barbançon *et al.*, 1997; Frank, 1997; Yahr *et al.*, 1997). The latter four proteins are responsible for altering cellular cytoskeleton rearrangement (Juris *et al.*, 2002).

ExoS and ExoT are bifunctional enzymes that have an N-terminal GAP domain and a C-terminal ADP ribosylation domain (Goehring *et al.*, 1999; Krall *et al.*, 2000). Intoxication with ExoS or ExoT causes cell rounding and detachment (Sato *et al.*, 2003). Both ExoS (453 aa) and ExoT (457 aa), which have 75% amino acid sequence identity, share high similarity to YopE at their N-termini (Frank, 1997). Different domains of ExoS activate distinct Toll like receptors (TLRs) (Epelman *et al.*, 2004). ExoS uses both TLR2 and the TLR4/MD-2/CD14 complex for cellular activation of monocytic cells. The TLR2 activity is related to the C-terminal domain of ExoS, whereas the TLR4 activity is related to the N-terminal domain (Epelman *et al.*, 2004). Recently, Deng *et al.* (2005) showed that ExoT uncouples the Crk signal transduction pathway. The cell rounding induced by ExoT can be reduced by overeexpression of the wild type Crk-I (Deng *et al.*, 2005).

ExoY is an adenylate cyclase (Yahr *et al.*, 1998). Intoxication with ExoY induces cell rounding but without causing a loss of viability (Vallis *et al.*, 1999; Lee *et al.*, 2005). ExoY also induces endothelial gap formation and increases the filtration coefficient in the isolated perfused lung (Sayner *et al.*, 2004). A recent study showed that both adenylate cyclase-active ExoY and its catalytic mutant form ExoYK81M disrupted the actin organization of epithelial cells at 2 h post-infection, accompanied by inhibition of bacterial invasion (Cowell *et al.*, 2005).

ExoU functions as a rapid and potent cytotoxin and has phospholipase activity which requires activation and modification by eukaryotic cells (Finck-Barbançon *et al.*, 1997; Phillips *et al.*, 2003; Sato *et al.*, 2003). ExoU expression causes a loss in yeast viability and low amounts of ExoU induce vacuolar fragmentation of yeast (Sato *et al.*, 2003). ExoU contains a patatin-like phospholipase domain in the N-terminal portion which is required for the cytotoxicity of ExoU (Sato *et al.*, 2003). The C-terminal portion of ExoU also plays an important role in ExoU-mediated toxicity and activity. Deletion of the C-terminal part or transposon inactivated C-terminus correlates with the loss of cytotoxicity against yeast and mammalian cells (Hauser *et al.*, 1998; Finck-Barbancon and Frank, 2001; Rabin and Hauser, 2003; Sato *et al.*, 2003).

Each of the four effectors contributes to the virulence of *P. aeruginosa*. Using tissue culture and *in vivo* infection model, Lee *et al.* (2005) showed that ExoS is the major cytotoxin required for colonization and dissemination during infection but ExoU is the most cytotoxic. They also demonstrated that ExoT prevents tissue culture cells from type III-dependent lysis, while ExoY seemed to have little effect on cytotoxicity (Lee *et al.*, 2005).

I.5.3.3 *Aeromonas salmonicida* TTSS

A. salmonicida is the etiological agent of furunculosis in salmonids (Munro and Hastings, 1993). Braun *et al.* (2002) cloned and sequenced a novel ADP-ribosyltransferase toxin called AexT. The amino acid sequence of AexT shared 57-62% identity to those of *P. aeruginosa* ExoT and ExoS (Braun *et al.*, 2002). The N-terminal domain amino acids of AexT were also 26-34% identical to those of YopE of *Yersinia*. Inactivation of AexT significantly diminished the cytotoxic effect of *A. salmonicida* on RTG-2 rainbow trout gonad cells, suggesting that AexT is directly involved in the toxicity of *A. salmonicida* for fish cells (Braun *et al.*, 2002). Following this study, Burr *et al.* (2002) identified a TTSS cluster in *A. salmonicida*. Inactivation of an apparatus gene (*ascV*) or a translocation apparatus gene (*aopB*) within this secretion system eliminated the cytotoxic effect of *A. salmonicida* on RTG-2 rainbow trout gonad cells, which indicated that the TTSS plays an important role in the virulence of this pathogen (Burr *et al.*, 2002). Subsequently, Burr *et al.* (2003) showed that the previously identified AexT was secreted into the culture supernatant and translocated into the cytosol of fish cells via a type III secretion pathway. As in *Yersinia*, an acrV (*Yersinia* PcrV homologue) mutant of *A. salmonicida* expresses and secretes significant amounts of AexT even in the presence of high concentrations of calcium (Bergman *et al.*, 1991**;** Burr *et al.*, 2003).

The genetic organization of a TTSS cluster of *A. salmonicida* is highly similar to that of *P. aeruginosa* (Yahr *et al.*, 1997; Burr *et al.*, 2003). It is also similar to that of *Yersinia* species, but differs from the gene order in *Yersinia* species where the homologous *ascU-aopD* regions are inverted (Yahr *et al.*, 1997; Burr *et al.*, 2003). Stuber *et al.* (2003) reported that the TTSS cluster is located on a large virulence plasmid of *A. salmonicida*,

which is the same as *Yersinia* species where the TTSS cluster is located on a 70 kb plasmid but different from *P. aeruginosa* where it is located on the chromosome (Michiels *et al*., 1990; Yahr *et al.*, 1996).

I.6 Objectives

Since multifactorial virulence factors are involved in *A. hydrophila* pathogenesis, it is necessary to explore the virulence-associated genes in *A. hydrophila* infection and elucidate the underlying molecular mechanisms of interaction between *A. hydrophila* and host so that suitable therapeutics can be designed.

Initially, a forward genetic approach was used to identify a phage-associated genomic island and a TTSS gene cluster from *A. hydrophila* PPD134/91 (Chapters III and V). We characterized these two virulence gene clusters and 22 unique DNA fragments (Zhang *et al.*, unpublished data) which are commonly present in virulent *A. hydrophila* strains (Chapter III). They were studied in a comparative manner to reveal the multifactorial pathogenicity of *A. hydrophila* and the genes which are most important in the pathogenesis of *A. hydrophila* (Chapter III).

The virulence factors can be present in the bacterial cytosol, the membrane, or secreted extracellularly. Extracellular proteins (ECPs) of pathogens play a vital role in the process of pathogen-host interaction since they may come into contact with host cells directly. Therefore, in Chapter IV, a reference map of the ECPs of *A. hydrophila* AH-1 is established. Such a reference map was further used to study the effect of temperature change on the secretion of virulence factors. In addition, functional correlations between different genes were deciphered by comparing the extracellular proteomes of several mutants with that of the wild type.

The TTSS was identified from our initial study and shown to be one of the most important virulence factors. In Chapter V, the TTSS of *A. hydrophila* AH-1 is studied in detail. The importance of this TTSS cluster in the virulence of *A. hydrophila* was revealed by infection of tissue culture cells and the blue gourami fish with the wild type and mutants of *A. hydrophila* AH-1. The distribution of this TTSS among *A. hydrophila* strains was surveyed by PCR screening.

Many Gram-negative bacteria were able to utilize the TTSSs to secrete effector proteins and deliver them into eukaryotic cytosol, interfering with the host signaling pathway (Cornelis and Gijsegem, 2000). In Chapter VI, some of the proteins secreted by the TTSS of *A. hydrophila* AH-1 are identified by examining the supernatants of two negative regulator mutants. The localization and functional mechanisms of two effector proteins inside host cytosols were investigated by immunofluorescence microscopy.

Chapter II Common materials and methods

II.1 Bacterial strains, plasmids and buffers

The bacterial strains and plasmids used in this study are listed in Table II.1. *A. hydrophila* strains were cultured on tryptic soy agar (TSA) (Difco, USA) or in tryptic soy broth (TSB) (Difco) at 25 °C or 30 °C. *E. coli* strains were cultured on L agar (Difco) or in Luria broth (LB) (Difco) at 37 °C. Stocks of *A. hydrophila* and *E. coli* were maintained at -80°C as a suspension in supplemented TSB and LB containing 25% (v/v) glycerol, respectively. When required, antibiotics (Sigma, USA), ampicillin (Amp), chloramphenicol (Chl), tetracycline (Tc) and kanamycin (Kan) were added at final concentrations of 100, 30, 10 and 50 μg/ml, respectively.

Phosphate buffered saline (PBS) was used for washing and resuspension of the bacteria. PBS contained 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4, and the pH was adjusted to 7.2.

II.2 Fish studies

II.2.1 Animal model and maintenance

The animal model used in this study was the three-spotted blue gourami, *Trichogaster trichopterus* (Pallas). This fish is easily available, cheap and hardy. They are of an average weight and length of 12 g and 10.0 cm, respectively. They were purchased from Mainland Tropical Fish Farm in Singapore.

Upon arrival, they were placed in water containing 0.5% (w/v) sodium chloride and 5 ppm acriflavine for one week, followed by another week in clean water. The fish were maintained in clean, de-chlorinated water at 25 to 28^{o}C and fed with

Table II.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant property[1]	Source[2] / Reference
A. hydrophila strains		
Aer-19	O:11, virulent	Human, CDHS
Aer-27	Virulent	Human, CDHS
Aer-184	O:34, virulent	Human, CDHS
Aer-186	O:16, virulent	Human, CDHS
Aer-205	O:11, virulent	Human, CDHS
Aer-211	O:18, virulent	Human, CDHS
ATCC7966	O:1, type strain	Milk, ATCC
AH-1	O:11, virulent, Col^r, Cm^s	Fish, UM
AH-3	O:34, virulent	Fish, UB
Ba5	O:34, virulent	Fish, UM
JCM3968	O:6	Snake, JCM
JCM3973	O:11	Unknown, JCM
JCM3976	O:14	Frog, JCM
JCM3978	O:16	Unknown, JCM
JCM3980	O:18	Unknown, JCM
JCM3981	O:19	Unknown, JCM
JCM3983	O:21	Unknown, JCM
JCM3984	O:22	Unknown, JCM
JCM3985	O:23	Unknown, JCM
JCM3996	O:34	Unknown, JCM
L15	O:51, avirulent	Fish, BAU
L31	O:91, virulent	Fish, BAU
L36	O:36, avirulent	Fish, BAU
LL1	O:11, virulent	Fish, UM
PPD35/85	O:7, avirulent	Fish, AVA
PPD11/90	O:21, virulent	Fish, AVA
PPD64/90	O:34, avirulent	Fish, AVA
PPD88/90	O:16, avirulent	Fish, AVA
PPD45/91	Avirulent	Fish, AVA
PPD70/91	O:5, virulent	Fish, AVA
PPD122/91	O:11, virulent	Fish, AVA
PPD134/91	O:18, virulent	Fish, AVA
Xs91/4/1	Virulent	Fish, China
E. coli strains		
JM109	Col^s, Amp^s	Promega
MC1061(λ*pir*)	(λ*pir*), *thi thr-1 leu6 proA2 his-4 argE2 lacY1 galK2 ara14 xyl5 supE44*, λ*pir*	Rubires *et al.*, 1997
S17-1(λ*pir*)	*thi pro hsdR hsdM*$^+$ *recA*[RP42-Tc::Mu-Km::Tn7 (Tp^r Sm^r)Tra+]	Simon *et al.*, 1983
Plasmids		
pGEM®-T Easy vector	Cloning vector; Amp^r	Promega
pRE112	Suicide vector; R6K ori *sacB* Cm^r	Edwards *et al.*, 1989
pDN19lacΩ	Promoterless *lacZ* fusion vector; $Sp^rSm^rTc^r$	Totten and Lory, 1990

[1]Virulent strains were defined as having a lower LD_{50} value in blue gourami or rainbow trout ($<10^{6.5}$) than the avirulent strains ($>10^{7.5}$).
[2]ATCC, American Type Culture Collection; AVA, Agri-Food and Veterinary Authority, Singapore; BAU, Bogor Agricultural University of Indonesia; CDHS, California Department of Health Services; JCM, Japan Collection of Microorganisms; UB, University of Barcelona; UM, University of Montreal.

commercial fish pellets at 1% of their body weight daily. During this period, they were allowed to acclimatize before the start of an experiment.

II.2.2 Fifty percent median lethal dose (LD_{50}) studies

Before injection, fish were anaesthetized in water containing 0.02% (v/v) 2-phenoxy-ethanol (Sigma) for ease of handling. Three groups of ten fish each were injected intramuscularly with 0.1 ml of PBS-washed bacterial cells adjusted to the required concentrations. The number of bacterial cells for each injected dose was confirmed by appropriate dilutions and plate count on TSA. Fish mortality was then observed over 14 days and the LD_{50} values were calculated according to the method of Reed and Muench (1938).

II.3 Statistical Analysis

All data were expressed as mean ± SEM. The data were analyzed using Student's *t* test. Values of $P < 0.05$ were considered significant.

II.4 Molecular biology techniques

II.4.1 Genome walking and cloning

Genome walking libraries were made according to the manual of Clontech Universal Genome Walker™ Kit. Briefly, 2.5 µg of purified genomic DNA was digested with different restriction enzymes including *EcoR*V, *Stu*I, *Pvu*II, *Rsa*I, *Sma*I and *Sca*I. The digested genomic DNA was purified and ligated to the genome walker adaptor as follows:

4.0 µl purified restriction enzyme digested genomic DNA (approx. 0.5 µg)

1.9 µl of Genome Walker Adapter (25 µM)

1.6 µl 5x ligation buffer

0.5 µl T4 DNA ligase (1 unit/µl)

The ligation mixture was incubated at 16°C overnight, followed by 5 min incubation at 70°C to stop the reaction. 72 µl of Tris-EDTA (10 mM/1 mM, pH 7.4) was then added to the ligation sample and mixed. The genome walking library was aliquoted 10 µl each into several microcentrifuge tubes and stored at -20°C.

PCR amplification was performed in an Applied Biosystems (ABI) PCR system 2400 or 2700 PCR machine using the Advantage 2 polymerase mix (BD Biosciences Clontech). The PCR conditions are: 1 min at 94^{o}C, 7cycles of 15 s at 94^{o}C, 4 min at 72^{o}C, 32 cycles of 15 s at 94^{o}C, 3 min at 67^{o}C. After the PCR reaction was completed, 5 µl of each PCR reaction was subjected to electrophoresis for analysis of the PCR product. For those PCR reactions in which multiple bands were obtained, a secondary PCR reaction was set up using nested primers and the appropriately diluted primary PCR reaction as the template. Conditions used for the secondary PCR were: 1 min at 94^{o}C, 5 cycles of 15 s at 94^{o}C, 3 min at 72^{o}C, 20 cycles of 15 s at 94^{o}C, 3 min at 67^{o}C and 5 min at 67^{o}C.

DNA fragments amplified were cloned into the pGEM-T Easy vector (Promega, USA) following the manufacturer's instructions and transformed into *E. coli* JM109 or *E. coli* DH5α. *E. coli* competent cells were prepared according to the procedure described by Sambrook and co-workers (1989). After transformation, the cells were plated on LBA containing ampicillin, isopropylthiogalactoside (IPTG, Bio-Rad), 5-bromo-4-chloro-3-indolyl-β-D galactopyranoside (X-gal, Bio-Rad), to allow for blue-white colony selection.

II.4.2 Analysis of plasmid DNA

The boiling lysis procedure described by Holmes and Quingley (1981) was used for the mini-preparation of plasmid DNA. Briefly, 20 µl of overnight bacterial culture was obtained and spun at 8,000 × *g* for 2 min. The bacterial pellet was resuspended in 3 µl of

sterile water, followed by addition of 8 µl of STET solution [0.1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 5% Triton X-100, 0.8 µg/µl lysozyme and 10 µg/µl RNase A]. Subsequently, the tubes were placed on boiling water bath for 30 seconds and allowed to cool down to room temperature. One µl of 10 × buffer of appropriate restriction enzyme and 0.1-0.2 units of restriction enzyme was added and the tubes were incubated at appropriate temperature for 30 min. The digested samples were analyzed by gel electrophoresis using 1% (w/v) agarose gel (Seakem®, BioWhittaker Molecular Applications, USA), followed by staining in ethidium bromide.

II.4.3 Purification of plasmid DNA

Plasmid DNA was isolated using QIAGEN Spin columns (QIAGEN GmbH, Germany). Bacterial strains containing plasmids were cultured in LB broth (with appropriate antibiotics) and incubated in an orbital shaker (Forma scientific, USA) with 225 rpm shaking at 37°C. After culturing for overnight (16 to 18 h), the plasmid DNA was extracted according to manufacturer's protocol and dissolved in 35 µl of elution buffer (10 mM Tris-HCl, pH 8.5). The quality and concentration of DNA was determined using spectrophotometer (Shimadzu, UV-1601, Japan).

II.4.4 Genomic DNA isolation

A. hydrophila strains were grown in 5 ml TSB at 25^{o}C, 200 rpm (MIR-262, Sanyo incubator, USA) for 18 h. Bacterial genomic DNA was extracted as described in the manuals of the Genomic DNA isolation/purification kits (Promega). For the mini-preparation, the purified genomic DNA was redissolved in 50 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5).

II.4.5 DNA sequencing

The ABI PRISM BigDye Terminator version 3.1 Cycle Sequencing Kit was used for the sequencing reaction. The sequencing reaction mix consists of: 1 µl of autoclaved water, 100 to 200 ng of the plasmid DNA or 40 to 50 ng of PCR product, 0.5 µl of 2.5 µM primer and 1 µl of BigDye. PCR was carried out in an ABI PCR system 2400 or 2700 using the conditions: 1 min at 96 ^{o}C, 25 cycles of denaturation 96^{o}C, 10 s, annealing 50^{o}C, 5 s, extension 60^{o}C, 1 min 45 s. The reaction was held at 16^{o}C until ready for purification.

The PCR product was purified by ethanol and sodium acetate precipitation. Briefly, the PCR product was transferred to a 1.5 ml microcentrifuge tube and 20 µl of 95% ethanol and 0.5 µl of 3 M sodium acetate (pH 4.6) were added. The mixture was gently vortexed for about 10 s and centrifuged for 15 min at 12000 × *g* in a conventional benchtop centrifuge (Eppendorf, Germany). The supernatant was carefully removed and the pellet was washed with 500 µl 70% ethanol and centrifuged for 5 min at 12000 × *g*. The wash step was repeated once and the supernatant was carefully removed. Residual 70% ethanol was removed after pulse-spinning of the tube. The pellet was air-dried and stored at -20^{o}C. The ABI 3100 Genetic Analyzer was used for automated DNA sequencing.

II.4.6 Sequence analysis

Sequence assembly and further editing were carried out with Vector NTI DNA analysis software (InforMax, USA). BLASTN, BLASTP and BLASTX sequence homology analyses and a protein conserved-domain database analysis (CDD search) were performed using the BLAST network server of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST; Altschul *et al*., 1990).

II.4.7 Southern hybridization

II.4.7.1 DNA preparation

Genomic DNA of *A. hydrophila* strains was digested with proper restriction enzymes prior to the Southern hybridization analysis. Approximately, 0.5 to 1.0 μg digested DNA was loaded into each well of precast 1% (w/v) Seakem® LE agarose gel (BMW, USA). Electrophoresis was carried out in 1 x Tris-acetate-EDTA (TAE) buffer at 100 V for 1 h. Subsequently, the gel was washed in solution I (0.25 M HCl) for 15 min, in solution II (0.4 M NaOH, 0.6 M NaCl) for 45 min and in solution III (0.6 M NaCl and 0.5 M Tris-HCl, pH 7.5) for 45 min. The washing was carried out in a plastic container placed on a BioDancer (New Brunswick Scientific, USA). The gel was transferred to a glass plate with a piece of nylon-based membrane (GeneScreen^{TM}) presoaked in solution III and placed on top of it. Caution was taken to remove any air bubbles between the gel and membrane. Three to four pieces of Whatman paper presoaked in 10 x SSC buffer were placed on the membrane. Paper towels and a heavy object were placed on top of the Whatman paper and the blotting process was performed overnight at room temperature. Lastly, the membrane was air-dried and the transferred DNA was cross-linked to the membrane by a UV-transilluminator (SPS-TF, Vilber Lourmat, France) for 5 min.

II.4.7.2 Probe preparation

The DIG (Digoxigenin) DNA labelling and detection kit (Roche Molecular Biochemicals, Germany) was used in Southern analysis. To label the DNA probe, the purified DNA (1-3 μg plasmid or PCR product) in a volume of 15 μl was denatured by boiling for 10 min and immediately kept on ice. The following reagents: 2 μl 10x hexanucleotide mix, 2 μl

dNTP labelling mix and 1 µl Klenow enzyme labelling grade were added to the freshly denatured probe. The labelling reaction was incubated overnight at 37^{o}C. The next day, the reaction was stopped by heating the tube at 65^{o}C for 10 min.

II.4.7.3 Hybridization analysis

For pre-hybridization, the membrane was incubated in an appropriate volume (20 ml/100 cm^{2} membrane) of pre-warmed (37^{o}C) DIG Easy Hyb solution in a hybridization bag (Kirkegaard & Perry Laboratories, USA), with gentle agitation for 30 min to 2 h. The DIG-labelled DNA probe was denatured by boiling for 5 min and rapidly cooled on ice. It was then added to an appropriate volume of pre-warmed DIG Easy Hyb solution and mixed (hybridization solution). The pre-hybridization solution was poured off and the membrane was incubated with the hybridization solution containing the probe overnight at 42^{o}C with gentle agitation.

II.4.7.4 Washing and visualization

After hybridization, the membrane was washed twice (5 min each) in 2x SSC, 0.1% (w/v) SDS, at room temperature with constant agitation. Then it was washed for another 2 times in 0.5x SSC, 0.1% (w/v) SDS (pre-warmed to wash temperature) at 65^{o}C with constant agitation. The subsequent steps were carried out at room temperature. The membrane was rinsed briefly in washing buffer [0.1 M Maleic acid, 0.15 M NaCl; pH 7.5; 0.3% (v/v) Tween 20] and incubated with freshly prepared 1x blocking solution (in Maleic acid buffer) for 30 min. Prior to use, the anti-DIG-AP was centrifuged at ~9200 *g* for 5 min. The required volume was carefully taken from the surface and diluted 1:5000 in blocking solution. The membrane was then incubated with the antibody solution for 30 min and washed twice (15 min each) in washing buffer, followed by equilibrating for 2-5 min in

detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5). For detection, the membrane was incubated in freshly prepared substrate solution (40 µl of NBT/BCIP to every 2 ml of detection buffer) in a hybridization bag (KPL) in the dark. When the desired band intensities were observed, the reaction was stopped by washing the membrane for 5 min with TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

II.5 Protein techniques

II.5.1 Preparation of extracellular proteins from *A. hydrophila*

A. hydrophila strains were grown in DMEM for 24 h [25°C in a 5% (v/v) CO_2 atmosphere]. The bacteria were adjusted to an OD value of 0.8 at 550 nm and inoculated into fresh DMEM at a 1:200 dilution. After 24 h, the extracellular protein (ECP) fraction was isolated by TCA–acetone precipitation (Shimizu *et al.*, 2002). Briefly, the bacterial cells were separated from the supernatant by centrifugation at 5000 *g* for 10 min at 4°C. The culture supernatant was passed through a 0.22 µm, low-protein-binding Millex filter (Millipore, USA) and then precipitated with 10% (w/v) TCA for 1 h at 4°C. The protein pellet was washed thrice with -20°C acetone and then air dried. The protein pellet was solubilized in ReadyPrep reagent 3 (5 M urea, 2 M thiourea, 2% [w/v] CHAPS, 2% [w/v] SB 3-10, 40 mM Tris, and 0.2% [w/v] Bio-Lyte 3/10 ampholyte; Bio-Rad, USA). Insoluble materials were removed by centrifugation at 18 000 × *g* for 15 min at room temperature. Protein sample was stored at -80°C until analysis.

The concentration of protein in the ECP samples was determined using the Bio-Rad Protein Assay. The protein solutions are assayed in duplicates. 200 µl of dye reagent concentrate (Bio-Rad) was added to each tube (799 µl H_2O + 1 µl protein) and the tubes were vortexed to mix. They were then incubated at room temperature for 10 min and

OD_{595} was recorded. The protein concentration of each ECP sample was obtained based on the standard curve plotted for the BSA protein of known concentrations.

II.5.2 One-dimensional polyacrylamide gel electrophoresis (1D-PAGE)

One-dimensional SDS-PAGE was performed according to a standard protocol (Sambrook *et al*., 1989). Briefly, the gel solution was poured into the gap between two glass plates and isopropanol was layered on top. After the resolving gel was polymerized, the isopropanol overlay was poured off and the gel was washed several times with milli-Q water. The 4% stacking gel was poured on top of the resolving gel and a clean Teflon comb was immediately inserted. After the stacking gel was polymerized, the Teflon comb was carefully removed and the wells were washed with milli-Q water from a squirt bottle to remove any traces of unpolymerized acrylamide. Prior to loading the protein samples, the sodium-dodecyl sulphate (SDS) gel-loading buffer (50 mM Tris-HCl, 100 mM DTT [Bio-Rad], 2% [w/v] SDS, 0.1% [w/v] bromophenol blue (Bio-Rad), 10% glycerol) was added and the samples were boiled for 5 min. After loading the samples, the electrophoresis apparatus was attached to an electric power supply and a constant current of 5 mA per gel was applied. After the dye front has moved into the resolving gel, the current was increased to 15 mA per gel.

II.5.3 Two-dimensional PAGE

II.5.3.1 Iso-electric focusing (IEF)

The first-dimension isoelectric focusing was performed with the Ettan™ IPGphor™ Isoelectric Focusing System (Amersham, USA) according to the manufacturer's instructions. Briefly, the 18 cm Immobiline Drystrips with a nonlinear gradient from pH range of 3 to 10 (Amersham) were passively rehydrated for 12 to 16 h at room temperature

with a mixture (340 µl) containing 8 M urea, 2% (w/v) CHAPS, 0.5% immobilized pH gradient (IPG) buffer, 50 mM dithiothreitol, and a trace amount of bromophenol blue. After rehydration, 15 µl of rehydration buffer was added to the desired amount of protein sample (Total volume < 30 µl) and cup loading was employed to load the protein sample just prior to IEF. IEF was performed using the following conditions: 50 V for 1 h, 500 V for 1.5 h, 4000 V for 1.5 h, 8000 V for 40000 Vh.

II.5.3.2 Second-dimensional PAGE

Before running the second dimension, the 18 cm IPG strips (Amersham) were equilibrated in equilibration buffer I [6 M urea, 2% (w/v) SDS, 50 mM Tris-HCl pH 8.8, 30% (v/v) glycerol and 130 mM DTT, trace amounts of bromophenol blue] for 10 min, followed by equilibration buffer II [6 M urea, 2% (w/v) SDS, 50 mM Tris-HCl (pH 8.8), 30% (v/v) glycerol and 135 mM iodoacetamide (Sigma), trace amounts of bromophenol blue] for another 10 min.

For second dimension SDS-PAGE, 12% polyacrylamide gels (20 x 20 cm, 1.0 mm thick) were used. The equilibrated-gel strips were placed on top of the 12% polyacrylamide gel and overlaid with 0.5% agarose (Seakem). The gels were mounted in a PROTEAN II XL Cell (Bio-Rad) and the Tris-glycine electrophoresis buffer [25 mM Tris, 250 mM glycine (pH 8.3), 0.1% (w/v) SDS] was added to the top and bottom of the gels between the glass plates. The proteins were then resolved with a constant current of 25 mA per gel.

II.5.4 Coomassie blue and silver staining of protein gels

The gels were stained with 0.1% (v/v) Coomassie Brilliant Blue R-250 (Bio-rad) (Meyer and Lamberts, 1965; Sambrook *et al*., 1989) for 2 h to overnight and de-stained the next day with 20% methanol (v/v) and 10% acetic acid (v/v).

For more sensitive detection of proteins in the gel, silver staining (Blum *et al*., 1987) was performed. The gel was first fixed in 50% (v/v) methanol and 10% (v/v) acetic acid for at least 30 min, followed by 15 min in 50% (v/v) methanol. After that, the gel was washed 5 times (5 min each) with milli-Q water, treated with fresh 0.02% (w/v) sodium thiosulfate (Sigma) for 1 to 2 min, and washed twice (1 min each) with milli-Q water. Freshly prepared, chilled 0.2% (w/v) silver nitrate (Merck, Germany) solution was then added and the gel was stained for 25 min. The gel was then washed twice (1 min) with milli-Q water and developed (3% [w/v] sodium carbonate, 0.025% [v/v] formaldehyde [Sigma]) for 5-10 min. The developing was stopped by placing the gel in 1.4% (w/v) EDTA for 10 min.

Chapter III

Identification and characterization of putative virulence genes and gene clusters in *Aeromonas hydrophila* PPD134/91

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Abstract

A. hydrophila is a Gram-negative opportunistic pathogen of animals and humans. The pathogenesis of *A. hydrophila* is multi-factorial. In the previous studies, two rounds of genomic subtraction led to the identification of twenty-two unique DNA fragments that are commonly present in the eight virulent strains examined and two genomic islands (GIs), O-antigen and capsule gene clusters. In this study, markers of GIs were used to identify two more GIs, namely, a phage-associated island and a TTSS gene cluster. All these putative virulence genes and gene clusters were positioned on a physical map of *A. hydrophila* PPD134/91 in order to determine their genetic organization in this bacterium. Further *in vivo* study of insertion and deletion mutants showed that the TTSS may be one of the important virulence factors in *A. hydrophila* pathogenesis. Furthermore, deletions of multiple virulence factors such as S-layer, serine protease and metalloprotease also increased the LD_{50} to the same level as the TTSS mutation (about 1 log) in a blue gourami infection model. This observation sheds light on the multi-factorial and concerted nature of pathogenicity in *A. hydrophila.* The large number of putative virulence genes identified in this study will form the basis for further investigation of this emerging pathogen and help to develop effective vaccines, diagnostics, and novel therapeutics.

III.1 Introduction

A. hydrophila is a ubiquitous Gram-negative bacterium of aquatic environments, which has been implicated as a causative agent of motile aeromonad septicemia in a variety of aquatic animals (especially freshwater fish species) (Thune *et al.*, 1993; Austin and Adams, 1996). They cause gastro- and extra-intestinal infections in humans, including septicemia, wound infections, and gastroenteritis (Janda, 2001). A number of virulence factors have been identified in *A. hydrophila*, namely pili and adhesins (Quinn *et al.*, 1993; Pepe *et al.*, 1996), O-antigens and capsules (Martinez *et al.*, 1995; Zhang *et al.*, 2002 & 2003), S-layers (Dooley and Trust, 1988), exotoxins such as hemolysins and enterotoxin (Chakraborty *et al.*, 1984; Howard *et al.*, 1996), and a repertoire of exoenzymes which digest cellular components such as proteases, amylases and lipases (Pemberton *et al.*, 1997; Leung and Stevenson, 1988).

The pathogenesis of *A. hydrophila* is multi-factorial. Most studies to date have concentrated on the characterization of a few virulence factors in different animal models using different strains (Dooley and Trust, 1988; Wong *et al.*, 1998; Sha *et al.*, 2002), making it very difficult to evaluate the significance of each gene in the virulence of *A. hydrophila*. This study aims to identify more putative virulence determinants and characterize them in an integrated manner.

Suppressive subtraction hybridization or genomic subtraction offers a genome-level approach to identifying the genetic differences between virulent and avirulent strains of bacteria (Mahairas *et al.*, 1996). In the previous studies, we have identified twenty-two unique DNA fragments encoding nineteen putative virulence factors and seven new open reading frames (ORFs), which are frequently present in a group of virulent strains of *A.*

hydrophila (Zhang *et al.*, 2000; Zhang *et al.*, unpublished data). In addition, two GIs which differ from the rest of the genome in G+C content and which carry mobility-associated genes (integrases or transposes) and putative virulence genes were found. They are a phage-associated gene cluster and a TTSS gene cluster. Subsequently, these putative virulence-associated genes GIs were located on a preliminary physical map of *A. hydrophila* PPD134/91 to determine their genetic organization in *A. hydrophila* which will provide insight into the molecular basis of pathogenicity of this bacterium.

We further studied the individual or combined roles of these putative virulence genes using a consistent animal model and in the same strains. Knockout mutations were constructed to elucidate the contributions of a number of these virulence genes to *A. hydrophila* pathogenesis. Of the single knockout mutations examined, only that in the TTSS (*ΔascN*) significantly affected the LD_{50}s in a blue gorami infection model. In addition, a triple deletion mutant of S layer, serine protease and metalloprotease (*ΔahsAΔserAΔmepA*) increased the LD_{50} to the same level as the *ascN* mutant.

III.2 Materials and methods

III.2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table III.1. The conjugative transfer of plasmids between *A. hydrophila* and *E. coli* strains was carried out by plate mating at 30^{o}C for 24 h.

III.2.2 Construction of defined insertion mutants and deletion mutants

To obtain defined insertion mutants, oligonucleotides containing flanking *Xba*I restriction sites (primers used are listed in Table III.2a & 2b) were used to amplify internal fragments from the targeted genes. The amplified fragment was ligated to pGEM-T easy vector and

Table III.1. Bacterial strains used in this study

Strain	Genotype and/or relevant property[1]	Source[2] / Reference
A. hydrophila strains		
ATCC7966	O:1, virulent, type strain	ATCC, USA
AH-1	O:11, virulent, Amp^r	UM, Canada
L31	O:91, virulent	BAU, Indonesia
PPD70/91	O:5, virulent	AVA, Singapore
PPD122/91	O:11, virulent	AVA, Singapore
PPD134/91	O:18, virulent	AVA, Singapore
PPD11/90	O:21, virulent	AVA, Singapore
Xs91/4/1	virulent	IHB, China
L15	O:51, avirulent	BAU, Indonesia
L36	O:36, avirulent	BAU, Indonesia
PPD35/85	O:7, avirulent	AVA, Singapore
PPD64/90	O:34, avirulent	AVA, Singapore
PPD88/90	O:16, avirulent	AVA, Singapore
PPD45/91	Avirulent	AVA, Singapore
AH-3	O:34, virulent, Amp^r	UB, Spain
Insertion mutants		
hlyA	*hlyA* insertion mutant from AH-1	This study
aerA	*aerA* insertion mutant from AH-1	This study
hup1	*hup1* insertion mutant from AH-1	This study
hup3	*hup3* insertion mutant from AH-3	This study
opdA	*opdA* insertion mutant from AH-1	This study
bvgA	*bvgA* insertion mutant from AH-3	This study
bvgS	*bvgS* insertion mutant from AH-3	This study
ompAI	*ompAI* insertion mutant from AH-1	This study
ascN	*ascN* insertion mutant from AH-1	This study
f85	*f85* insertion mutant from AH-1	This study
flhA1	*flhA1* insertion mutant from AH-1	This study
flhA3	*flhA3* insertion mutant from AH-3	This study
Deletion mutants		
ΔbvgA	*bvgA* deletion mutant from AH-3	This study
ΔahsA	*ahsA* deletion mutant from AH-1	This study
ΔahsA ΔserA	double deletion mutant from AH-1	This study
ΔahsA ΔmepA	double deletion mutant from AH-1	This study
ΔascN	*ascN* deletion mutant from AH-1	This study
ΔompAI	*ompAI* deletion mutant from AH-1	This study
ΔahsA ΔahyR	double deletion mutant from AH-1	This study
ΔahsAserAmepA	triple mutants from AH-1	This study

[1]Virulent strains were defined as having a lower LD_{50} value in blue gourami or rainbow trout ($<10^{6.5}$) than the avirulent strains ($>10^{7.5}$).
[2]ATCC, American Type Culture Collection; IHB, Institute of Hydrobiology, China; AVA, Agri-Food and Veterinary Authority, Singapore; BAU, Bogor Agricultural University of Indonesia; JCM, Japan Collection of Microorgamisms; UB, University of Barcelona; UM, University of Montreal.

Table III.2a. Primers used for construction of insertion and deletion mutants

	Primers used for construction of insertion mutants	
Gene	Primer pairs	Primer sequence (5' to 3')
hlyA	hlyA-For	GCATCTAGAGAACAGGGTCTGATTTACCT
	hlyA-Rev	AATCTAGAATGTTCTCGCTCCAGCTCAGA
aerA	aerA-For	GCTCTAGAGAAGGTGACCACCAAGAA
	aerA-Rev	GCTCTAGAGATGACGAAGGTGTGGTT
hup1	hup1-For	TGCTCTAGAGCGAACAAAGCTCAACTTGTC
	hup1-Rev	GCTCTAGAGCCTTTACCAGTTTGCGGATT
hup3	hup3-For	GCTCTAGAAGCTCAACTTGTCGATGC
	hup3-Rev	GCTCTAGACACAAAGGACGGCACATT
opdA	opdA-For	GGTCTAGAACTCCGTACTCAACAGCGAA
	opdA-Rev	CGTCTAGAAGATCGGTGAGGAAGTTCAC
bvgA	bvgA-For	GAGCTCTAGAGAATGGCAGTGAATATGCTG
	bvgA-Rev	AAAGCTCTAGAAGCTTGGACATGAATCCATC
bvgS	bvgS-For	GCTCTAGAGAAGAAGTTACCTTCTCGAT
	bvgS-Rev	GCTCTAGACCAGCAACTGTGTCAAACGT
ompAI	ompAI-For	ACGTCTAGATTACGCTGGTACTGAAGACG
	ompAI-Rev	CGTTCTAGACAACAGCGTTGCCATCTTTC
ascN	ascN-For	GAGCTCTAGAGCTCCTTGCAAGCAGAAGTGAT
	ascN-Rev	AAGCTCTAGAGCGAATCCATCAACAGCAGCA
f85	f85-For	CTGCTCTAGAGCGATGTTAAGAAAGCGAGTGC
	f85-Rev	GAGCTCTAGAGCCAAACAGCCTCTTTTACGCA
flhA1	flhA1-For	GCTCTAGATGATGCTGGATATCCTGTT
	flhA1-Rev	GCTCTAGAGGATGTTGATGAAGAGGAT
flhA3	flhA3-For	GCTCTAGAGCCATCTCCTTATCATGGTA
	flhA3-Rev	GCTCTAGAACGATCCTGATGATCATCA

Table III.2b. Primers used for construction of insertion and deletion mutants

	Primers used for construction of deletion mutants	
Gene	Primer pairs	Primer sequence (5' to 3')
bvgA	DC-bvgA-F	GTAGCTCTAGAGCTGGATTTGCCATCCAGGTGGTT
	DC-bvgA-R	GATGCTCTAGAGCAAACCACTGCTAACAGCTAT
	Inv-bvgA-L	GGGATGATCATCGACTATCAATATCCTTG
	Inv-bvgA-R	ACCAACATGGTTGACTTGATTAACCTGGCCAA
ahsA	DC-S-For	GAGGCTCTAGAGCGGTTGTTGATCTGACAATTAGT
	DC-S-Rev	GGAGGCTCTAGAGCCCAAAACTCACGAAAATA
	Inv-S-L	GGCAGGCCTCATTAGAATCATTCTCCAAA
	Inv-S-R	GAAAGGCCTAACGCTCTGAACACCATGAA
mepA	DC-endo-F	GAAGCTCTAGAGCCATCTCTGTCATCGGTGTTGA
	DC-endo-R	TAGCTCTAGAGCAAGGCGAGTGGATCACCAAACGA
	Inv-endo-L	GGGAGTCGCTTTCATCATGTTTCCTCA
	Inv-endo-R	GCCGAGAATACGCCGAGCGAAAACTGATT
serA	DC-ser-F	GAAGCTCTAGAGCGATCTTGTGCTGCCCAAGCACA
	DC-ser-R	AGCTCTAGAGCGTTTGGCTGCAGGCAGGTTAACAA
	Inv-ser-L	CCGAGATTGCCAACGCTAACGATGTTTTTCTCA
	Inv-ser-R	AACAACAGCCAACCGGGTCAACTGCTCAA
ascN	DC-ascN-F	GAAGCTCTAGAGCTGTTGCTGTCGCTTAGCTGTA
	DC-ascN-R	GATGCTCTAGAGCTTCTCCACCAATGCAATCA
	Inv-ascN-L	CAGTGGCTCAAACAAGGCACCCATGAA
	Inv-ascN-R	GCCTGCAATGTGGTCGAAGGAAAGATTCAT
ompAI	DC-omp-F	GTAGCTCTAGAGCGTGCAGTTTTACACCCTTACT
	DC-omp-R	GATGCTCTAGAGCATGGTTCCTGGATACTCACGA
	Inv-omp-L	GATGGCGATCAGGGAAGGAGCCATTTTCATCAT
	Inv-omp-R	GTGAAAGCCAAAGCTCAGCTGATCTCCTGCCT
ahyR	DC-ahyR-F	GAAGCTCTAGAGCGTCGCCTTTATTCTGTGA
	DC-ahyR-R	AAAGCTCTAGAGCAGGCTGTAGAACCAGACCAG
	Inv-ahyR-L	CAGTTGGTCTTGTTTCATATGCTAGCC
	Inv-ahyR-R	CCCAAGCTGATGCAATAAAACCCGATGA

transformed into *E. coli* JM109. The internal fragment was recovered by *Xba*I restriction digestion, and ligated to *Xba*I-digested and dephosphorylated suicide vector pRE112 (Edwards *et al.*, 1998). The recombinant plasmid was transformed into *E. coli* MC1061 (λ*pir*), selecting for Cm^r to isolate the pRE-112-derived plasmids. These Plasmids were transformed into *E. coli* S17-1(λ*pir*) and transferred by conjugation to *A. hydrophila* AH-1 (Ap^r), selecting for Cm^r and Ap^r colonies. The insertion of plasmids into the chromosomes of these mutants was confirmed by PCR using appropriate primers.

Non-polar deletion mutants were constructed according to the method based on suicide plasmid pRE112 (Edwards *et al.*, 1998). Briefly, the targeted gene and at least 300 bp flanking sequences were amplified with oligonucleotides containing *Xba*I restriction sites and then cloned into pGEM-T easy vector, followed by inverse PCR using these constructs as templates. The inverse PCR products were purified and self-ligated to get the deleted constructs of targeted genes. The deletion constructs were digested using *Xba*I and ligated to *Xba*I-digested and dephosphorylated pRE112. This construct was transformed into *E. coli* S17-1(λ*pir*). The single cross-over mutants were obtained by conjugal transfer into *A. hydrophila* AH-1. Double cross-over mutants were obtained by selecting against the presence of the *sacB* gene carried by the vector. Mutants resistant to sucrose were isolated by plating onto LB-sucrose agar (1% tryptone, 0.5% yeast tract, 1.5% agar, 12% sucrose). The double cross-over mutants were confirmed by PCR.

III.2.3 Preparation of *A. hydrophila* genomic DNA

Genomic DNA plugs of *A. hydrophila* strains were prepared according to the CHEF genomic DNA plug kits (Bio-Rad, United Kingdom) instruction manual. *A. hydrophila* strains were inoculated into 50 ml of TSB Broth and grew with agitation to an OD_{600} of

0.8-1.0 at 25^{o}C. Chloramphenicol was added to a final concentration of 180 μg/ml and the incubation was continued up to 1 h. Meanwhile, a 20 time dilution of this bacterial suspension was made using 1 ml bacteria, 1 ml Gram Crystal Violet, and 18 ml PBS. The bacterial concentration was measured by placing a small amount of the bacterial suspension on a haemocytometer and counting at 400 × magnification under an inverted microscope. After incubation with chloramphenicol for 1 h, 5 ×10^{8} cells in agarose plugs were prepared. The bacteria were resuspended in 0.5 ml Cell Suspension Buffer (10 mM Tris-HCl, pH 7.6; 1 M NaCl) for each 1 ml of agarose plugs and combined with 0.5 ml 2% CleanCut agarose which was melted and equilibrated to 50^{o}C in a water bath. The mixture was gently and thoroughly mixed. It was kept at 50^{o}C in a water bath and then transferred to plug moulds. The agarose was allowed to solidify at 4^{o}C for 10-15 min. Meanwhile, lysozyme solution was prepared by adding 100 μl of Lysozyme stock to 2.5 ml of Lysozyme Buffer (10 mM Tris-HCl, pH 8.0) for each 1 ml of agarose plug in a 50 ml conical centrifuge tube. The solidified agarose plugs were pushed into the tube and incubated for 2 h at 37^{o}C. The lysozyme solution was removed at the end of 2 h and the plugs were rinsed with sterile water. 2.5 ml of Proteinase K Reaction Buffer (0.5 M EDTA, pH 9.0; 1% Sarkosyl) and 100 μl of Proteinase K stock were added for each 1 ml of agarose plug and incubated at 50^{o}C overnight without agitation. The plugs were washed using 1 ml of 1x wash Buffer [10 mM Tris-HCl; 1 mM EDTA, pH 8.0] per ml agarose plug for four times, 1 h each at room temperature with gentle agitation the next day. 1 mM PMSF was added during the second or third wash to inactivate residual Proteinase K. The plugs were then stored in 1x wash Buffer at 4^{o}C.

III.2.4 Restriction enzyme digestion of *A. hydrophila* genomic DNA plugs

One plug was taken out and placed in a sterile 1.5 ml microcentrifuge tube. The plug was washed in 1 ml 0.1x wash Buffer for 1 h with gentle agitation. The buffer was decanted and the plug was rinsed with 0.5 ml 0.1x wash Buffer to reduce the EDTA concentration. 1 ml of the appropriate 1x restriction enzyme buffer was then added. The plug was incubated at room temperature for one h. The buffer was aspirated off after incubation and 0.4 ml of fresh 1x restriction enzyme buffer was added and followed by an addition of 30-50 Unit of restriction enzymes and incubation at the appropriate temperature overnight. The buffer was removed and the plug was incubated in 1 ml of 1x wash Buffer for about 30 min with gentle agitation.

III.2.5 Pulse field gel electrophoresis (PFGE)

The restriction digested genomic DNA plugs were loaded into the well of precast 1% agarose gel (Pulse Field Gel Electrophoresis Agarose, Bio-Rad) using long gel cast (21 ×14 cm, Bio-Rad), followed by sealing of wells with 0.8% low melting point agarose (GIBCOBRL). CHEF DNA Size Standard, Lambda Ladder (Bio-Rad) with a range of 0.05-1 Mb was used as a size marker to determine the size of DNA fragments. The running buffer was 0.5x TBE. The gel was run in the CHEF-DRII PFGE system (Bio-Rad) at 5~60 seconds, 200V for 22 h. The PFGE system was connected to a model 1000 Mini chiller (Bio-Rad) and a variable speed pump (Bio-Rad) to maintain the temperature of running buffer at 14^{o}C. DNA was visualized by staining gels in TBE buffer containing 10ng ethidium bromide per ml for 10 min. The size of each digested fragment was estimated by comparing with the Lambda ladder (Bio-Rad).

III.2.6 Nucleotide sequence accession numbers

Thirteen genes of *A. hydrophila* AH-1 or AH-3 for construction of knockouts were assigned with corresponding accession numbers: *aerA* (AY442276), *bvgA* and *bvgS* (AY841798), *f85* (AY442275), *flhA1* (AY841793), *flhA3* (AY841794), *hlyA* (AY442273), *hup1* (AY442272), *hup3* (AY841797), *mepA* (AY841796), *ompAI* (AY442271), *opdA* (AY442274), and *serA* (AY841795).

III.3 Results and discussion

III.3.1 Summarization of putative virulence genes identified from two rounds of genomic subtraction

Genomic subtraction approach has been used to identify common virulence genes based on screening genetic differences between virulent and avirulent strains of *A. hydrophila* (Zhang *et al.*, 2000). Two rounds of genomic subtraction have led to the identification of 22 unique DNA fragments (F97 and PB45 are in the same DNA fragment) which were commonly present in the eight virulent strains (≥ 4) (Table III.3; Zhang *et al.*, 2000; Zhang *et al.*, unpublished data). These twenty-two DNA fragments were assumed to encode potential virulence genes and were subjected to further analysis.

III.3.2 Sequence analysis of the twenty-two unique DNA fragments

Genome walking was carried out to obtain complete ORFs as well as upstream and downstream sequences of these 22 DNA fragments in order to predict their genetic organizations and functions (Table III.3). Fifteen of the DNA fragments encode nineteen proteins with homologues in other bacteria. Eleven of these homologues showed significant homology to virulence-associated factors (Table III.3). These included known

Table III.3. Summary of putative virulence genes identified in *A. hydrophila* PPD134/91

Clone	Size (aa)	Position (bp)	Accession no.	Homologies to predicted encoded protein	E value	% identity (olh[a])	Homologue accession no.	Location on physical map
F2/F3	344	172-1203	AF146597	Outer membrane protein (OmpAI) of *A. salmonicida*	e-135	78(344)	CAA63036	C5
†F11	266	993-1790*	AY376445	VsdC protein of *S. typhimurium*	e-55	52(593)	P24419	C3
	266	1790-2587		Type III secretion protein (*E. carotovora* subsp. *atroseptica* SCRI 1043)	2e-93	62(265)	YP_050200	
†F20	ND	2-712	AY376446	Probable acinetobactin biosynthesis protein (*A. baumannii*)	2e-44	40 (697)	BAC87898	C19
†F32	711	282-2414	AY378288	Unknown	--	--	--	C14
†F34	54	35-196	AY452280	Unknown	--	--	--	C14
F52	98	1061-1354	AF146598	HU alpha protein (HU) of *S. marcescens*	4e-29	82(90)	P52680	C14
F58	103	774-1082	AY378289	Unknown	--	--	--	C14
F61/F72/ F88/F109	680	1-2040	AF146608	Oligopeptidase A (OpdA) of *Y. pestis*	0	64(678)	NP_407415	C14
†F85	79	198-434	AY378290	Unknown	--	--	--	C14
†F87	606	5-1822	AY378291	Unknown	--	--	--	C14
F89	150	562-1011	AY378292	YaiI/YqxD family protein of *S. oneidensis*	2e-46	64(146)	NP_718466	C6
F92	ND	73-615	AY378293	Partial ORF of Para-aminobenzoate synthase component I of *V. vulnificus*	e-31	49(155)	NP_761116	C7
F93	102	112-417	AY378294	Putative exported protein of *Y. pestis*	3e-17	49(81)	NP_404091	C8
†F97/PB45	316	8-955	AY378295	Reverse transcriptase like protein of *Pirellula* sp.	e-38	32(321)	NP_866340	C18
F99/F106	409	267-1493	AF146029	Transporter transmembrane protein of *R. solanacearum*	2e-40	27(376)	NP_521519	C15
F108	557	2675-4345	AF146599	Hemolysin (HlyA) of *A. hydrophila*	0	76(577)	A61372	C16
†PA1/PA4/ PB62	263	1171-1959	AY378297	Unknown	--	--	--	C8
PA6/PA98/ †PB78/PB38	190	449-1018	AY378298	Unknown	--	--	--	C19
PA91	207	5841-6461	AY378300	Putative positive transcription regulator BvgA of *B. pertussis*	3e-45	44(209)	NP_880570	C15
	1252	6467-10222*		Virulence sensory protein BvgS of *B. pertussis*	e-156	32(1238)	P16575	
†PB28/PB35	160	96-575	AY378301	Topoisomerase of *P. aeruginosa*	8e-13	57(56)	AAD20003	C18
				Transmembrane protein of *R. solanacearum*	3e-07	48(50)	NP_521535	
†PB60	314	54-995	AY378302	Sensory box/GGDEF family protein of *C. crescentus*	9e-42	34(311)	NP_421888	C16
PB80/PA26	488	1128-2591*	AY378303	Aerolysin (AerA) of *A. hydrophila*	0	84(488)	AAL04124	C15
	527	2856-4436		Arylsulfotransferase of *E. rectale*	e-13	26(352)	AAK14940	

*For clones which have two ORFs, these fragments are used for Southern hybridization to show they are common in eight virulent but not in six avirulent strains.
"/"denotes that these subtracted clones are overlapping with each other; [a]the overall length of homologous proteins; "†"Clones chosen for genome walking;
ND, not determined

virulence factors of *Aeromonas* species such as hemolysin (HlyA, F108) and aerolysin (AerA, PA26/PB80).

The other nine genes identified are new putative virulence factors for *A. hydrophila* and have high similarities to known virulence proteins of other pathogens, including the homologues of a histone-like protein (HU, F52), oligopeptidase A (OpdA, F88/F109/F61/F72), outer membrane protein (OmpAI, F2/F3), VsdC and a type III secretion protein homologue (F11), acinetobactin biosynthesis protein (F20), a two component BvgA and BvgS system, and arylsulfotransferase.

VsdC is essential for virulence in *S. dublin* (Krause *et al.*, 1991). The VsdC homologue in *A. hydrophila* contains a VIP2 domain which belongs to a family of actin-ADP-ribosylating toxins. The presence of a type III secretion protein homologue in *A. hydrophila* PPD134/91 correlates well with the identification of a TTSS in this strain, as discussed further below. The acinetobactin biosynthesis protein F20 is involved in iron acquisition and may have an important role in the pathogenesis of *Acinetobacter baumannii* (Mihara *et al.*, 2004). In *B. pertussis*, the *bvg*A and *bvg*S two-component system controls the expression of many virulence genes, including those encoding pili (Mooi *et al.*, 1987) and adenylate cyclase (Glaser *et al.*, 1988). These *bvgA* and *bvgS* homologues may thus also be involved in the regulation of pathogenesis in *A. hydrophila* PPD134/91. On the other hand, arylsulfotransferase has been suggested to be a virulence factor of the *Campylobacter-Wolinella* family of many oral bacteria and is widely distributed in *Campylobacter* species (Wyss, 1989; Yao and Guerry, 1996).

The rest of the eight proteins showed high homology to proteins in other bacteria that have not yet been linked to virulence. They are topoisomerase (PB28/PB35), GGDEF family

protein (PB60), YaiI/YqxD family protein (F89), para-aminobenzoate synthase (F92), a putative exported protein (F93), a tranporter transmembrane protein (F99/F106), a transmembrane protein (PB28/PB35) and a reverse transcriptase-like protein (F97). The functions of these proteins in *A. hydrophila* pathogenesis remain to be clarified.

For the last seven DNA fragments (F32, F34, F58, F85, F87, PA1/PA4/ PB62, PA6/PA98/PB38/PB78), the ORFs encoded have no significant matches with entries in the GenBank database and may represent novel virulence determinants. These presumptive virulence factors will be studied in future experiments.

III.3.3 Identification of a phage-associated genomic island

The second approach adopted to search for putative virulence genes was to look for pathogenicity island (PAI) markers. PAIs are specific genomic islands with a large unstable chromosomal region that encodes virulence genes, and are present in most of the pathogenic bacteria (Hacker and Kaper, 2000). Recently, *ssrA*, a small stable RNA molecule (tmRNA) has been reported to reside in or near the junction point of a mosaic of *Salmonella*-specific sequences (Conner *et al.*, 1998) and serves as the insertion site for acquired sequences such as the cryptic phage CP4-57 in *E. coli* and PAIs in *V. cholerae* (Karaolis *et al.*, 1998) and *Dichelobacter nodusus* (Billington *et al.*, 1996).

Primers *ssrA*-F (5-CAAACGACGAAAACTACGC-3) and *ssrA*-R (5-GGTACTACATGCTTAGTC) within the conserved *ssrA* region were designed and an *ssrA* gene was identified in *A. hydrophila* PPD134/91 via a PCR reaction using these primers. Subsequently, a series of genome walking led to the identification of a 23 kb DNA region. Flanked by *ssrA* at the left end, this region exhibits significantly lower G+C content (49.7%) than the average genome G+C content of *A. hydrophila* (57-63%) which

is a common characteristic of GIs (Hacker and Kaper, 2000). It also shows a mosaic distribution of G +C content (Table III.4, Fig.III.1). Furthermore, two ORFs (ORF1 and ORF2, Table III.4) of this region show high similarities to the insertion element IS1650 and are adjacent to *ssrA*. The G + C content of thirteen ORFs (Table III.4) did not differ significantly from that of *A. hydrophila*, indicating that they might have been acquired from species with G + C content similar to that of *A. hydrophila* or the base composition of these acquired DNA has gradually adapted to the host genome (Lawrence and Ochman, 1996).

Many putative virulence factors are bacteriophage-encoded (Boyd and Brussow, 2002), and like insertion elements, bacteriophages are often associated with GIs and are involved in the transfer of virulence determinants between bacterial species (Hueck, 1998). This island carries several homologues to bacteriophage-encoded proteins (Table III.4), suggesting they are good virulence factor candidates. Furthermore, a conserved protein domain database search indicated that ORF6 contains an AAA domain which belongs to an AAA-superfamily of ATPases which are associated with a wide variety of cellular activities, including membrane fusion, proteolysis, and DNA replication. More interestingly, both ORF16 and ORF19 contain a Helix-turn-helix XRE domain, suggesting they may function as transcriptional regulators. Sixteen out of the twenty-four ORFs did not show any good homology in the GenBank database, suggesting that they may encode novel proteins. This putative GI thus consists of a mosaic of segments similar to those found in various bacteriophages and is therefore referred to as phage-associated island.

The distribution of this putative GI among 14 *A. hydrophila* strains was surveyed by Southern analysis using probes from four regions of the DNA (Fig.III.1,). The four probes

Table III.4. Homology and G+C content for open reading frames of phage-associated island (Accession no. AY442269)

ORF	Name	Homology	G+C %	E value	% identity (olh[a])	Homologue accession No
	ssrA	*ssrA* gene (*A. salmonicida)*)	52.1	e-144		AF440330
1	Transposase	IS1650 orf B (*S. flexneri*)	54.3	e-29	46(171)	AAK18513
2	Transposase	IS1650 orf A (*S. flexneri*)	55.2	4e-25	46(149)	AAK18514
3	Hypothetical protein	Hypothetical protein (Bacteriophage VT2-Sa)	59.7	0.03	25(2806)	NP_050570
4	Putative phage nin-region protein	Putative phage nin-region protein (*Y. pestis* KIM)	59.9	7e-37	54(148)	NP_993273
5	Hypothetical protein	Hypothetical protein (*N. punctiforme*)	49.5	e-05	37(216)	ZP_00109981
6	Hypothetical protein	Hypothetical protein (*T. maritime*)	33.6	3e-04	22(718)	NP_228994
7	No good homology		31.3			
8	Unknown protein	Unknown (*Z. mobilis*)	56.7	2e-30	43(318)	AAD19409
9	No good homology		33.6			
10	Putative phage protein	Putative phage protein (*S. typhimurium* LT2)	37.8	5e-22	28(443)	NP_461181
11	No good homology		50.0			
12	No good homology		32.8			
13	No good homology		62.0			
14	Replication Protein	Replication prtotein (*S. flexneri* bacteriophage V)	59.3	e-31	55(272)	AAL89441
15	Hypothetical protein	Gp12 (*B. cenocepacia* phage Bcep1)	59.8	e-26	36(181)	NP_944320
16	Putative phage repressor	Putative phage repressor (*B. bronchiseptica* RB50)	51.5	4e-05	34(227)	NP_888223
17	No good homology		60.8			
18	No good homology		50.9			
19	Transcriptional prtotein	Predicted transcriptional regulators (*A. pleuropneumoniae* serovar 1 strain 4074)	60.3	e-04	38(112)	ZP_00135591
20	No good homology		52.8			
21	No good homology		64.0			
22	Exodeoxyribonuclease VIII	Gifsy-2 prophage; exodeoxyribonuclease (*S. typhimurium* LT2)	55.9	3e-38	31(975)	T03004
23	Hypothetical protein	Hypothetical protein (*S. enterica* subsp. *enterica* serovar Typhi)	55.7	2e-61	62(350)	NP_456435
24	Hypothetical protein	Hypothetical protein (*B. cereus* ZK)	46.2	0.003	27(613)	YP_082425

[a]overall length of homologous protein

Base composition: Window size =100 reference line: 49.7% G+C content

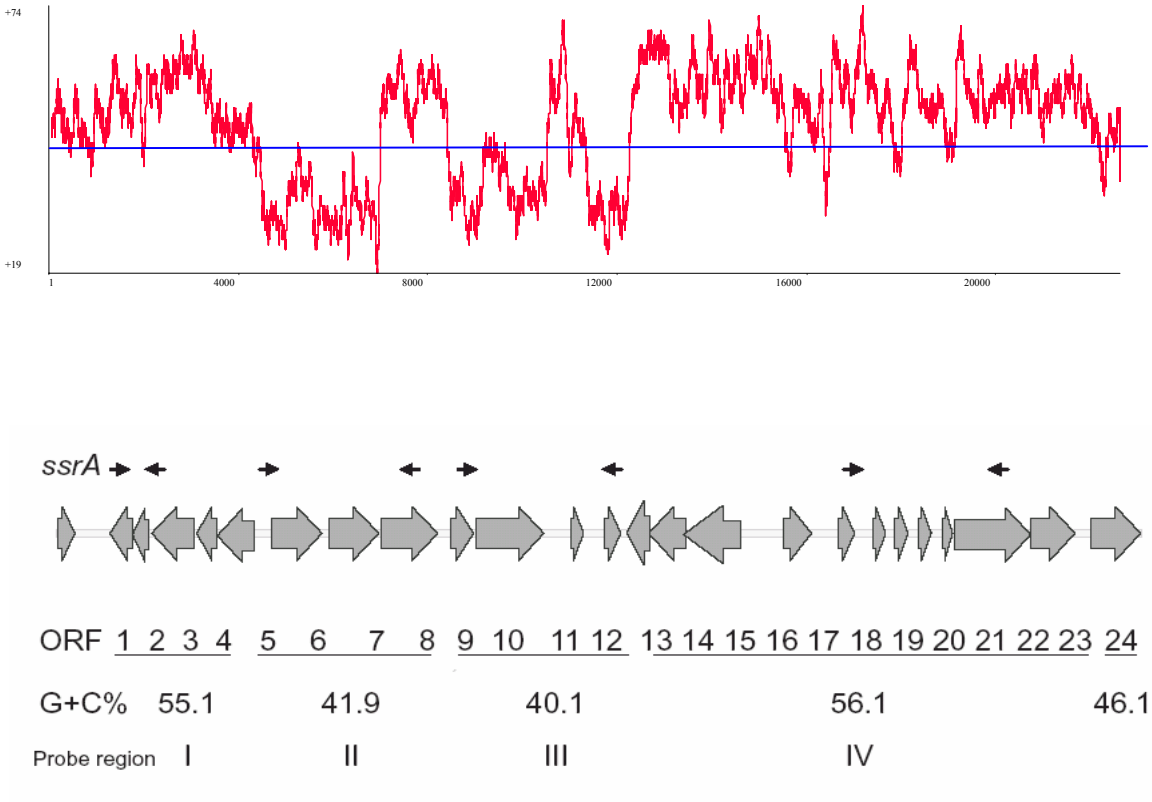


Fig. III.1. G + C content of each region for the phage-associated island and the location of probes used for southern blot. "→ ←" indicates the location of primer pairs for the probes.

hybridized with most of the eight virulent strains but only hybridized with one out of the six avirulent strains tested (Fig.III.2, Table III.5). The hybridization signals are strong in virulent strains ATCC7966, PPD134/91 and Xs91-4-1, and the avirulent strain L36 but weak in the other five virulent strains, suggesting some genetic variations among the virulent strains. The results therefore indicate that this putative GI is PPD134/91-specific and/or virulent strain specific. Identification and sequencing of this GI in other virulent strains will help to clarify this issue.

Phage-mediated integration events may be involved in the acquisition of this phage-associated island. Phages contribute to the evolution of bacterial pathogens through gene transfer at the time of infection (Wagner and Waldor, 2002). It has been reported that removal of a portion of a *Salmonella*-specific region which is near *ssrA* and contains a number of mobile elements such as IS and bacteriophages conferred the greatest defect in virulence (Conner *et al.*, 1998). This phage-associated island may also contribute to differences in host specificity or disease manifestation.

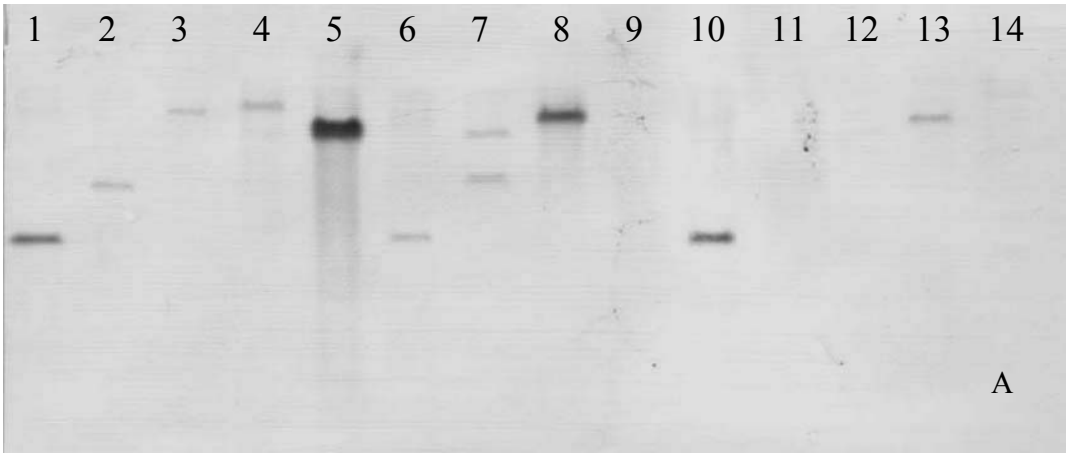
III.3.4 Identification of a TTSS gene cluster

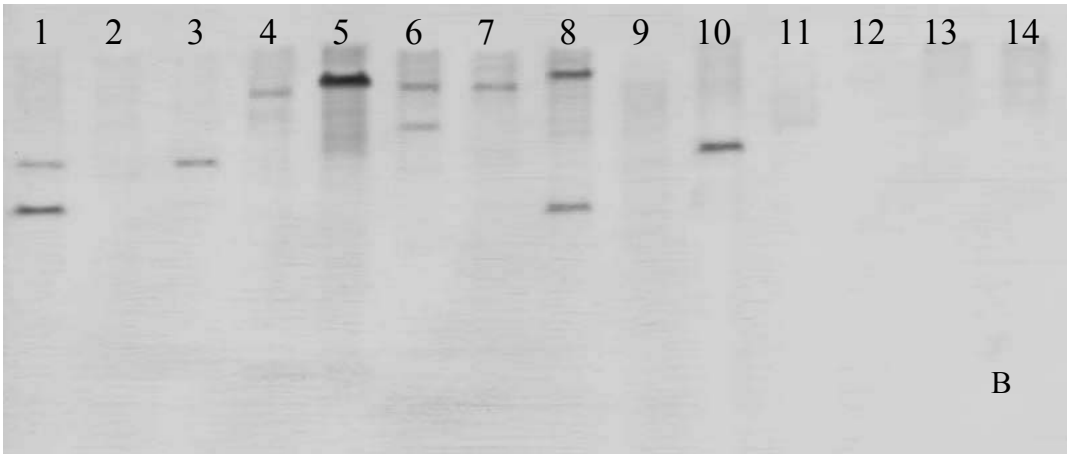
TTSSs have been reported in many Gram-negative animal and plant pathogens (Cornelis and Gijsegem, 2000; Chacon *et al.*, 2004). As reported later (Chapter V), an *ascV* homologue is present in *A. hydrophila* PPD134/91. Furthermore, an *ascU* homologue, similar to the one near one end of the TTSS in *A. hydrophila* AH-1, was also identified in *A. hydrophila* PPD134/91 by a PCR reaction (*ascU*-F: 5-GGTGATCGCCATCGCCGA-3; *ascU*-R: 5-ACGGCGCTTGCTCTTGAT-3). The identification of these two TTSS homologues strongly indicated a TTSS cluster is also present in *A. hydrophila* PPD134/91. This TTSS cluster was located on the chromosome of *A. hydrophila* PPD134/91 (Fig.III.3). The

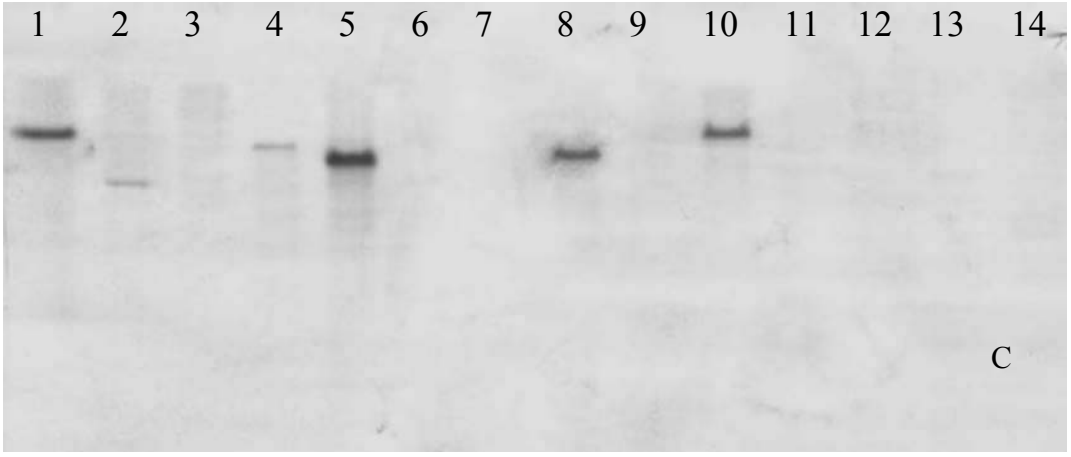
Table III.5. Distribution of the ORFs from phage-associated island in different *A. hydrophila* strains

Strain	Probes used for Southern analysis			
Virulent strain	I	II	III	IV
ATCC7966	+	+	+	+
L31	±	-	±	±
PPD11/90	±	±	-	-
PPD70/91	±	±	±	-
PPD134/91	+	+	+	+
PPD122/91	±	±	-	±
AH-1	±	±	-	±
Xs91-4-1	+	+	+	+
Avirulent strain				
L15	-	-	-	-
L36	+	+	+	+
PPD35/86	-	-	-	-
PPD45/91	-	-	-	-
PPD64/90	±	-	-	-
PPD88/90	-	-	-	-

Hybridization results: +, strong signal; ±, weak signal; -, no signal


1 2 3 4 5 6 7 8 9 10 11 12 13 14
A


1 2 3 4 5 6 7 8 9 10 11 12 13 14
B


1 2 3 4 5 6 7 8 9 10 11 12 13 14
C

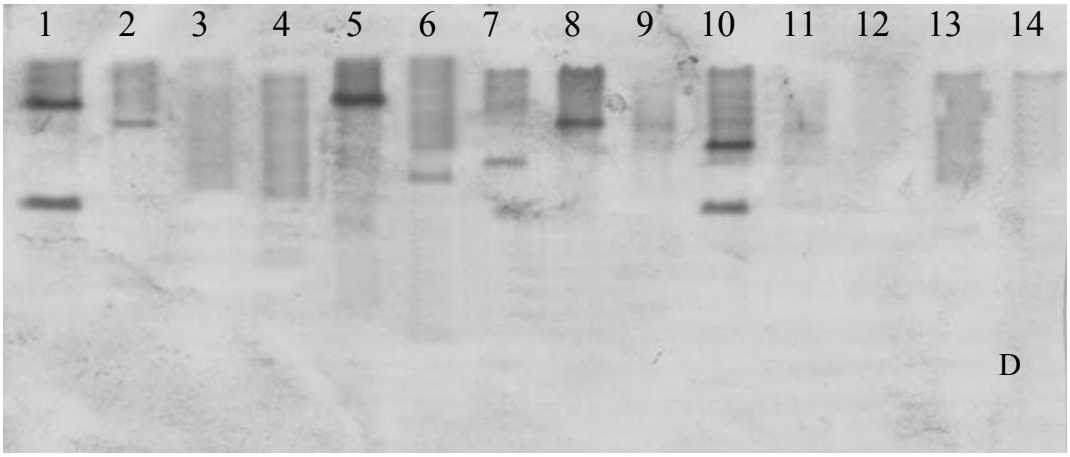


Fig. III.2. Distribution of phage-associated island genes among 14 virulent and avirulent *A. hydrophila* strains. Southern hybridization was carried out using different genes as probes. Lane 1~14 are genomic DNA from ATCC7966, L31, PPD11/90, PPD70/91, PPD134/91, PPD122/91, AH-1, Xs91-4-1, L15, L36, PPD35/86, PPD45/91, PPD64/90 and PPD88/90, respectively. A. Probe from region I; B, Probe from region II; C, Probe from region III; D, Probe from region IV.

complete TTSS sequence of *A. hydrophila* PPD134/91 is not yet available. However, analysis of the TTSS gene cluster of *A. hydrophila* strain AH-1 revealed an ORF found near *ascU* showing high homology to the P4-family integrase of a variety of bacteria. Hueck (1998) reported that the TTSS may be acquired as intact genetic blocks by horizontal gene transfer during evolution. It is thus reasonable to speculate that the integrase may have been involved in the original mobilization of the TTSS into the chromosome of *A. hydrophila*. Downstream sequencing of *ascU* in *A. hydrophila* PPD134/91 will facilitate the understanding of the evolutionary history of this TTSS. Moreover, complete TTSS gene clusters have been recently identified in the two *A. hydrophila* strains AH-3 and SSU (Vilches *et al.*, 2004; Sha *et al.*, 2005), which further suggests a complete TTSS gene cluster may be also present in PPD134/91.

III.3.5 Mapping of putative virulence genes on the physical map of *A. hydrophila* PPD134/91

With a variety of novel putative virulence genes identified in this study, it was necessary to understand their genetic organization in the genome of *A. hydrophila* PPD134/91, in order to provide insight into understand the mechanisms of gene regulation and the molecular basis of pathogenicity of this bacterium. A physical map of *A. hydrophila* was therefore constructed (Fig.III.3).

In the previous studies, seven unique DNA fragments (out of the total 22 DNA fragments), O-antigen cluster and capsule cluster have been positioned on the physical map (Zhang *et al.*, unpublished data). Similarly, we located the rest fifteen unique DNA fragments, the phage-associated island and the TTSS gene cluster on the physical map by Southern hybridization (Fig.III.3 and 4). The results showed O-antigen and capsule gene clusters

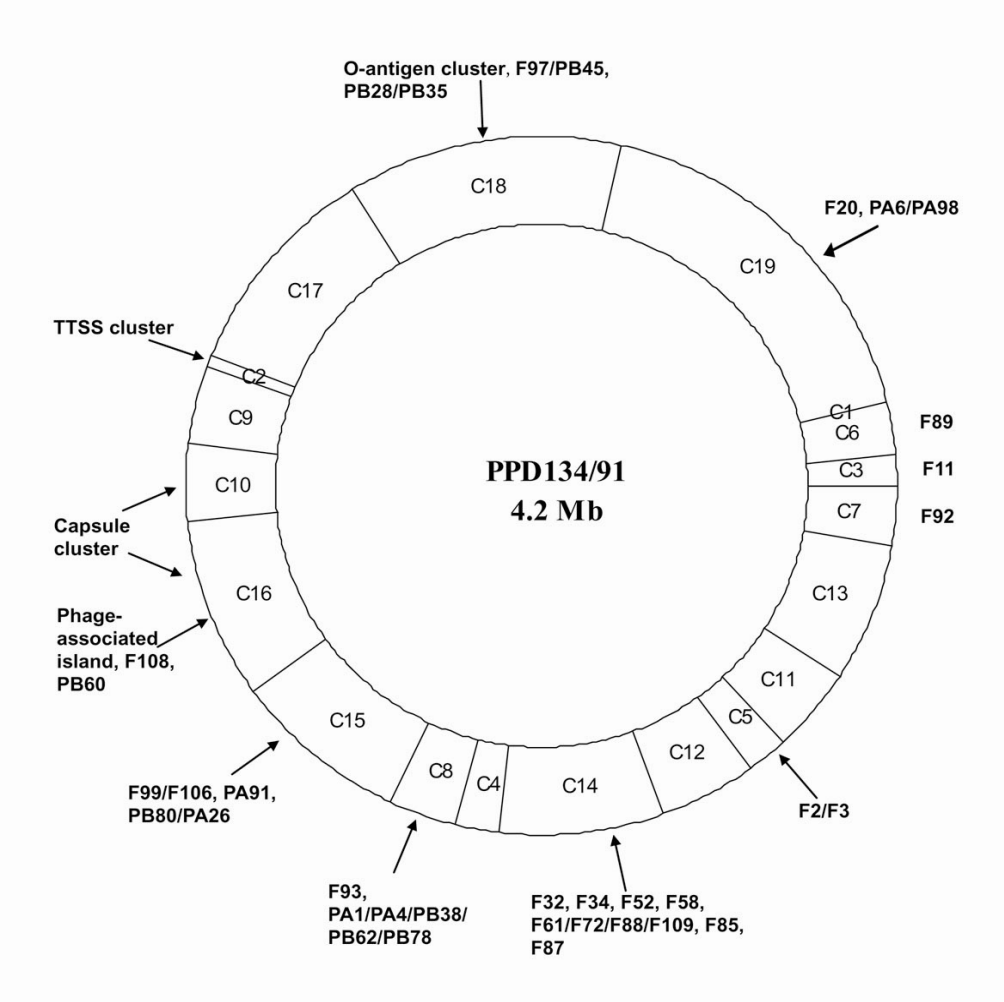


Fig. III.3. Physical map of *A. hydrophila* PPD134/91. The locations of virulence genes and clusters were determined with respect to *Pac*I fragments.

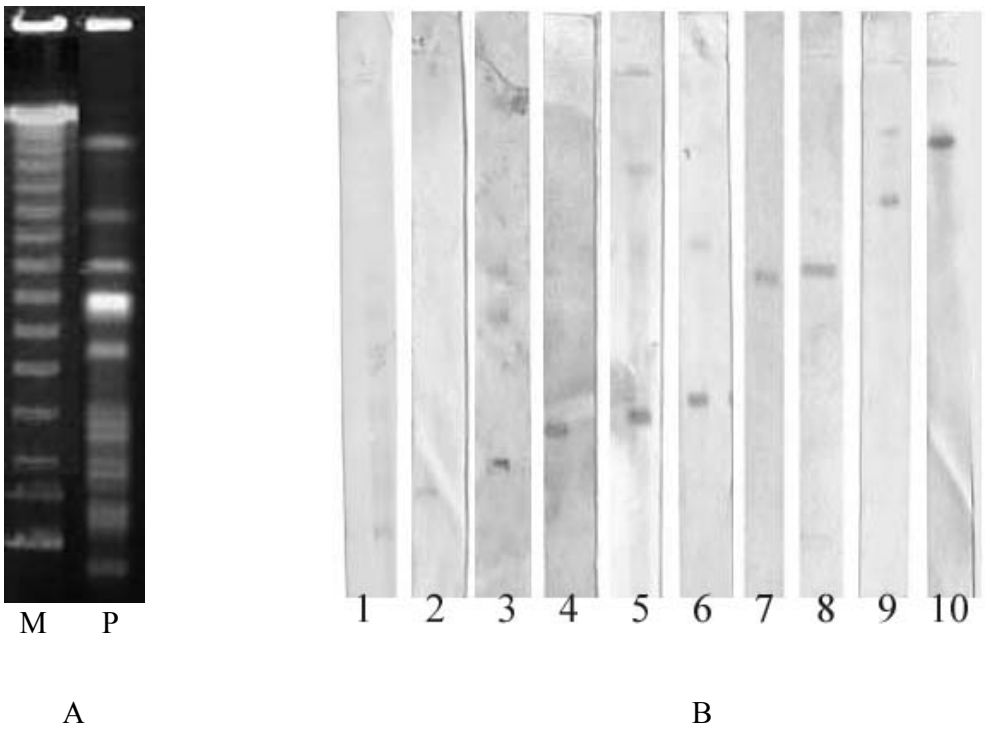


Fig. III. 4. PFGE analysis of *Pac*I digested genomic DNA of *A. hydrophila* PPD134/91 (A) and Southern hybridization analysis of the locations of virulence-related fragments in *Pac*I digested genomic fragments of *A. hydrophila* PPD134/91 (B).
A: M, λ ladder; P, PPD134/91 digested with PacI
B: Lane 1, TTSS cluster (C2); lane 2, F11 (C3); lane 3, F2/3 (C5); lane 4, F89 (C6); lane 5, F92 (C7); lane 6, F93, PA1/PA4/PB38/PB62/PB78 (C8); lane 7, F99/F106, PA91, PB80/PA26 (C15); lane 8, phage-associated island, F108, PB60 (C16); lane 9, F97/PB45, PB28/PB35 (C18); lane 10, F20, PA6/PA98 (C19).

were located at different regions of the chromosome (Fig.III.3), while these two clusters were located near each other in *E. coli* (Schnaitman and Klena, 1993). The locations of the TTSS cluster and the F11 fragment which contains a type III secretion protein homologue are quite far away from each other. This was not surprising as the genes encoding a secretion apparatus are usually clustered while the genes encoding the secreted proteins and their transcriptional regulators are often located in unlinked positions (Hueck, 1998).

Ten subtracted clones representing seven unique DNA fragments were located in the same *Pac*I digested fragment C14 (Fig.III.3) of strain PPD134/91. However, now, there is no indication that these virulence genes are located in a PAI. If a PAI is present in C14, all of the common subtracted DNA fragments that are present in this region should also be clustered in one region in other virulent strains of *A. hydrophila*. Therefore, six of these common fragments (F32, F34, F52, F58, F85 and F87) were used as probes to hybridize with *Pac*I digests of eight pathogenic genomic DNA. Most of these genes were distributed in two to four DNA fragments rather than clustered in the same *Pac*I digested fragments in the genomes of seven other virulent strains using Southern analysis (Fig.III.5, Table III.6).

III.3.6 Construction and characterization of mutants

One strategy to examine whether the putative virulence genes identified in this study are involved in pathogenesis is to construct knockouts and test their virulence in the blue gourami fish model. Previous attempts to introduce plasmids or transposons into *A. hydrophila* PPD134/91 were not successful. A genetic barrier may exist in strain PPD134/91 and prevent the construction of knockouts in this strain. Therefore, other well studied pathogenic strains of *A. hydrophila* such as AH-1 and AH-3 (Merino *et al.*, 1999) were used as the hosts for the construction of mutants. Fifteen genes in *A. hydrophila*

Table III.6. The location of six putative virulence genes of *A. hydrophila* PPD134/91 on the chromosomes of other *A. hydrophila* virulent strains.

DNA fragments	F32	F34	F52	F58	F85	F87
PPD134/91	C14	C14	C14	C14	C14	C14
ATCC7966	C18	C18	C18	C17	C17	C17
L31	C9	C8	C8	C13	C16	C13
PPD11/90	-	C12	C15	C15	C13	C13
PPD70/91	-	C11	C11	-	C17	C17
PPD122/91	-	C11	C17	-	C17	C17
AH-1	C12	-	C14	C13	C15	C14
XS-91-4-1	-	-	C18	C10	C18	-

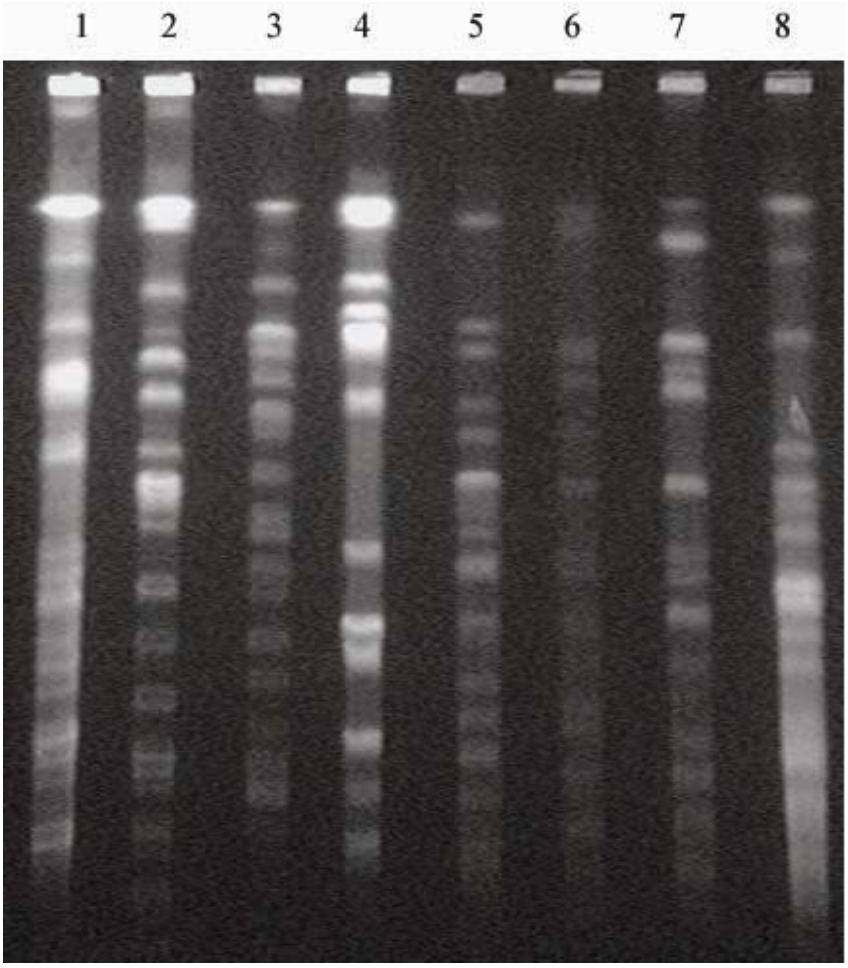


Fig. III. 5. PFGE analysis of *A. hydrophila* (virulent strains) genomic DNA digested with *Pac*I. Lanes 1 to 8 are genomic DNA plugs from strain PPD134/91, ATCC7966, L31, PPD11/90, PPD70/91, PPD122/91, AH-1, and Xs91-4-1, respectively.

AH-1 and/or AH-3 which are homologous to their corresponding genes in *A. hydrophila* PPD134/91 were successfully cloned for the construction of knockouts. In general, insertion mutants were constructed first to examine the effect of the target genes. If the LD_{50} values of insertion mutants increased by at least 0.5 logs, deletion mutants were then constructed in the same genes to confirm that the attenuation was not due to polar effects. We also included several deletion mutants such as *ΔompAI*, *ΔascN* and *ΔbvgA* to confirm there are no differences in the LD_{50}s between insertion and deletion mutations. *vsdC*, the putative type III secretion protein (F11), and the phage-associated island sequences could not be successfully cloned from strains AH-1 and AH-3, and these genes were thus not characterized in this study. The construction of knockouts in these PPD134/91-specific genes will shed light on their roles in PPD134/91 pathogenesis.

The genes used for construction of mutants can be classified into three groups (Table III.7). Group I includes hemolysin (*hlyA*) and aerolysin (*aerA*) which have been reported to be associated with the virulence of *A. hydrophila* (Allan and Stevenson, 1981; Wong *et al.*, 1998). Group II includes histone-like protein (*hup1* for AH-1 and *hup3* for AH-3) (Stinson *et al.*, 1998), oligopeptidase A (*opdA*) (Novak *et al.*, 1986), *bvgA*, *bvgS*, *ascN* (Chapter V) and outer-membrane protein (*ompAI*) (Weiser and Gotschlich, 1991) which are homologous to known virulence factors of other pathogens. Group III includes a novel putative virulence protein (*f85*) and a recently identified polar flagellar assembly protein homologue FlhA (*flhA1* for AH-1 and *flhA3* for AH-3). In addition, *ahyR*, a gene encoding quorum sensing regulator, was also mutated to evaluate its possible role in the pathogenesis of *A. hydrophila*.

Table III.7. LD_{50} of mutants and wild types of *A. hydrophila*

strain	LD_{50}	strain	LD_{50}
AH-1	$10^{5.3}$	AH-3	$10^{6.5}$
aerA	$10^{5.5}$	*bvgA*	$10^{6.5}$
ascN	$*10^{6.2}$	*bvgS*	$10^{6.2}$
f85	$10^{5.3}$	*flhA3*	$10^{6.3}$
flhA1	$10^{5.2}$	*hup3*	$10^{6.2}$
hlyA	$10^{5.4}$		
hup1	$10^{5.5}$	Δ*bvgA*	$10^{6.2}$
ompAI	$10^{5.1}$		
opdA	$10^{5.2}$		
Δ*ascN*	$*10^{6.3}$		
Δ*ompAI*	$10^{5.4}$		
Δ*ahsA*	$10^{5.3}$		
Δ*ahsA*Δ*ahyR*	$10^{5.3}$		
Δ*ahsA*Δ*mepA*	$10^{5.7}$		
Δ*ahsA*Δ*serA*	$10^{5.7}$		
Δ*ahsA*Δ*serA*Δ*mepA*	$*10^{6.5}$		

*denotes that P value < 0.05. For all the other mutants, P value > 0.05.

Our results showed that the LD_{50}s of all the mutants except *ascN* were comparable to that of the wild type strains using blue gourami fish as the infection model (Table III.7). The LD_{50}s of the wild types AH-1 and AH-3 were $10^{5.3}$ and $10^{6.5}$, respectively. LD_{50}s of both insertion and deletion mutants of *ascN* in AH-1 were determined to be $10^{6.3}$. Wong and coworkers (Wong *et al.*, 1998) reported that inactivation of *hlyA* or *aerA* alone showed no statistically significant attenuation in a suckling mouse model when compared to the wild type. Our virulence assay showed similar results for these genes, and extended this finding to most of the other virulence genes which we analyzed. Interestingly, fish infected with an *ahyR* mutant appears to die a little faster than those infected with the wild type although this mutant exhibited the same LD_{50} as wild type. AhyR, a member of LuxR-type response regulators which have been reported to be involved in positive regulation of

exoprotease production of *A. hydrophila* AH-1 and negative regulation of TTSS in *Pseudomonas aeruginosa*, *Vibrio harveyi*, *Vibrio parahaemolyticus* (Swift *et al.*, 1999; Henke and Bassler, 2004; Bleves *et al.*, 2005). Therefore, it will be interesting to investigate the relationship between quorum sensing and TTSS in *A. hydrophila* AH-1. In all the mutants tested, only *ascN* exhibited a one log higher LD_{50} than that of the wild type strain AH-1. AscN, a homologue of the YscN of *Yersinia* species (Woestyn *et al.*, 1994), possibly interacts with membrane bound components of the TTSS apparatus to energize secretion or to provide the energy for the assembly of the secretion apparatus. Disruption or deletion of *ascN* may thus render the TTSS non-functional, leading to the increase in LD_{50} and consistent with our study described in Chapter V. The fact that most of the virulence genes did not attenuate the mutants suggested that the pathogenesis of *A. hydrophila* is multi-factorial in nature. Disruption of more than one gene or a whole gene cluster as in the case of TTSS appears to be necessary to observe differences in the LD_{50}s. On the other hand, the virulence of *A. hydrophila* may be also bacterial strain, infection route and animal model dependent. It has been reported that a single mutation in cytotoxic enterotoxin of *A. hydrophila* SSU resulted in a ~300 fold increase in LD_{50} by intraperitoneal injection in Swiss-Webster mice (Xu *et al.*, 1998) and a single mutation in elastase of *A. hydrophila* AG2 resulted in a ~100 fold increase in LD_{50} by intraperitoneal challenge in rainbow trout (Cascon *et al.*, 2000).

In addition, double deletions mutations were made in strain AH-1, such as S-layer and metalloprotease (*ΔahsAΔmepA*, $LD_{50} = 10^{5.7}$), S-layer and serine protease (*ΔahsAΔserA*, $LD_{50} = 10^{5.7}$), as well as a triple deletion mutant of S-layer, metalloprotease and serine protease (*ΔahsAΔserAΔmepA*, $LD_{50} = 10^{6.5}$). Different proteases have been shown to be

involved in *A. hydrophila* virulence (Cascon *et al.*, 2000). The double deletion mutants resulted in about half of a log increase in LD_{50}s but the attenuation was not statistically significant ($P>0.05$). However, fish infected with these double mutants survived 1 to 2 days longer when compared to those infected with the wild type at the same lethal dosage (Fig.III.6). Statistically significant attenuation was observed for the triple mutant whose LD_{50} increased about one log (Table III.7). More apparently, the fish infected with this triple mutant survived significantly longer (Fig.III.6). In addition, the fish infected with these mutants survived much longer compared to those infected with the wild type at the same lethal dosage (Fig.III.6). Our results therefore strongly support the idea that virulence factors in *A. hydrophila* pathogenesis work in a concerted manner and multiple factors are required to produce the observed deleterious effect.

III.4 Conclusion

A variety of putative virulence genes in *A. hydrophila* have been identified by both genomic subtraction and GI analysis in this study. These include known *A. hydrophila* virulence genes (encoding for hemolysin and aerolysin) as well as other genes showing homologies to known virulence factors, such as *bvgA*, *bvgS*, *vsdC* and *ompAI*, which have not yet been examined in *A. hydrophila*. In addition, the putative virulence gene clusters, such as the presence of a phage-associated island and a TTSS in *A. hydrophila* PPD134/91 were established. Subsequent positioning of these putative genes and gene clusters on a physical map of strain PPD134/91 has helped us in better understanding the chromosome organization and the molecular mechanisms of pathogenicity in this bacterium.

This is the first report to present a comparative study of different virulence factors in *A. hydrophila* pathogenesis by constructing knockouts in the same strains and infecting the

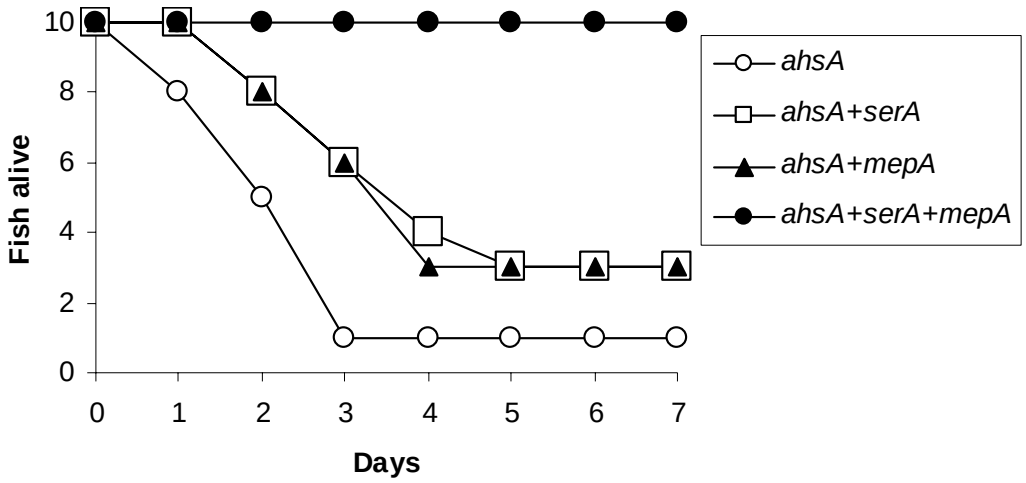


Fig. III.6. Survival of blue gourami fish after intramuscularly injection of *A. hydrophila* AH-1 and deletion mutants. Three groups of ten fish each were injected separately with the wild type (not shown in the graph as the result is the same as *ΔahsA*), *ΔahsA*, *ΔahsAΔmepA*, *ΔahsA ΔserA*, and *ΔahsA ΔserA ΔmepA* deletion mutants at a dosage of 1×10^6 CFU. Surviving fish were counted in a period of seven days.

same host. Our virulence assay of these mutants demonstrated that, as is increasingly observed for other pathogens, virulence in *A. hydrophila is* complex and involves multiple virulence factors which may work in concert. Construction of more multiple mutants, infection of different animal models and inclusion of other functional assays are underway to elucidate the mechanisms of these putative virulence factors. The putative virulence genes presented in this work will thus form the basis for further investigation of the pathogenesis of *A. hydrophila* and will be useful for data-mining for the development of effective vaccines, diagnostic and novel therapeutics against animal and human infection caused by motile aeromonads.

Chapter IV

Characterization of major secreted proteins of *Aeromonas hydrophila* AH-1

The results from this chapter are included in the following manuscript:

Yu, H. B., K. M. S. Rasvinder, S. M. Lim, X. H. Wang and K.Y. Leung. Characterization of major secreted proteins of *Aeromonas hydrophila* AH-1. (Submitted)

Abstract

A. hydrophila is a ubiquitous Gram-negative bacterium which can cause motile aeromonad septicemia in both fish and human. The pathogenesis of *A. hydrophila* is multi-factorial. *A. hydrophila* secretes many extracellular proteins associated with pathogenicity and environmental adaptability. In this study, an extracellular proteome map of *A. hydrophila* AH-1 was constructed. The major extracellular virulence factors were characterized by comparing the proteomes of various deletion mutants with that of the wild type. Our results suggest that the serine protease may be involved in the processing of secreted enzymes such as hemolysin, GCAT and metalloprotease. Using the proteomic approach and *lacZ* transcriptional fusion study, we also demonstrated that expressions of polar and lateral flagellins are under the control of temperature and other flagellar regulatory proteins. Most interestingly, a cross-talk between the lateral flagellar secretion system and the TTSS was discovered. In addition, many potential novel effector proteins secreted via the TTSS were revealed by comparing the extracellular proteomes of TTSS negative regulator mutants and the wild type.

IV.1 Introduction

A. hydrophila secretes many ECPs which contribute significantly to their wide distribution and great adaptability to environmental changes. Some virulence factors, such as the S-layers, polar flagella and lateral flagella are secreted and attached to the cell surface (Tomas and Trust, 1995; Altarriba *et al.*, 2003). Other extracellular virulence factors include proteases, GCAT, hemolysin, aerolysin and lipases (Pemberton *et al.*, 1997; Wong *et al.*, 1998; Janda, 2001). These virulence factors are secreted into the media via different secretion pathways (Pemberton *et al.*, 1997). However, the numbers and quantities of these secreted proteins remain unclear.

The TTSSs play very important roles in host-pathogen interactions (Cornelis and Gijsegem, 2000). This system can efficiently deliver anti-host virulence determinants into the host cells, directly interfering with and altering host processes. The TTSS has recently been shown to be required for the pathogenesis of *Aeromonas* species (Burr *et al.*, 2002, 2003a and 2003b; Vilches *et al.*, 2004). In *A. salmonicida*, one effector protein AexT was secreted into culture media via a TTSS (Burr *et al.*, 2003a). The identification of other effector proteins secreted by the TTSSs will provide insights into the signaling pathways involved in the pathogen-host interaction of aeromonads.

To identify proteins in complex mixtures, the proteomic approach has been widely adopted. Recently, a proteomic approach was used to identify outer membrane proteins in response to different conditions in *A. salmonicida* and to screen highly efficient vaccine candidates in *A. hydrophila* (Ebanks *et al.*, 2003; Chen *et al.*, 2004). Proteomic studies have also been performed to analyze the secreted proteins by various bacteria, such as

Erwinia chrysanthemi (Kazemi-Pour *et al.*, 2004), *Bacillus subtilis* (Hirose *et al.*, 2004), *H. pylori* (Bumann *et al.*, 2002) and *P. aeruginosa* (Nouwens *et al.*, 2002 and 2003).

This study was to establish an extracellular proteome map of *A. hydrophila* for a better understanding of the pathogenesis of *A. hydrophila*. The extracellular proteomes of several deletion mutants were also compared with that of the wild type to reveal the underlying mechanisms involved in the regulation of protein expressions or modifications. More interestingly, the proteomes of these TTSS negative regulators mutants were used to identify potential effector proteins for future study. In addition, *lacZ* transcriptional studies were used to confirm the proteomic results.

IV.2 Materials and methods

IV.2.1 Bacterial strains and culture conditions

The bacterial strains and plasmids used in this study are listed in Table IV.1.

IV.2.2 Primers used in this study

The primers used for the construction of deletion mutants, reporter fusions, and degenerate PCR are listed in Tables IV.2a and b.

IV.2.3 Construction of LacZ reporter fusions

For the construction of LacZ reporter plasmids, the putative promoter regions were amplified from *A. hydrophila* genomic DNA with the primers listed in Table IV.2a. The resulting PCR products were cut with *Eco*RI and *Bam*HI and cloned into pDN19lacΩ digested with *Eco*RI and *Bam*HI (Totten and Lory, 1990).

IV.2.4 β-galactosidase assays

A. hydrophila strains were grown in DMEM for 24 h at 25°C or 37°C in a humidified atmosphere of 5% CO_2. β-galactosidase activities were determined with cells

Table IV.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant property	Source / Reference
A. hydrophila strains		
AH-1	O:11, virulent, Col^r, Cm^s	Fish, UM
AH-1S	S-layer (*ahsA*) deletion mutant from AH-1	Table III.1
ΔserA	In-frame deletion of *serA* from AH-1S	Table III.1
ΔmepA	In-frame deletion of *mepA* from AH-1S	Table III.1
ΔserAmepA	In-frame deletion of *serA* and *mepA* from AH-1S	Table III.1
ΔflhA	In-frame deletion of *flhA* from AH-1S	This study
ΔlafK	In-frame deletion of *lafK* from AH-1S	This study
ΔrpoN	In-frame deletion of *rpoN* from AH-1S	This study
ΔaopN	In-frame deletion of *aopN* from AH-1S	This study
ΔexsD	In-frame deletion of *exsD* from AH-1S	This study
ΔaopNΔexsA	In-frame deletion of *exsA* from *ΔaopN*	This study
ΔlafA1	In-frame deletion of *lafA1* from AH-1S	This study
ΔlafA2	In-frame deletion of *lafA2* from AH-1S	This study
ΔexsA	In-frame deletion of *exsA* from AH-1S	This study
Plasmids		
pRE112	Suicide vector; R6K ori *sacB* Cm^r	Edwards *et al.*, 1989
PRE*ΔflhA*	pRE112 with *flhA* fragment deleted from 8 to 641 amino acids	This study
PRE*ΔlafK*	pRE112 with *lafK* fragment deleted from 27 to 452 amino acids	This study
PRE*ΔrpoN*	pRE112 with *rpoN* fragment deleted from 3 to 457 amino acids	This study
PRE*ΔaopN*	pRE112 with *aopN* fragment deleted from 8 to 259 amino acids	This study
pRE*ΔexsD*	pRE112 with *exsD* fragment deleted from 4 to 270 amino acids	This study
pRE*ΔexsA*	pRE112 with *exsA* fragment deleted from 9 to 268 amino acids	This study
pDN19lacΩ	Promoterless *lacZ* fusion vector; $Sp^rSm^rTc^r$	Totten and Lory, 1990
pDN*flaA*	pDN19lacΩ containing -462 to +63 of *flaA* relative to putative translational start site	This study
pDN*flaB*	pDN19lacΩ containing -684 to +124 of *flaB* relative to putative translational start site	This study
pDN*lafA1*	pDN19lacΩ containing -402 to +183 of *lafA1* relative to putative translational start site	This study
pDN*lafA2*	pDN19lacΩ containing -616 to +55 of *lafA2* relative to putative translational start site	This study

Table IV.2a. Primers used for construction of deletion mutants and reporter fusions

	Primers used for construction of deletion mutants	
Gene	Primer pairs	Primer sequence (5' to 3')
flhA	DC-flhA-F	GTAGCTCTAGAGCGATCAGGCGTTCGATCCCAAT
	DC-flhA-R	GAAGCTCTAGAGCGTAGTTGTCGGTAGTGATGAG
	Inv-flhA-L	CAATGATGCCTGGAGTTTCATCGTTTTTCTCA
	Inv-flhA-R	GAAGCCACTCAACGACAAGAACTGGAA
lafK	DC-lafK-F	GAAGCTCTAGAGCTGGTGAAGGAGCAATTGCAACA
	DC-lafK-R	GTTGCTCTAGAGAGCTCTTCTCCAGCCTGAATGGTA
	Inv-lafk-L	GCCAATCAATAACAAATGGGGAGCACTCTCA
	Inv-lafK-R	GAGCAGCAGCTTCAAAACTAAGGGATAAACCGAGT
rpoN	DC-rpoN-F	GATGCTCTAGAGCTATGAGCTGAAGAACCGCTTGGTGA
	DC-rpoN-R	GAAGCTCTAGAGCTTGTCATACTGGCTCTCGACCGAGACGAT
	Inv-rpoN-L	CTTCATCTACGTCGGGATATCCTTATGTTCAGCGA
	Inv-rpoN-R	CGCACGATCGCAAAGTACCGTGAATCCTTGTTGAT
aopN	DC-aopN-F	GTAGCTCTAGAGGGTCATGTCATCACCTTCTA
	DC-aopN-R	GAAGCTCTAGAGCGGTCATACCTTGTGCAACAGA
	Inv-aopN-L	GTTGCTCTGGATAATGGCCATGGAAACT
	Inv-aopN-R	GACATGCACAAGCTGCGGTTGCTCAATACCCTCT
exsD	DC-exsD-F	GAAGCTCTAGAGCAATCCTGTCTTTGCTGAGGCGTCA
	DC-exsD-R	GAAGCTCTAGAGCATCGCGATACTGAATGGAACGAT
	Inv-exsD-L	CTGACTCATGCTATGCCTTGCTCATC
	Inv-exsD-R	CGCTAGATAACCCGAGGAACTATGCAA
exsA	DC-exsA-F	GAAGCTCTAGAGCTCGTCAGCTGGCCAGTTTCAA
	DC-exsA-R	GTAGCTCTAGAGCCTGGCTTCCAGTTGCAGATT
	Inv-exsA-L	GGAAGGCCTCTCTGTGGTTGTAATGCCTTTCA
	Inv-exsA-R	GGAAGGCCTGGCAAAGATTAACGTCGGACTAA
lafA1	DC-lafA1-F	GAAGCTCTAGAGCGTTTGCATTGCCATCGGATCGAGCAA
	DC-lafA1-R	GCTGCTCTAGAGCCATCATCCTTCAGCTCCAGTTTCT
	Inv-lafA1-L	GGTCAGAGAAGCGAAGTTGGTGTGAATGGACAA
	Inv-lafA1-R	CAGATGACTGGTATGGTGACTTCCCTGCTGCGTTAA
lafA2	DC-lafA2-F	GATGCTCTAGAGCGAGCCAGCTCAATACCACCAACAAGAT
	DC-lafA2-R	GAAGCTCTAGAGCTCATCGATCAGCTCGTTATAGCTGT
	Inv-lafA2-L	GGAAGCGAAATTGGTGTGAATGGACAAACCCATGAT
	Inv-lafA2-R	GGCATGGTGACTTCCCTGCTGCGTTAATCTGTT
	Primers used for construction of reporter fusions	
flaA	P-flaA-F	GCGTGAGAATTCAGTACCAAACATGTCGCTGATGTAAA
	P-flaA-R	GATAAGGATCCATCATGTTACGCTGAGCGTTGAGTGA
flaB	P-flaB-F	GCGACAGAATTCTGACCAAGCAGAATATTCTGCAACA
	P-flaB-R	GACAAGGATCCTTGCACTATTGATGCGCAAACCAGA
lafA1	P-lafA1-F	GCGAGAGAATTCCATTGCTCTGTTTGACCTTGCGGATAT
	P-lafA1-R	GAAATGGATCCCTGACCACTGGACTGAGATTGCAGA
lafA2	P-lafA2-F	GGAGAGAATTCGTTTCCAGGAAGATGTGACCTTCCAGA
	P-lafA2-R	GAAATGGATCCTCAACTGGCTCTGAGTGGTCAGGGAA

Table IV.2b. Degenerate primers used in this study

Gene	Primer pairs	Primer sequence (5' to 3')
flaA/B	flaA-deg-F	TGGATACYTCYTACACCCGTCTGGC
	flaB-deg-R	GAGGTCARACGGTTGGAGATCTGCA
LafA1/A2	laf-1	GGTCTGCGCATCCAACTC
	laf-5	ATCGCTGGAGGTCATCTTG
lafK	lafK-deg-F	AACTGTGCYGCSATTCCTGAATC
	lafK-deg-R	CGCATGGCYGCSAGCTTGTA
rpoN	rpoN-deg-F	ATGACGCCRCARYTRCARCA
	rpoN-deg-R	ACCAKBGGYTTCATGGCTTCTTC

permeabilized with SDS and chloroform as described by Miller (1972). Briefly, OD_{600} values for each strain were measured using the spectrophotometer (Shimadzu, UV-1601, Japan). 1.0 ml of the bacterial cultures was then transferred to a 1.5 ml eppendorf tube and centrifuged at 12,000 × *g* for 2 min at room temperature. After the removal of the supernatant, the cell pellet was resuspended in 600 µl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM MgSO4; pH 7.0) by vortexing. 20 µl of 0.05% SDS and 20 µl of $CHCl_3$ were then added. The mixture was vortexed for 10 min, followed by incubation at room temperature for 10 min. To start the assay reaction, 100 µl ONPG (4 mg per ml in Z-buffer) were added into the above mixture. When the suspension started to turn yellow color, the reaction was terminated by adding 250 µl 1M Na_2CO_3. The time of reaction was also recorded. The supernatant was then obtained by centrifuging the tube at 12,000 × *g* for 2 min and the OD_{420} value was recorded. Specific units of β-galactosidase activity were calculated as per the following formula: $OD_{420} \times 10^3 \times 6/T_{reaction}\,(min)/OD_{600}$. One unit (U) of enzyme activity corresponds to the hydrolysis of 1 nmol ONPG min^{-1} (mg protein)$^{-1}$.

IV.2.5 Cell culture and morphological changes induced by *A. hydrophila* AH-1

HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) at 37^{o}C in a humidified atmosphere of 5% CO_2. Studies on morphological changes were conducted by seeding 1×10^5 HeLa cells into each well of a 24-well tissue culture plate (Falcon). An *A.hydrophila* single colony was cultured in TSB for 18 h at 37 ^{o}C. 2.5 h prior to the infection of HeLa cells, mid-exponential-phase cultures were prepared by diluting the overnight culture 1:20 in fresh TSB and incubated at 37°C. Bacterial cells were pelleted and washed three times in PBS. Before infection, HeLa cells were washed

three times with DMEM to remove FBS and antibiotics. HeLa cells were infected with *A. hydrophila* cells suspended in PBS at a multiplicity of infection (MOI) of 1. Changes in the cell morphology were observed over a period of 4 h. The HeLa cells were photographed under an Axiovert 25CFL phase-contrast inverted microscope (Carl-Zeiss) at 40 × magnification after 2.5 h of infection at 37^{o}C.

IV.2.6 Preparation of extracellular proteins

To prepare ECPs *of A. hydrophila*, overnight precultures in DMEM were diluted 1:200 in DMEM and were incubated for 24 h, at 25ºC or 37ºC in a 5% (v/v) CO_2 atmosphere. Bacterial cells were removed from the culture by centrifugation (5,500 × *g*, 10 min, 4°C), and the supernatant was filtered through a 0.22-μm-pore-size small-protein binding filter (Millipore). The ECP fraction was isolated by trichloroacetic acid precipitation (Shimizu *et al.*, 2002), and the protein pellet was washed thrice with -20°C acetone and then air dried. The protein pellet was solubilized in ReadyPrep reagent 3 (5 M urea, 2 M thiourea, 2% [w/v] CHAPS, 2% [w/v] SB 3-10, 40 mM Tris, and 0.2% [w/v] Bio-Lyte 3/10 ampholyte; Bio-Rad) and was stored at –20°C until analysis. The protein concentration was determined by use of a Bio-Rad protein assay kit, with bovine serum albumin as a standard.

IV.2.7 Two-dimensional gel electrophoresis (2-DE)

ECPs in amounts of 15 to 30 μg that gave similar profiles to those of the background proteins were used to generate the extracellular proteomes of different strains for a comparative analysis. 2-DE was performed by use of the Ettan IPGphor isoelectric focusing system (Amersham) according to the manufacturer's instructions. Dry gel strips were rehydrated for 12 h at room temperature with a mixture containing 8 M urea, 2%

(w/v) CHAPS, 0.5% immobilized pH gradient (IPG) buffer, 50 mM dithiothreitol, and a trace amount of bromophenol blue. For the first dimension, the ECP samples were separated on 18-cm-long rehydrated Immobiline DryStrips with a nonlinear gradient from pH 3 to 10 (Amersham) by use of a cup-loading system and were focused at 500 V for 1.5 h, 4,000 V for 2 h, and 8,000 V for 40,000 Vh. After isoelectric focusing, the IPG strips were reduced, alkylated, and exchanged with detergent. 2-DE was carried out in 12.5% SDS gels (20 by 20 cm), and the proteins were visualized by silver staining. Computer-assisted gel analysis with PD-QUEST, version 7.1.0 (Bio-Rad), was performed on images captured with a Molecular Dynamics Densitometer (Bio-Rad). ECP samples were isolated from at least three independent cultures. More than three separate gels were analyzed for each sample. Protein spots that displayed dominant and consistent patterns were selected for further identification.

IV.2.8 Tryptic in-gel digestion and MALDI-TOF/TOF MS analysis

The protein spots of interest were excised from the 2-DE gels and digested with porcine sequencing-grade modified trypsin (Promega) according to a procedure described by Shevchenko *et al.* (1996). The extracted peptides were re-dissolved in 1 µl of matrix solution [5 mg ml of a-cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA, 50% ACN in MilliQ water]. All the solution that could be pipetted out from the well was used to spot onto the MALDI target plate, and allowed to dry in air, prior to analysis by mass spectrometry.

Peptide mass fingerprint maps of trypsin digested peptides were generated by Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems) mass spectrometer. All of the spectra were obtained in reflectron mode, with an accelerating

voltage of 20 kV and a delayed extraction of 150 ns. Mass calibration was performed with a peptide mixture (angiotensin I) (1,296.6853 $[M + H]^+$) and adrenocorticotropin (18-39 clip) (2,465.1989 $[M + H]^+$) as an external standard. Internal calibration with two peptides arising from trypsin autoproteolysis of 842.51$[M + H]^+$ and 2,211.10 $[M + H]^+$ was performed whenever possible. Peptide masses were searched against the NCBInr database or *A. hydrophila* AH-1 database by use of the MS-Fit program (http://prospector.ucsf.edu) and Mascot software (Matrix Science), with the mass tolerance set to 0.2 Da (internal calibration) or 200 ppm (external calibration). Proteins with a minimum of four matching peptides and with sequence coverage exceeding 20% with the matched proteins were considered positive.

IV.2.9 Nucleotide sequence accession numbers

The DNA sequences of *A. hydrophila* AH-1 polar flagellar operon, lateral flagellar operon, *lafK* and *rpoN* were deposited in GenBank under accession numbers#, #, # and #, respectively.

IV.3 Results and discussion

IV.3.1 Analysis of *A. hydrophila* extracellular proteins

ECPs of pathogens play a vital role in the process of pathogen-host interaction. The ECPs can be resolved by 2-DE according to the isoelectric point (pI) and the molecular weight of the proteins. The establishment of an extracellular proteome map will allow us to examine the expression levels of different proteins in an integrated manner.

To generate a two dimensional extracellular proteome map for *A. hydrophila* secreted proteins, *A. hydrophila* was grown in a protein-free culture medium (DMEM). Proteins were collected by TCA precipitation and the concentrations of proteins were quantified by

Bio-Rad assay. 10 to 30 μg proteins were loaded onto the 2-DE gels for analysis. Although ECPs of *A. hydrophila* AH-1 are distributed within a wide range of isoelectric points, most of them are present within pH 3 to 10.

Twenty-four protein spots were consistently and dominantly detected in the culture supernatant of *A. hydrophila* AH-1 by 2-DE and silver staining (Fig. IV.1, Table IV.3). Half of the spots were identified in the extracellular proteome map, while the other half could not be identified due to the incomplete genome sequence data of *A. hydrophila* (Table IV.3).

As shown in Fig. IV.1, a large proportion of the ECPs contains the S-layer proteins (spot 5). This is not surprising since the S-layer typically consists of up to 10% of the total cellular proteins (Tomas and Trust, 1995). In order to detect the less abundant proteins present in the ECPs secreted by *A. hydrophila* AH-1, an S-layer deletion mutant (AH-1S) was constructed. The other mutants characterized in this study were constructed in an AH-1S background (Table IV.1). The extracellular proteomes of AH-1 and AH-1S are similar except for the absence of S-layer proteins in the AH-1S mutant (Fig. IV.2).

Four spots (spots 20 to 23 in Fig. IV.1) were identified as metalloproteases in the supernatant of *A. hydrophila* AH-1, but they exhibited smaller molecular masses than predicted (37.7 kDa). This suggests that the metalloproteases may be extensively processed by a yet undefined mechanism. On the other hand, a serine protease (spot 2) was observed in the extracellular proteome map (Fig. IV.1). The secretion of these two proteases may be important for the degradation of host tissues, allowing bacteria to obtain nutrients and to invade host tissues. It is quite common that serine proteases and

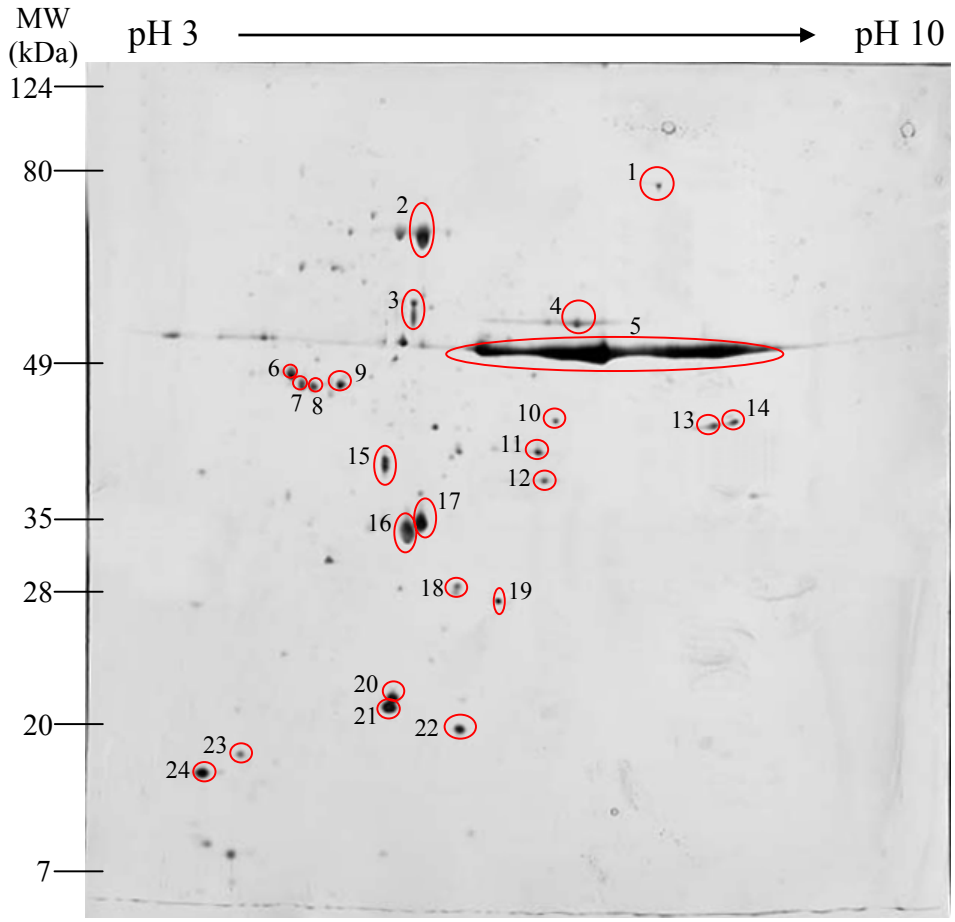


Fig. IV.1. ECPs of *A. hydrophila* AH-1. The proteins were separated by 2-DE using IPG at pH 3 to 10. The circled proteins were identified by MALDI-TOF/TOF MS. Numbers represent the spot identification numbers listed in Table IV.3.

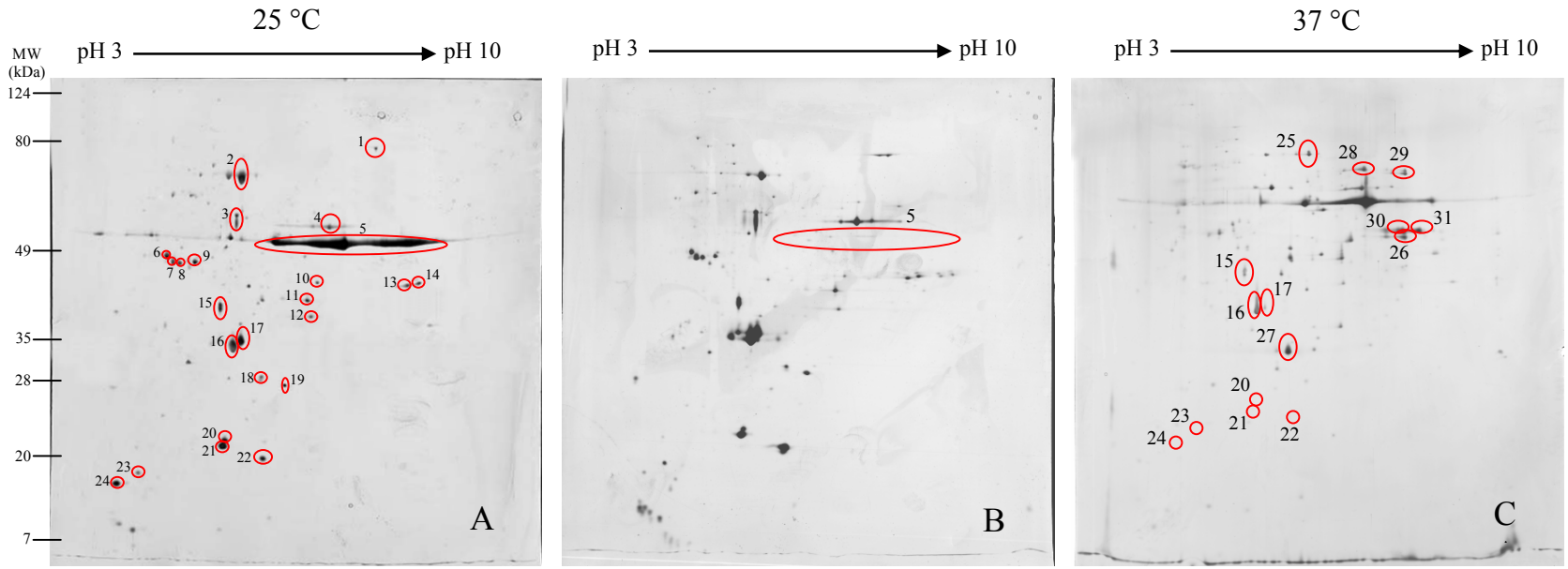


Fig. IV.2. Comparative extracellular proteome analysis of *A. hydrophila* wild type AH-1 grown at 25°C (A) and 37°C (C). The extracellular profile of AH-1S grown at 25°C (B) is also compared with that of *A. hydrophila* wild type AH-1. The circled proteins indicate missing, up-regulated or down-regulated proteins when compared to the wild type AH-1 grown at 25°C. Numbers represent the spot identifications listed in Tables IV.3 and 4.

Table IV.3. Summary of extracellular proteins of *A. hydrophila* AH-1 identified by MALDI-TOF/TOF MS

Spot no.	Protein identification from NCBI	NCBI accession no.	Sequence coverage (%)	Protein size (kDa)/pI	Protein identification from *A. hydrophila* AH-1 database	Sequence coverage (%)	Protein size (kDa)/pI
2[a]	Serine protease	AAP22653	47	31.411/4.72	Serine protease	45	66.5/5.65
3	ChiY protein	CAC83040	11	53.733/5.72		-	
4	hemolysin	BAD90679	46	69.179/6.02	hemolysin		68.854/6.19
5	S-layer	AAA67043	21	47.617/8.37			
15[a]	FlaB	AAF19180	36	31.716/9.21	FlaB	52	31.414/5.30
16[a]	LafA2	AAK57645	25	29.961/4.74	LafA2	62	30.278/5.33
17[a]	LafA1	AAK57644	26	29.734/5.10	LafA1	60	30.382/5.37
19[a]	GCAT	CAA30260	31	33.454/8.85	GCAT	32	37.385/5.80
20[a]	Metalloprotease	AAX49385	38	37.732/5.12			
21[a]			44				
22[a]			45				
23[a]			39				

Spots 1, 6, 7, 8, 9, 10, 11, 12, 13[a], 14[a], 18, 24[a] did not match any proteins in the NCBI database.
[a]Spots down-regulated in the extracellular proteome of *A. hydrophila* AH-1 grown at 37°C when compared to that grown at 25°C.

metalloproteases are purified from ECPs of *A. hydrophila* (Rivero *etal.*, 1990 and 1991; Rodriguez *et al.*, 1992; Cascon *et al.*, 2000).

Hemolysin and aerolysin have been reported to be secreted into the supernatant of *A. hydrophila* (Rodriguez *et al.*, 1992; Wong *et al.*, 1998). Accordingly, hemolysin (spot 4) was also detected in the extracellular proteome map of AH-1 (Fig. IV.1 and Table IV.3). Unlike hemolysin, aerolysin was not identified from this study although the aerolysin gene has been cloned in *A. hydrophila* AH-1 (accession no. AY442276). It has been reported that the amount of aerolysin secreted into the supernatant of *A. hydrophila* reaches to a maximum level during active growth but decreases quickly thereafter (Bernheimer and Avigad, 1974; Ljungh *et al.*, 1981). Hence, we speculate that the absence of the aerolysin spot in our extracellar proteome is due to little or no secretion of aerolysin into the medium under the current growth condition.

Aeromonas spp. produces several distinct extracellular chitinases (Shiro *et al.*, 1996; Sitrit, 1995). The chitinase, a chitin-binding protein, can hydrolyze chitin to provide the resource of carbon, nitrogen and energy for *A. hydrophila*. Consistent with these reports, a chitin-binding protein homologue (spot 3) was identified in the extracellular proteome of AH-1 (Fig. IV.1 and Table IV.3). The MS-MS sequences of this protein showed high homology with ChiY of *Y. enterocolitica* (Iwobi *et al.*, 2003). ChiY, a putative chitin-binding protein, was identified downstream of a novel type II cluster in the high-pathogenicity *Y. enterocolitica* WA-314 strain and was regarded as a potential virulence factor secreted via the type II secretion system (Iwobi *et al.*, 2003). The presence of the ChiY homolog in the extracellular proteome of *A. hydrophila* AH-1 suggests that this protein may be also important for the pathogenicity of *A. hydrophila*.

The flagella, attached to the cell surface, can be easily disrupted during the processes of centrifugation and filtration. Both polar and lateral flagella have been shown to be required for adherence of *A. hydrophila* to epithelial cells and for the formation of biofilm (Rabaan *et al.*, 2001; Gavin *et al.*, 2002; Kirov *et al.*, 2002; Canals *et al.*, 2006a and b). They may serve as important colonization factors. The sequences of polar and lateral flagellins of *A. hydrophila* AH-1 were obtained by a degenerate PCR in this study (Table IV.2b). Two tandem polar flagellin genes (*flaA* and *flaB*) and two tandem lateral flagellin genes (*lafA1* and *lafA2*) were present in *A. hydrophila* AH-1, which is similar to the genetic organization of polar and lateral flagellin loci in *A. caviae* (Rabaan *et al.*, 2001; Gavin *et al.*, 2002). The two flagellin genes in polar and lateral flagellin loci of *A. hydrophila* AH-1 shared 96% and 89% similarity, respectively, at the amino acid level. As shown in Fig. IV.1, polar (spot 15) and lateral (spots 16 and 17) flagellins are abundantly present in the extracellular proteome of *A. hydrophila* AH-1 (Table IV.3). Although the polar flagellin (FlaA) was not detected in the ECPs of the wild type, the expression of *flaA* and *flaB* was studied in parallel in another study. This was based on the assumption that the expression of *flaA* and *flaB* may be coordinately regulated by some factors, since *flaA* and *flaB* of *A. hydrophila* AH-1 were genetically linked to each other and transcribed in the same direction. The LafA1 and LafA2 spots were missing in the extracellular proteomes of *ΔlafA2* and *ΔlafA1* mutants, respectively, which allowed us to differentiate these two spots in the ECPs of the wild type (Fig. IV.3). The secretion of flagella proteins into the supernatant has also been observed in *S. typhimurium* and *P. aeruginosa*

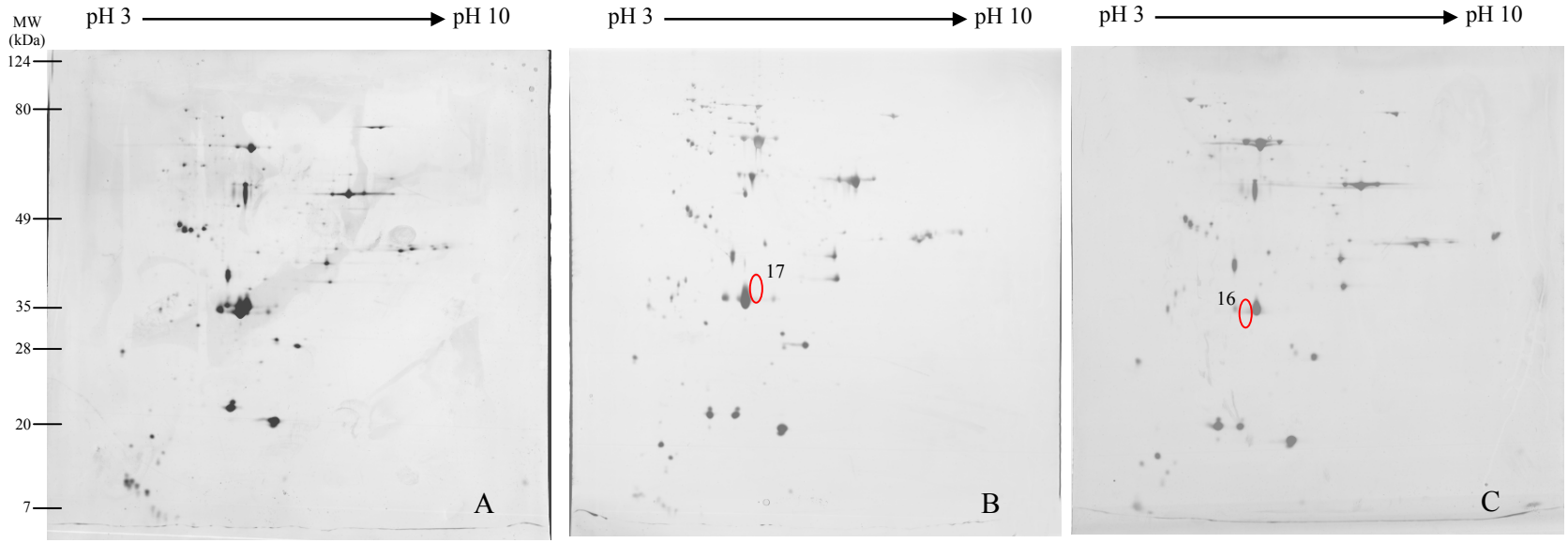


Fig. IV.3. Comparative extracellular proteome analysis of AH-1S (A), *ΔlafA1* (B) and *ΔlafA2* mutants (C). The circled proteins were missing in *lafA1* and *lafA2* mutants. Numbers represent the spot identifications listed in Table IV.5.

(Komoriya *et al.*, 1999; Nouwens *et al.*, 2002). The predicted molecular masses of FlaA, FlaB, LafA1 and LafA2 of *A. hydrophila* AH-1 were 31.3, 31.4, 30.4 and 30.3 kDa, respectively. All the polar and lateral flagellins in the extracellular proteome of AH-1 exhibited higher estimated molecular masses than those predicted from the nucleotide sequences. This is similar to the reports that both polar and lateral flagellins exhibited aberrant migration on SDS-PAGE due to the post-translational modification of flagellins (Rabaan *et al.*, 2001; Gavin *et al.*, 2002).

Twelve protein spots in the extracellular proteome map did not match with any proteins in the NCBI database (Table IV.3, Fig. IV.1). These proteins could be identified after the completion of *A. hydrophila* genome sequencing. Further study is required to confirm that these proteins are truly secreted into the culture supernatant. These proteins may be good candidates for the study of *A. hydrophila* pathogenicity and may be suitable targets for *A. hydrophila* vaccines.

No type III secreted proteins were identified in the extracellular proteome map. As described previously (Chapter III 3.6), the disruption of *ascN* may render the TTSS non-funtional. The *ΔascN* mutant showed a similar ECP profile to that of AH-1S, indicating that those proteins in the extracellular proteome map are not secreted by the TTSS (Fig. IV.4). This is not surprising since the activation of TTSS normally requires host-cell contact or growth under specific conditions.

The extracellular proteome of *A. hydrophila* AH-1 is clearly less than complete due to the lack of genome data of *A. hydrophila*. However, this does not hamper further studies of the pathogenesis of *A. hydrophila* using this extracellular proteome as a reference map. In fact, the major ECPs (proteases, GCAT, flagella and hemolysin) which are shown to play

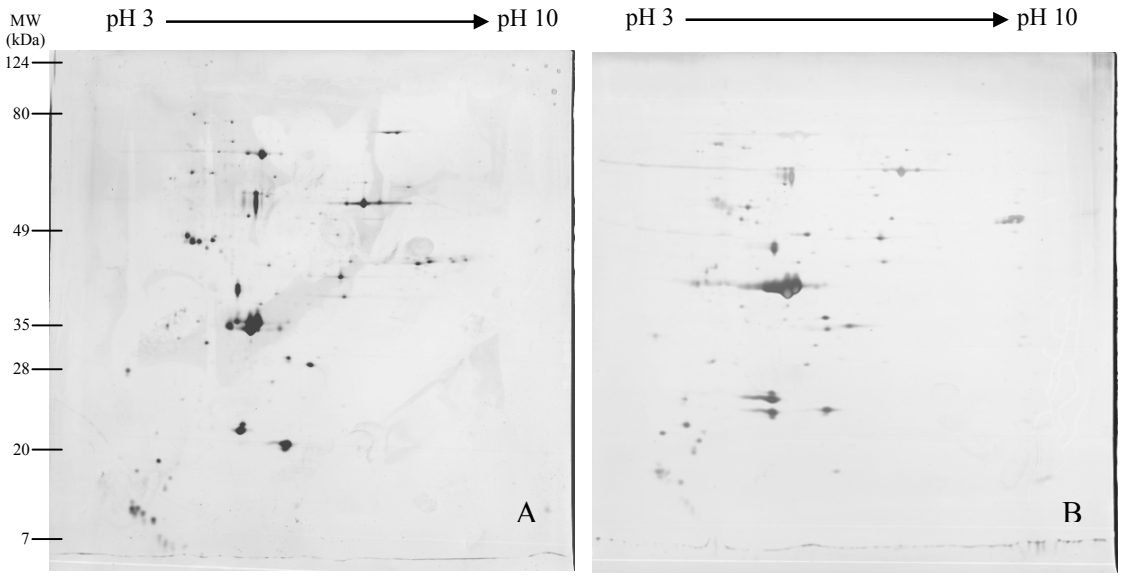


Fig. IV.4. The ECP profile of *ΔascN* (B) is similar to that of AH-1S (A).

very important roles in the pathogenesis of *A. hydrophila* have been detected in this reference map (Janda, 2001).

IV.3.2 Influence of temperature on the extracellular proteome

A. hydrophila AH-1 is cytotoxic to HeLa cells (Fig. IV.5), suggesting that this strain can be regarded as a potential pathogen to humans. This prompted us to examine the extracellular proteome of *A. hydrophila* AH-1 at the human body temperature (*i.e.* 37ºC).

The expression levels of both serine protease (spot 2) and metalloprotease (spots 20 to 23) at 37ºC decreased significantly as compared to those at 25ºC (Fig. IV.2). This is consistent with other studies which showed that the production of proteases in *A. hydrophila* was inhibited at 37ºC (O'Reilly and Day, 1983; Mateos *et al.*, 1993). In the ECPs of *A. hydrophila* AH-1 grown at 37ºC, two additional hemolysin spots (spots 25 and 26) were present (Fig. IV.2, Table IV.4). The presence of spot 25 could be due to the decreased expression of serine protease at 37ºC, which in turn led to the up-regulation of a hemolysin precursor as discussed in section IV.3.3. The expression of protein spots 27 to 31 was also up-regulated at 37ºC when compared to that at 25ºC (Fig. IV.2). However, these spots could not be identified and may represent novel virulence factors which need to be studied further.

In addition, the expression levels of both polar (FlaB, spot 15) and lateral flagellins (spots 16 and 17) were also down-regulated at 37ºC when compared to those at 25ºC (Fig. IV.2). The expression of *lafA1* (spot 17) appeared to decrease more significantly when compared to that of *lafA2* (spot 16), suggesting that the expression of *lafA1* and *lafA2* is finely regulated by temperature at different levels. To examine whether the down-regulation of polar and lateral flagellins is at the transcriptional level, plasmids pDN*flaA*, pDN*flaB*,

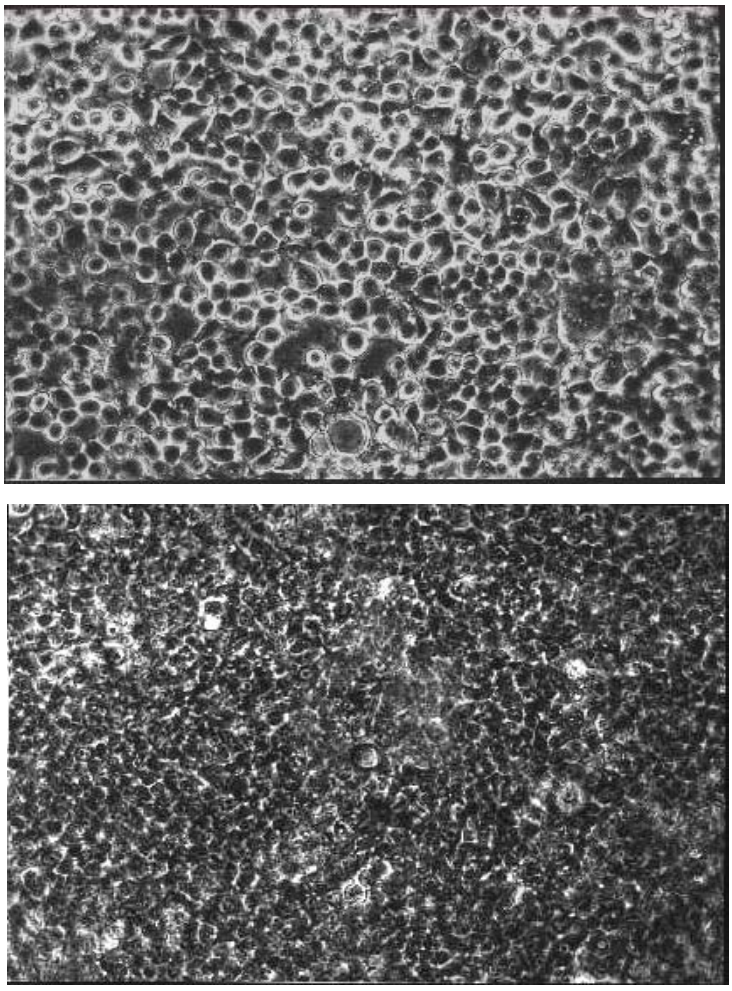


Fig. IV.5. Phase-contrast micrographs of HeLa cells infected with AH-1 (A), *ΔexsA* mutant (B) at 2.5 h post-infection (MOI of 1).

Table IV.4. Identification of ECPs of *A. hydrophila* AH-1 which are up-regulated at 37°C when compared to 25°C

Spot no.	Protein identification	NCBI accession no.	Sequence coverage (%)	Predicted Protein size (kDa)/pI
25	hemolysin	BAD90679	55	69.179/6.02
26	hemolysin	BAD90679	38	69.179/6.02
27	No match	-	-	-
28	No match	-	-	-
29	No match	-	-	-
30	No match	-	-	-
31	No match	-	-	-

pDN*lafA1* and pDN*lafA2*, carrying the *lacZ* transcriptional fusions to the putative promoter regions of *flaA*, *flaB*, *lafA1* and *lafA2*, respectively, were constructed and introduced into AH-1S (Fig. IV.6). Results showed that the expression of *lacZ* of *flaA-lacZ*, *flaB-lacZ*, *lafA1-lacZ* and *lafA2-lacZ* at 37ºC decreased about 6- to 23-fold when compared to those at 25ºC. The down-regulation of the transcription of both polar and lateral flagellins at 37ºC may allow *A. hydrophila* to avoid host recognition and evade the host immune response, thus surviving and replicating inside specific hosts (such as humans). Similarly, Grundling *et al.* (2004) also reported that the flagellar motility gene is transcriptionally down-regulated at 37ºC in *Listeria monocytogenes*.

IV.3.3 Characterization of protease-deficient mutants

The proteomic approaches have been applied to compare the extracellular proteomes of the wild type and mutants to reveal the underlying mechanisms involved in the pathogenesis of bacteria (Nouwens *et al.*, 2003; Kazemi-Pour *et al.*, 2004). Proteases are required for the proteolytic activation of protoxins in aeromonads (Howard and Buckley, 1985; Song *et al.*, 2004). The presence of proteases in the supernatant may affect the extracellular proteome. Thus, the ECP profiles of AH-1S, *ΔserA*, *ΔmepA* and *ΔserAΔmepA* mutants were compared in this study (Fig. IV.7).

Although the metalloprotease may be involved in the degradation or proteolytic processing of other proteins, the extracellular proteomes of AH-1S and the *ΔmepA* mutant are quite similar except for the absence of metalloproteases (spots 20 to 23) in the *ΔmepA* mutant (Table IV.5, Fig. IV.7). In contrast with the *ΔmepA* mutant, several major changes including the absence of serine protease were observed in the ECPs of the *ΔserA* mutant (Table IV.5, Fig. IV.7).

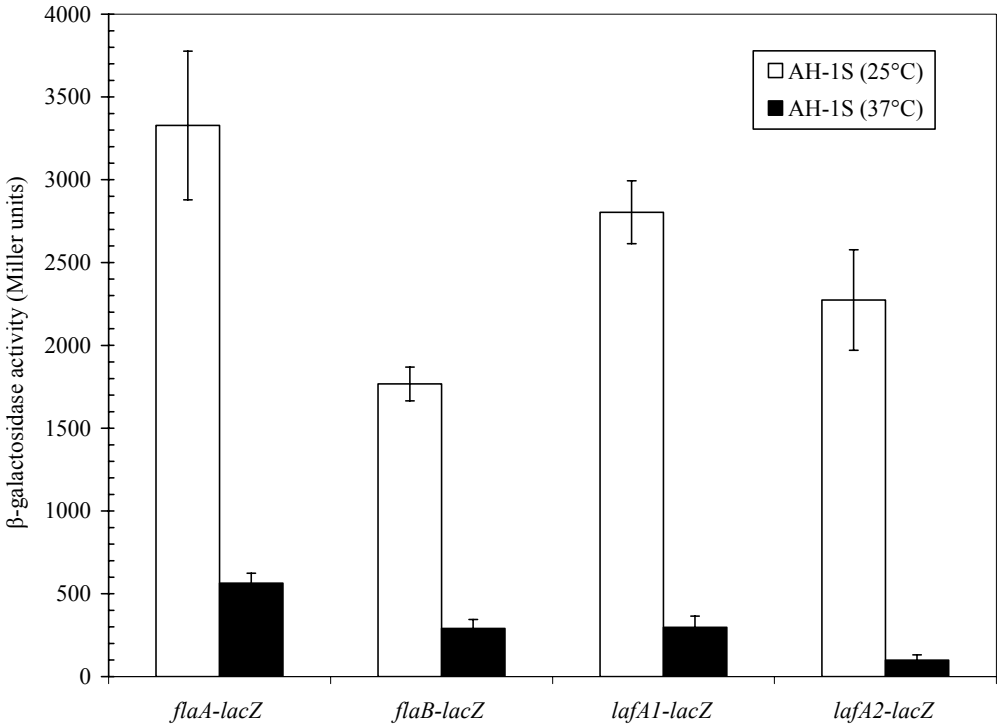


Fig. IV.6. Effect of temperature on the expression of *flaA*, *flaB*, *lafA1* and *lafA2*. *A. hydrophila* strains with different reporter fusions were cultured in 25°C or 37°C in DMEM for 24 h. The transcription levels of different reporter fusions were measured by assaying β-galactosidase activities in bacterial cell lysates. The values represent the mean ± SD from three separate tests.

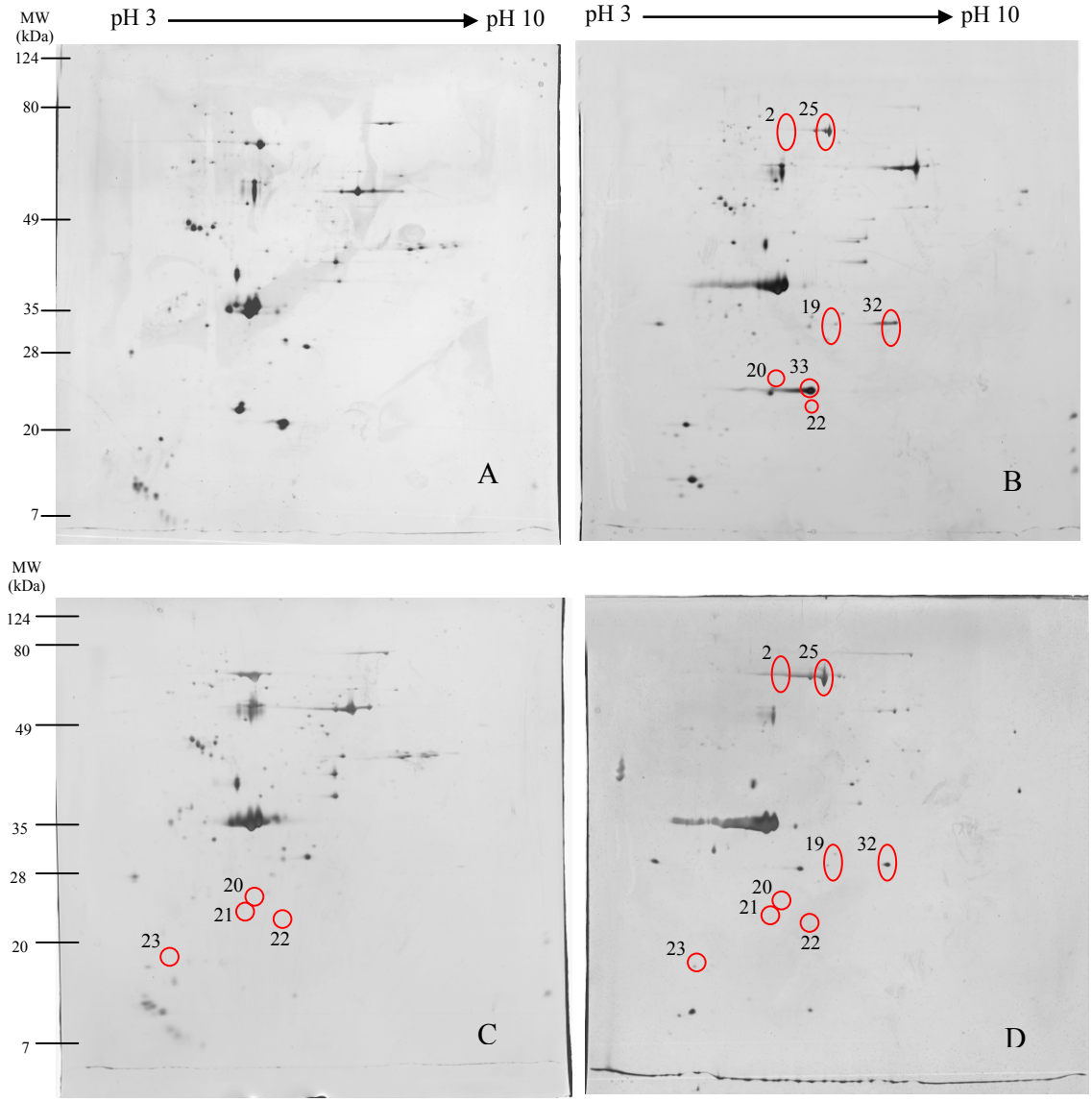


Fig. IV.7. Comparative extracellular proteome analysis of AH-1S (A), *ΔserA* (B), *ΔmepA* (C) and *ΔserAΔmepA* mutants (D). The circled proteins were missing or up-regulated in *ΔserA*, *ΔmepA* or *ΔserAΔmepA* mutants. Numbers represent the spot identifications listed in Table IV.5.

Table IV.5. Identification of proteins with altered expression levels in different mutants

Spot no.	Protein identification	NCBI accession no.	Sequence coverage (%)	Protein size (kDa)/pI	Mutant
(A)					
25	Hemolysin	BAD90679	51	69.179/6.02	*ΔserA*/*ΔserAΔmepA*
32	GCAT	CAA50021	14	37.547/6.97	*ΔserA*/*ΔserAΔmepA*
33	metalloprotease	AAX49385	49	37.732/5.12	*ΔserA*
34	AcrV	AAV67437	62	41.263/6.43	*ΔaopN*/*ΔexsD*
35	No match				*ΔaopN*
I[b]	ND				*ΔaopN*
(B)					
2	Serine protease				*ΔserA*/*ΔserAΔmepA*
15	FlaB				*ΔflhA*/*ΔrpoN*
16	LafA2				*ΔlafA2*/*ΔlafK*/*ΔrpoN*/*ΔaopN*/*ΔexsD*
17	LafA1				*ΔlafA1*/*ΔlafK*/*ΔrpoN*/*ΔaopN*/*ΔexsD*
19	GCAT				*ΔserA*/*ΔserAΔmepA*
20	metalloprotease				*ΔserA*/*ΔmepA* /*ΔserAΔmepA*
21					*ΔmepA* /*ΔserAΔmepA*
22					*ΔserA*/*ΔmepA* /*ΔserAΔmepA*
23					*ΔmepA* /*ΔserAΔmepA*

(A) Proteins up-regulated or present in mutants; (B) Proteins down-regulated or absent in mutants when compared to the extracellular proteome of AH-1S.

I[b], a large amount of proteins not identified by MALDI-TOF/TOF MS. ND, not determined.

Spots 19 and 32, exhibiting similar molecular masses but different pIs, were identified as GCAT (Tables IV.3 and 5, Figs. IV.1 and 7). The presence of different forms of GCAT in AH-1S and the *ΔserA* mutant suggested that the serine protease may be involved in the post-translational modification of GCAT. Spot 19 was present in both the *ΔmepA* mutant and AH-1S and no other spots were identified to be GCAT from the ECPs of the *ΔmepA* mutant, indicating that the metalloprotease may not be required for the post-translational modification of GCAT (Fig. IV.7). In *A. salmonicida*, the processing of pro-GCAT is also mediated by a serine protease (Vipond *et al.*, 1998).

Cason *et al.* (2000) demonstrated that a serine protease (AhpA) was partially involved in the processing of an elastic protease (AhpB). Similar to this report, the distribution of different forms of metalloproteases was slightly altered in the *ΔserA* mutant when compared to AH-1S (Fig. IV.7). Although spots 20 and 22 were missing, a new spot (spot 33, metalloprotease) which exhibited different molecular mass and pI from other metalloprotease spots appeared in the ECPs of the *ΔserA* mutant (Table IV.5, Fig. IV.7). This suggests that a serine protease is also involved in the processing of metalloproteases, but the mechanism needs to be further investigated.

Another major difference between AH-1S and the *ΔserA* mutant was the presence of spot 25 in the ECPs of the *ΔserA* mutant (Fig. IV.7). This spot was identified as hemolysin which migrated as an about 66 kDa protein, similar to the predicted molecular mass of hemolysin of *A. hydrophila* AH-1 (accession no. AY442273), indicating that spot 25 may be the precursor form of hemolysin. As the putative precursor of hemolyin, spot 25 is absent from the *ΔmepA* mutant but present in both *ΔserA* and *ΔserAΔmepA* mutants (Fig. IV.7). Thus, at the genetic level, our results revealed that the serine protease, rather than

metalloprotease, was responsible for the activation of the hemolysin precursor. This may also explain the results from Titball *et al.* (1985) who demonstrated that an inactive form of hemolysin can be activated by autogenous caseinase and, with less efficiency, by other serine proteases in *A. salmonicida*.

IV.3.4 Characterization of flagellar regulatory proteins

The flagella-mediated motility plays very important roles in bacteria-host interactions. The flagellar assembly is regulated in a hierarchical cascade and is subjected to transcriptional, translational and post-translational regulation (Aldridge and Hughs, 2002; Soutourina and Bertin, 2003).

FlhA, a polar flagella assembly protein, has been reported to be involved in swarming differentiation, flagellin export and the secretion of virulence factors in *Bacillus thuringiensis* (Ghelardi *et al.*, 2002). It is also required for virulence in *Campylobacter jejuni* (Carrillo *et al.*, 2004). This led us to examine the ECPs of the *ΔflhA* mutant (Fig. IV.8). Spot 15 (FlaB) was absent in the ECPs of the *ΔflhA* mutant while the other ECPs appeared to be unaffected. To investigate whether FlhA can affect the expression of *flaA* and *flaB* at the transcriptional level, plasmids pDN*flaA* and pDN*flaB* carrying the *lacZ* transcriptional fusion to putative promoter regions of *flaA* and *flaB* were constructed and introduced into the AH-1S and *ΔflhA* mutants (Fig. IV.9). As shown in Fig. IV.9, the expression of *lacZ* in the *ΔflhA* mutant carrying pDN*flaA* and pDN*flaB* decreased about 15 times and 5 times, respectively, when compared to that in AH-1S carrying pDN*flaA* and pDN*flaB*. This indicates that the transcription of *flaA* and *flaB* is regulated by FlhA in *A. hydrophila* AH-1, which is quite different from *B. thuringiensis* where mutation of *flhA* only affects the export of flagellin but does not influence the production of flagellin

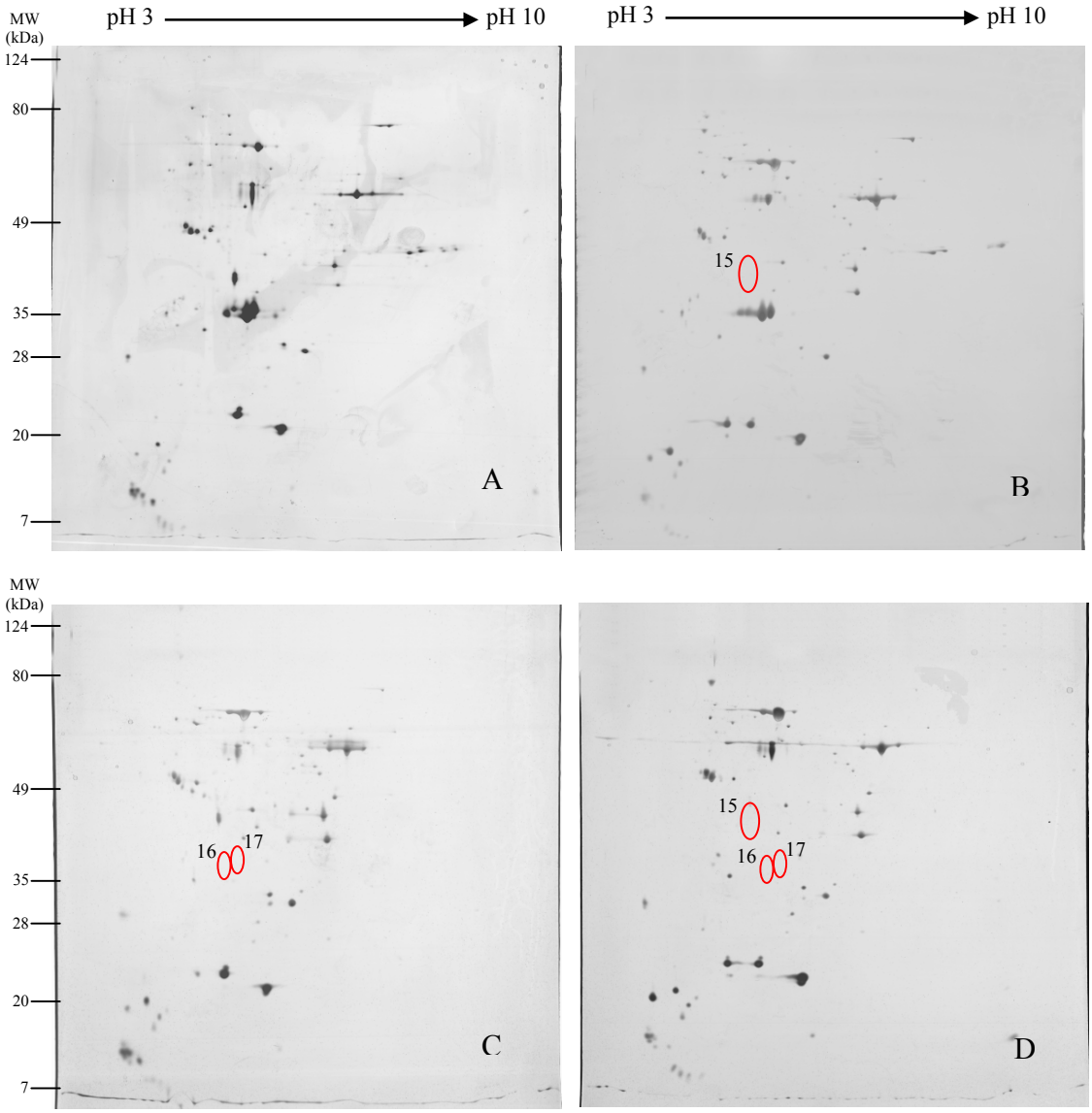


Fig. IV.8. Comparative extracellular proteome analysis of AH-1S (A), *ΔflhA* (B), *ΔlafK* (C) and *ΔrpoN* mutants (D). The circled proteins were missing in *ΔflhA*, *ΔlafK* and *ΔrpoN* mutants. Numbers represent the spot identifications listed in Table IV.5.

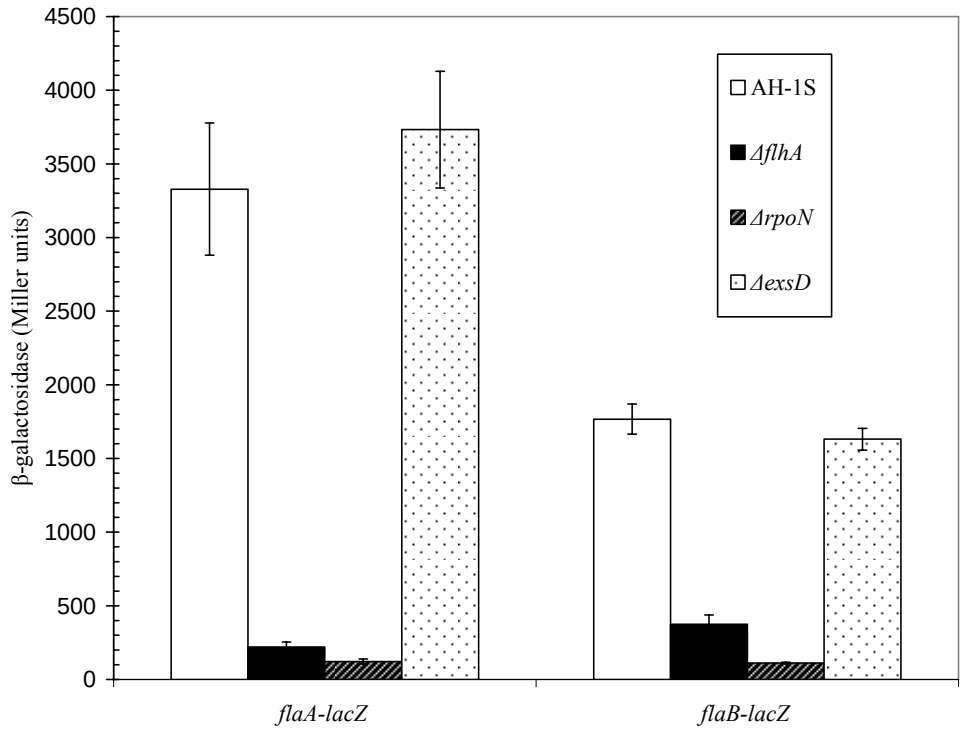


Fig. IV.9. Effect of *ΔflhA*, *ΔrpoN* and *ΔexsD* mutants on the expression of *flaA* and *flaB*. *A. hydrophila* strains with different reporter fusions were cultured in 25°C in DMEM for 24 h. The transcriptional levels of different reporter fusions in different *A. hydrophila* genetic backgrounds were measured by assaying β-galactosidase activities in bacterial cell lysates. The values represent the mean ± SD from three separate tests.

(Ghelardi *et al.*, 2002). In the previous study, the deletion of *flhA* had no effect on virulence when the mutant was intramuscularly injected into blue gourami fish (Chapter III), suggesting that *flaA* and *flaB* may not be essential for the virulence of *A. hydrophila*. In addition, although the polar flagellar system may control the synthesis of the lateral flagellar system in *A. hydrophila* (Gavin *et al.*, 2002), the mutation of *flhA* did not abolish the expression of lateral flagellins in *A. hydrophila* AH-1(Fig. IV.8).

In *Vibrio parahaemolyticus*, a *laf*-specific regulator called LafK was shown to induce the lateral flagellar gene expression (Stewart and McCarter, 2003). The homolog of *V. parahaemolyticus* LafK in *A. hydrophila* AH-1 was identified by a degenerate PCR and the ECPs of the *ΔlafK* mutant were examined (Fig. IV.8). The two lateral flagellin spots (spots 16 and 17) were absent in the ECP profile of the *ΔlafK* mutant while other ECPs remained unaffected. To investigate whether LafK controls the transcription of lateral flagellin genes, the plasmids pDN*lafA1* and pDN*lafA2* were introduced into a *ΔlafK* mutant and the expression of *lacZ* was monitored. As shown in Fig. IV.10, the expression of *lacZ* in the *ΔlafK* mutant carrying either pDN*lafA1* or pDN*lafA2* decreased significantly. Thus, LafK is also required for the expression of lateral flagellar genes in *A. hydrophila* AH-1.

Sigma factor σ^{54}, encoded by *rpoN*, is another important flagellar regulatory protein and distributed widely among bacteria. It has also been shown to regulate bioluminescence, pili, and other virulence factors (Totten *et al.*, 1990; Stewart and McCarter, 2003). Both polar and lateral flagellins are absent in the ECPs of the *ΔrpoN* mutant (Fig. IV.8). A transcriptional study also confirmed that the expression of *lacZ* of *flaA-lacZ*, *flaB-lacZ*, *lafA1-lacZ* and *lafA2-lacZ* was greatly reduced in the absence of *rpoN* (Figs. IV.9 and 10),

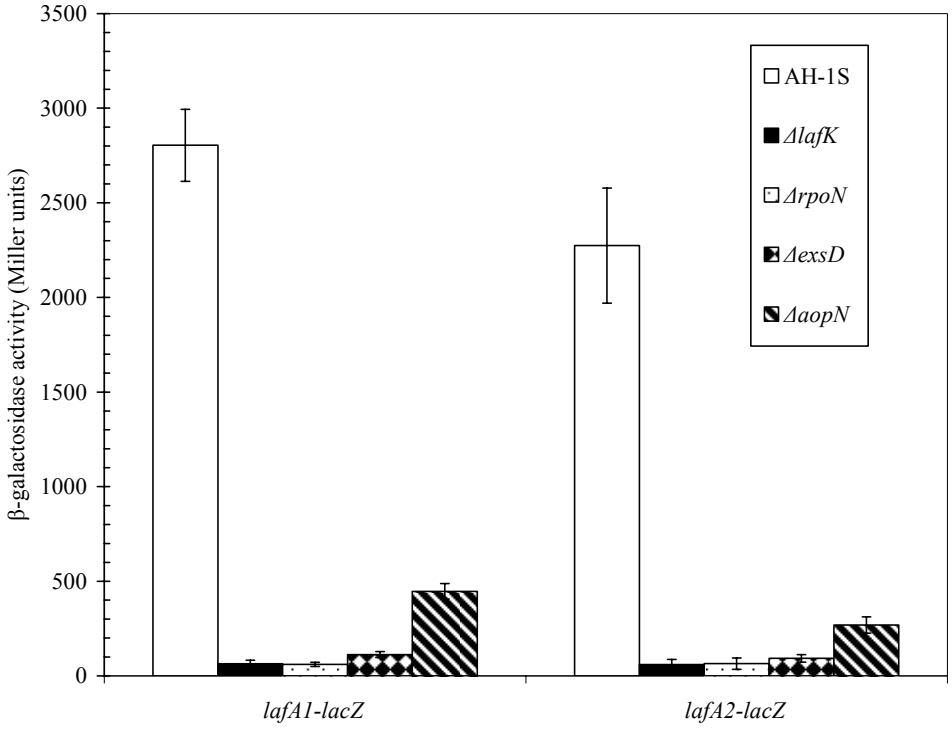


Fig. IV.10. Effect of *ΔlafK*, *ΔrpoN*, *ΔexsD* and *ΔaopN* mutants on the expression of *lafA1* and *lafA2*. *A. hydrophila* strains with different reporter fusions were cultured in 25°C in DMEM for 24 h. The transcriptional levels of different reporter fusions in different *A. hydrophila* genetic backgrounds were measured by assaying β-galactosidase activities in bacterial cell lysates. The values represent the mean ± SD from three separate tests.

suggesting that the expression of both polar and lateral flagellins is σ^{54}-dependent in *A. hydrophila* AH-1. A similar result was also observed in *V. parahaemolyticus* where the polar and lateral gene expression requires σ^{28} and σ^{54} (McCarter, 2004). Although RpoN may play a global regulatory role in the expression of a variety of genes, the mutation of *rpoN* did not appear to affect the secretion of other proteins in the extracellular proteome. A comparative analysis of the total cell protein profiles of the wild type AH-1 and the *ΔrpoN* mutant may allow us to find additional virulence factors regulated by RpoN. In fact, this method has been used to reveal the global role of σ^{54} in the gene expression of *Pseudomonas alcaligenes* (Zhao *et al.*, 2005).

The regulatory roles of FlhA, LafK and RpoN in *A. hydrophila* AH-1 are similar to those in *A. hydrophila* AH-3 (Canals *et al.*, 2006a and b). Here, our results provide additional evidence on the regulatory roles of FlhA, LafK and RpoN by proteomic and transcriptional fusion studies.

IV.3.5 Characterization of TTSS negative regulator mutants

In *Yersinia* spp. and *P. aeruginosa*, the secretion of effector proteins can be induced by growing bacteria under low calcium conditions (Portnoy *et al.*, 1984; Yahr *et al.*, 1997). The genetic organization of the TTSS of *A. hydrophila* shares great similarity to both *Yersinia* spp. and *P. aeruginosa* (Chapter V), suggesting that the functional similarities may also be present among these pathogens. However, the initial trials to induce the secretion of TTSS-dependent proteins of *A. hydrophila* AH-1 by adding EGTA into the culture medium did not reveal any promising effector proteins. Therefore, an alternative strategy was used to identify potential effectors. It has been reported that an insertional inactivation of the *yopN* gene resulted in a derepressed transcription of *yop* genes in

Yersinia spp. (Forsberg *et al.*, 1991). Recently, it has been shown that a *ΔexsD* mutant is competent for type III secretion and translocation of the ExoU cytotoxin to eukaryotic host cells in *P. aeruginosa* even in the presence of calcium (McCaw *et al.*, 2002). In *A. hydrophila* AH-1, AopN and AscZ/ExsD are homologous to YopN and ExsD, respectively. Accordingly, non-polar deletion mutants were created in these two genes and the extracellular proteomes of the *ΔaopN* and *ΔexsD* mutants were examined to identify potential effectors secreted by the TTSS of *A. hydrophila* AH-1.

As shown in Figs. IV.11B and C, AcrV (spot 34), a protein encoded within the TTSS cluster, was abundantly present in the ECPs of both *ΔaopN* and *ΔexsD* mutants (Table IV.5). It was also reported that the AcrV homologs, LcrV in *Yersinia* spp. and PcrV in *P. aeruginosa* were secreted into the supernatant via TTSSs (Skrzypek and Straley, 1993; Yahr *et al.*, 1997). This supports the notion that the TTSSs of *A. hydrophila*, *Yersinia* spp. and *P. aeruginosa* share many functional similarities. It is noteworthy that another intense spot (spot 35) is present in the ECPs of the *ΔaopN* mutant (Fig. IV.11B). MALDI-TOF/TOF analysis showed that this protein (spot 35) did not match any proteins in the NCBI database. This may be attributed to the fact that secreted effector proteins are usually species specific and/or genetically diverse (Hueck, 1998). Thus, it may encode a novel secreted effector protein. In addition, several protein spots were also present in the ECPs of a *ΔaopN* mutant (spots region "I", Table IV.5, Fig. IV.11B). These spots cannot be identified by MALDI-TOF/TOF and await further study. ExsA, an AraC family regulator, functions as a central transcriptional regulator of the type III regulon in *P. aeruginosa* (Hovey and Frank, 1995; Yahr *et al.*, 1995). The AscA/ExsA of *A. hydrophila* AH-1 shares 64% identity with ExsA of *P. aeruginosa*, indicating ExsA homologs may

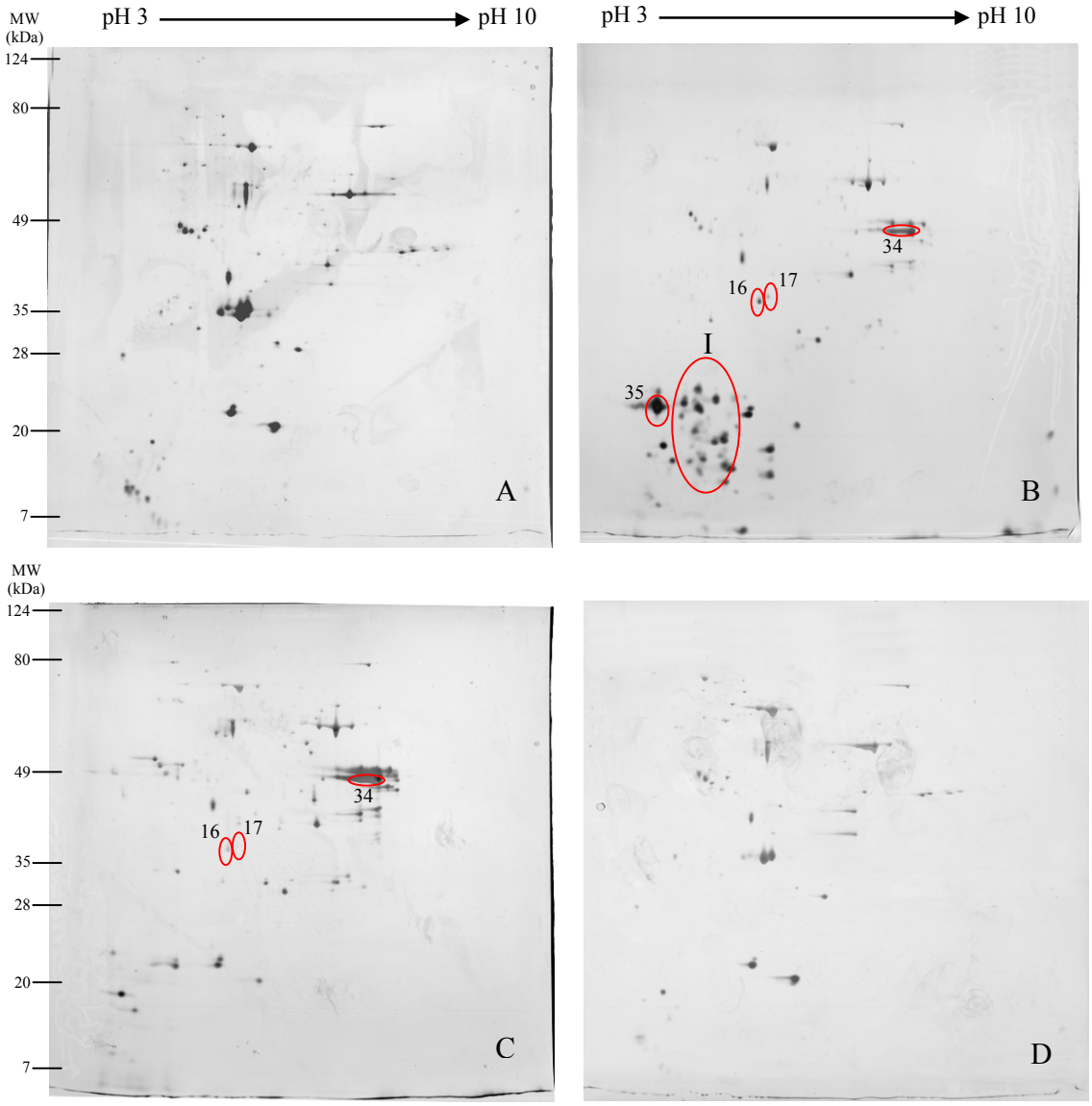


Fig. IV.11. Comparative extracellular proteome analysis of AH-1S (A), *ΔaopN* (B), *ΔexsD* (C) and *ΔaopNΔexsA* mutants (D). The circled proteins were down-regulated or up-regulated in *ΔaopN* or *ΔexsD* mutants. Numbers represent the spot identifications listed in Table IV.5.

function similarly in these two bacteria. As expected, the deletion of *exsA* in the *ΔaopN* background abolished the secretion of AcrV, spot 35 and spots in region I (Fig. IV.11D), suggesting that these proteins are secreted via the TTSS of *A. hydrophila* AH-1.

More interestingly, the secretion of LafA1 and LafA2 was significantly decreased in the ECPs of both *ΔaopN* and *ΔexsD* mutants (Figs. IV. 11B and C). To investigate whether the transcription of *lafA1* and *lafA2* is affected in these two mutants, plasmids pDN*lafA1* and pDN*lafA2* carrying the *lacZ* transcriptional fusion to the putative promoter region of *lafA1* and *lafA2*, respectively, were introduced into *ΔaopN* and *ΔexsD* mutants (Fig. IV.10). The expression of *lacZ* of *lafA1-lacZ* and *lafA2-lacZ* in both *ΔaopN* and *ΔexsD* mutants was reduced significantly (Fig. IV.10). The deletion of *exsA* in the *ΔaopN* mutant background can restore the secretion (expression) of lateral flagellins (Fig. IV.11D). These results suggest that there may be a cross-talk between the TTSS and the lateral flagellar secretion system. The TTSS appears to control the expression of lateral flagella via a TTSS central regulator (ExsA) as the deletion of *exsA* abolishes the regulatory effect of the TTSS on the lateral flagellar secretion system. However, whether ExsA controls the expression of lateral flagella directly or indirectly needs further study. The TTSS did not have a cross-talk with the polar flagellar secretion system, as the transcription levels of polar flagellins were not affected in a *ΔexsD* mutant (Fig. IV.9). The cross-talk between the lateral flagella and type III secretion systems may play a vital role in coordinating the regulation of these two secretion systems during the process of infection. Before contact with the host cells, the TTSS may not be activated while lateral flagella may be expressed to help bacteria colonize host cells. On contact with host cells, the TTSS may be activated while the expression of lateral flagella may be inhibited due to the increased level of ExsA

protein. Thus, bacteria may be able to evade host response but consistently secrete and inject effector proteins into host cells, interfering with the host signaling pathways and facilitating their survival in host cells.

IV.4 Conclusion

This is the first study on characterization of extracellular proteins from *A. hydrophila*. A reference map of ECPs of *A. hydrophila* AH-1 has been established. The major ECPs are identified in this proteome map.

A comparison of the extracellular proteomes of protease-deficient mutants and the wild type suggests that the serine protease, rather than the metalloprotease, could be involved in the processing of several secreted enzymes, such as GCAT, metalloprotease and hemolysin. Using a proteomic approach, in combination with a transcriptional fusion study, we have also showed that the expression of polar and lateral flagellins is under the control of multiple factors, such as temperature and different flagellar regulatory proteins. Most intriguingly, a cross-talk between the lateral flagellar secretion system and the TTSS has been discovered. In addition, many potential novel effector proteins secreted via the TTSS have been revealed by comparing the ECPs of TTSS negative regulator mutants with those of the wild type.

The advent of genome sequencing of a variety of bacteria, especially that of *A. hydrophila*, should readily lead to a complete extracellular proteome map of all the secreted proteins. Such an extracellular proteome map will help in our understanding of the exact roles the secreted proteins play in the pathogenesis of *A. hydrophila*.

Chapter V

Type III secretion system is required for *Aeromonas hydrophila* AH-1 pathogenesis

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Abstract

A. hydrophila is a Gram-negative opportunistic pathogen in fish and humans. Many bacterial pathogens of animals and plants have been shown to inject anti-host-virulence-determinants via a TTSS into the hosts. Degenerate primers based on *lcrD* family genes that are present in every known TTSS allowed us to locate the TTSS gene cluster in *A. hydrophila* AH-1. A series of genome walking helped in the identification of 25 open reading frames (AscU to AscB) that encode the proteins homologous to TTSS in other bacteria. PCR based analysis showed the presence of *lcrD* homologs (*ascV*) in all the 33 strains of *A. hydrophila* isolated from various sources. Insertional inactivation of two of the TTSS genes (*aopB* and *aopD*) led to decreased cytotoxicity in carp epithelial cells, increased phagocytosis and reduced virulence in blue gourami. These results show that a TTSS is required for *A. hydrophila* pathogenesis. This is the first report of sequencing and characterization of TTSS gene clusters from *A. hydrophila*. The TTSS identified here may help in developing suitable vaccines as well as the further understanding of the pathogenesis of *A. hydrophila*.

V.1 Introduction

The pathogenesis of *A. hydrophila* is multi-factorial. These include O-antigens, capsules (Merino *et al.*, 1996; Zhang *et al.*, 2002), the S layer (Dooley and Trust, 1988), exotoxins such as hemolysins and enterotoxin (Chakraborty *et al.*, 1984; Howard *et al.*, 1996), and a repertoire of exoenzymes which digests cellular components such as proteases, amylases and lipases (Leung and Stevenson, 1988; Pemberton *et al.*, 1997). These virulence determinants are involved sequentially for the bacteria to colonize, gain entry to, establish, replicate and cause damage in host tissues, and evade the host defense system and spread, eventually killing the host. The mechanisms of action by most of these virulence factors remain unknown.

Recent studies have shown that the virulence mechanisms of various pathogens are highly similar (Finlay and Falkow, 1997), one of which is a TTSS that has played crucial roles in host-pathogen interactions (Cornelis and Gijsegem, 2000). The TTSS is found in many Gram-negative animal and plant pathogens. This system can efficiently deliver anti-host virulence determinants into the host cells, directly interfering with and altering host processes. More recently, a fish pathogen, *A. salmonicida*, has been reported to have a functional TTSS located on a large thermolabile virulence plasmid (Burr *et al.*, 2002; Stuber *et al.*, 2003). Since motile aeromonads and *A. salmonicida* are in the same genus, it is possible that the TTSS is also present in other *Aeromonas* species including *A. hydrophila*.

A. hydrophila AH-1 is pathogenic to several fish such as the rainbow trout ($10^{4.5}$ as the LD_{50} value; J. M. Tomas, personal communication) and blue gourami ($10^{5.6}$ as reported in this study). It belongs to the O:11 serotype, which is one of the four dominant sero-groups

(O:11, O:16, O:18 and O:34) that are associated with gastroenteritis and septicemia in clinical studies (Kokka *et al.*, 1991). In the present study, we successfully located the TTSS gene cluster in *A. hydrophila* AH-1 using homology based analysis. This is the first report of cloning and characterization of partial TTSS gene cluster in *A. hydrophila* AH-1. Further inactivation of two of the TTSS genes (*aopB* and *aopD*) resulted in a delayed cytotoxic effect on carp epithelial cells, increased phagocytosis, and reduced virulence in gourami fish, showing that a TTSS is required for *A. hydrophila* pathogenesis.

V.2 Materials and methods

V.2.1 Plasmids, bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are listed in Table V.1 and Table II.1.When required, media were supplemented with chloramphenicol (Cm, 5 or 25 μg/ml), gentamicin (100 μg/ml) and colistin (Col, 6.25 μg/ml). The conjugation transfer of plasmids between *A. hydrophila* and *E. coli* strains was carried out by plate mating at 30^{o}C for 36 h.

V.2.2 Sequence analysis

Tansmembrane helices in proteins were predicted by analysis at http/www.cbs.dtu.dk/services/TMHMM (Krogh *et al*, 2001; Sonnhammer *et al.*, 1998). Terminator programs from the Genetics Computer Group package (GCG, Madison,Wis.) on a VAX4300 was used to confirm terminator sequences in the TTSS gene cluster.

V.2.3 PFGE and S1 nuclease digestion of genomic plugs

A. hydrophila AH-1 cells were embedded in low-melting-point agarose (Bio-Rad) to prepare the agar plugs using the CHEF Genomic DNA Plug Kit (Bio-Rad) according to the manufacturer's instruction. For *Pac*I restriction digestion, agarose plugs were

Table V.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant property	Source/Reference
AH-1	O:11, virulent, Col^r, Cm^s	Fish, UM
aopB	*aopB* insertion mutant from AH-1	This study
aopD	*aopD* insertion mutant from AH-1	This study
Plasmids		
pFS100	pGP704 suicide plasmid, λ*pir* dependent, Km^r	Rubires et al., 1997
pCM100	pGP704 suicide plasmid, λ*pir* dependent, Cm^r	This study
pCM-AOPB	pCM with an internal fragment of *aopB* (605 bp)	This study
pCM-AOPD	pCM with an internal fragment of *aopD* (565 bp)	This study
COS-TTSS	Cosmid pLA2917 containing partial TTSS gene cluster of AH-1	from J. M. Tomas

equilibrated with 500 µl of suitable restriction enzyme buffer for 30 min, exchanged with 400 µl fresh buffer containing 10 U of *Pac*I restriction enzyme, and incubated for overnight at 37°C. DNA fragments were separated by using a Bio-Rad CHEF-DRII apparatus. Electrophoresis was carried out at 200 V, 14°C for 22 h, with pulse times ranging from 5 to 60 s. For detecting and sizing large plasmids in *A. hydrophila* AH-1, plugs were incubated with S1 nuclease and subjected to PFGE as described previously (Barton *et al.*, 1995).

V.2.4 Construction of defined insertion mutants

Defined insertion mutants in *aopB* and *aopD* were constructed using the protocol described earlier with slight modifications (Rubires *et al.*, 1997). For the better selection of insertion mutants in *A. hydrophila* AH-1, we removed the kanamycin resistance cassette in pFS100 and replaced it with the chloramphenicol resistance (Cm^r) cassette from pACYC184 and generated the plasmid pCM100. Then, oligonucleotides *aopB*-F and *aopB*-R, *aopD*-F and *aopD*-R were used to amplify internal fragments from the *aop*B and *aop*D genes, respectively (Table V.2). Amplified fragment was ligated to pGEM-T Easy vector (Promega) and transformed into *E. coli* JM109. The internal fragment was recovered by *Eco*RV restriction digestion (*Eco*RV sites in primers are underlined), and finally ligated to *Eco*RV-digested, and dephosphorylated pCM100 and transformed into *E. coli* MC1061(λ*pir*), selecting Cm^r to generate plasmid pCM-AOPB and pCM-AOPD (Table V.1). The recombinant plasmid was isolated and transformed into *E. coli* S17-1(λ*pir*). Plasmids pCM-AOPB and pCM-AOPD were transferred by conjugation to *A. hydrophila* AH-1 (Col^r) to obtain defined mutants by selecting Cm^r and Col^r and insertion

Table V.2. Primers used in the detection of *ascV* gene and the construction of *aopB* and *aopD* mutants

Primer name	Sequence (5'-3')[1]	Amplified size (bp)
ascV-F	GTAARCAGATGAGTATCGATGG	331
ascV-R	GAGACSCGGGTGACGATAAT	
aopB-F	GTG**GATATC**TTGATCAATTGAGGAAGACGG	605
aopB-R	GGA**GATATC**GGTACCAATATCAACTACCAG	
aopD-F	GAA**GATATC**GATTCGAGCCTGCTGAGCAA	565
aopD-R	GGA**GATATC**GCACTTCATCTTCCTTGGCATT	

[1]Letters in bold indicate the introduction of *Eco*RV site in the primers.

mutants of *aopB* and *aopD* were generated, respectively. The insertion of plasmids on the chromosomes of these mutants was confirmed by PCR using appropriate primers.

V.2.5 Cell culture and morphological changes induced by *A. hydrophila*

All tissue culture reagents were obtained from Invitrogen. Epithelioma papillosum of carp, *Cyprinus carpio*, (EPC cells) (Wolf and Mann, 1980) was grown in minimal essential medium (MEM) with Hank's salts, 10 mM HEPES (pH 7.3), 2 mM glutamine, 0.23% $NaHCO_3$ and 10% heat-inactivated fetal bovine serum (FBS) at 25^{o}C in a 5% (v/v) CO_2 atmosphere. Cells were grown in 75 cm^2 flasks and split at least once a week by trypsin/EDTA treatment and dilution at 1:10 in fresh media. Studies on morphological changes were conducted by seeding 5×10^5 fish cells into each well of a 24-well tissue culture plate (Falcon) and then proceeding as described previously (Tan *et al.*, 1998). EPC monolayers were infected with *A. hydrophila* cells suspended in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 [pH 7.2]) at a multiplicity of infection (MOI; number of bacteria per cultured cell) of 1. Changes in the cell morphology were observed over a period of 6 h. The fish cells were photographed under an Axiovert 25CFL phase-contrast inverted microscope (Carl-Zeiss) at 40× magnification after 2.5 h and 5.5 h of infection at 25^{o}C.

V.2.6 Phagocyte isolation

Healthy blue gourami (*T. trichopterus* Pallas) were obtained from a commercial fish farm and maintained in well-aerated, dechlorinated water at 25 ± 2 ^{o}C. Phagocytes were isolated from the head kidney of naïve gourami and purified following the procedure of Secombs (1990). Purified phagocytic cells (4×10^6 to 5×10^6 cells/well) were allowed to adhere to 24- or 48-well tissue culture plates (Falcon) in 5% FBS supplemented L-15 medium

(Sigma). After 3 h of incubation at 25°C in a 5% CO_2 atmosphere, the cells were washed twice using Hanks balanced salt solution (HBSS) (Sigma) to remove unattached cells. The remaining monolayer of phagocytes was infected with the wild type and mutant strains of *A. hydrophila* at a MOI of 10 in all the experiments.

V.2.7 Microscopic examination and phagocytosis assay

Glass coverslips were placed into each well of the 24-well tissue culture plate, and the wells were seeded with blue gourami phagocytes and incubated for 3 h at 25 °C in a 5% CO_2 atmosphere as described above. Three hours after infection, the phagocytes were washed three times with HBSS and later stained with Giemsa (Merck) for 30 min. After they were washed three times with PBS, the stained samples were examined under an Axiovert 25CFL phase-contrast inverted microscope (Carl-Zeiss) at 100× magnification. For the phagocytosis assay, the phagocytes were infected for 3 h, washed twice with HBSS, and then incubated for 1.5 h in 5% FBS supplemented fresh L-15 medium with 100 μg/ml of gentamicin. The gentamicin treatment killed extracellular bacteria but did not affect the viability of intracellular organisms. The phagocytes were then washed three times with HBSS to remove gentamicin and lysed with 1% (v/v) Triton X-100. This was followed by a 1-min incubation, which released intracellular bacteria. Intracellular bacteria were quantified on triplicate TSA plates. The percentage of phagocytosis was calculated by dividing the viable bacterial count after gentamicin treatment by the initial bacterial count. Three independent experiments with duplicate wells were performed.

V.2.8 Nucleotide sequence accession number

The nucleotide sequence data of the *A. hydrophila* AH-1 TTSS gene cluster has been deposited in GenBank under accession no. AY394563.

V.3 Results and discussion

V.3.1 Sequencing and genetic organization of a TTSS gene cluster in AH-1

TTSSs are present in many Gram-negative bacteria (Hueck, 1998; Galan and Collmer, 1999). Various components of the type III secretion genes are highly conserved. *lcrD* homologs are one of those components that are present in all known TTSSs, and sequence similarities between individual members of the LcrD family vary between 36% and 66% (Hueck, 1998). Multiple sequence alignment of proteins of the LcrD family from *A. salmonicida* (Burr *et al.*, 2002), *P. aeruginosa* (Yahr *et al.*, 1997), and *Y. enterocolitica* (accession no AF102990) helped in locating two regions which are highly conserved at both amino acid and nucleotide levels (Fig. V.1). A pair of degenerate primers, *ascV*-F and *ascV*-R (Table V.2), were designed and the truncated *lcrD* homologous gene (renamed as *ascV* in this study; 331-bp in size) was successfully amplified in *A. hydrophila* AH-1. The 331-bp fragment showed 92% and 99% identity to the *ascV* gene of *A. salmonicida*, at nucleotide level and amino acid level, respectively. Subsequently, the TTSS gene cluster was partially sequenced by a series of genome walking. This led to the identification of 25 open reading frames (ORFs) which showed high homology with the TTSS proteins in *A. salmonicida* (Burr *et al.*, 2002), *Photorhabdus luminescens* (Waterfield *et al.*, 2002), *P. aeruginosa* (Yahr *et al.*, 1997), and *Yersinia* species (Bergman *et al.*, 1994 ; Michiels *et al.*, 1991) (Table V.3). Most of the putative TTSS proteins in *A. hydrophila* were designated according to the *Yersinia* nomenclature which was also applied to designate the ORFs of *A. salmonicida* TTSS (Table V.3). The genetic organization of this cluster is highly similar to that of *P. aeruginosa* (Fig.V.2). It is also similar to that of *A. salmonicida, P. luminescens* and *Yersinia* species, but differs from the

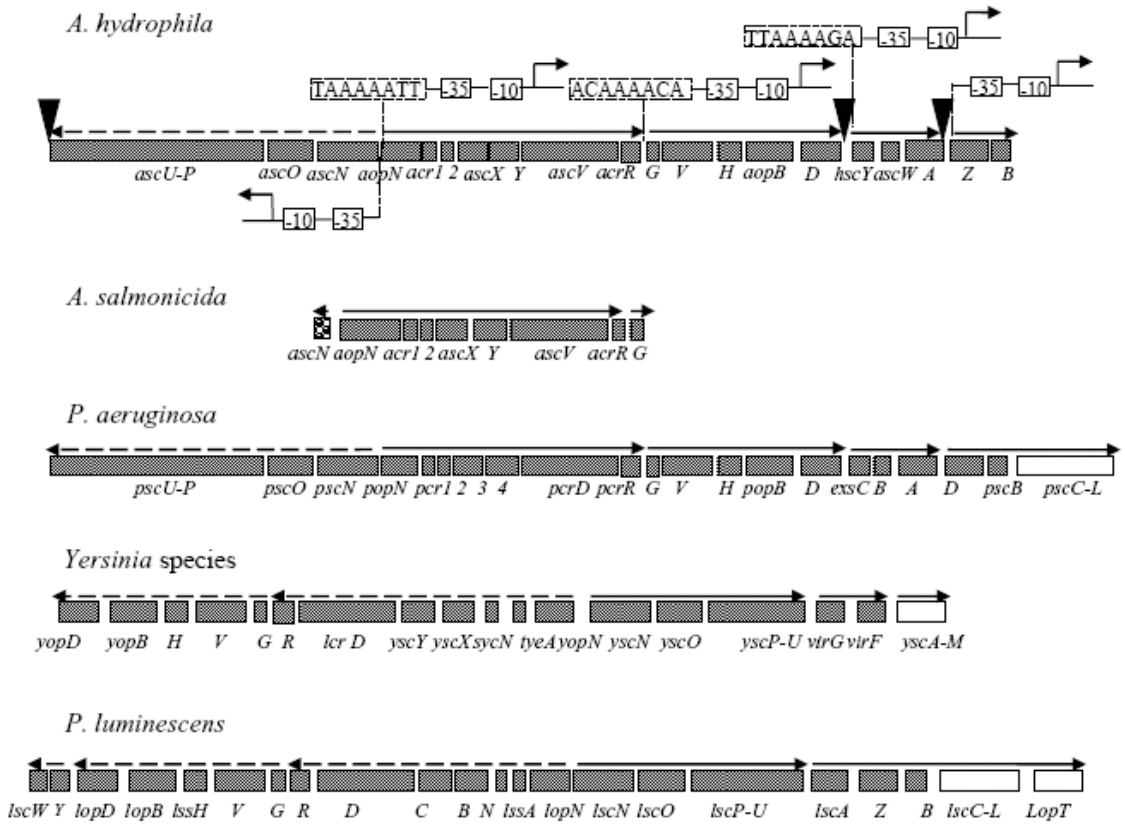
(A) Amino acid alignment

```
     AscV  (151) GSERVAEVSARFSLDAMPGKQMSIDGDMRAGVIDVHEARDRRGVIEKESQ
     YscV  (135) GSERVAEVSARFSLDAMPGKQMSIDGDMRAGVIDVNEARERRATIEKESQ
     PcrD  (134) GAERVAEVSARFSLDAMPGKQMSIDGDMRAGVIDVNEARARRAVIEKESQ
Consensus  (151) GSERVAEVSARFSLDAMPGKQMSIDGDMRAGVIDVNEARDRRAVIEKESQ

     AscV  (251) ILTVGDGMVSQVPALLIAITAGIIVTRVSSEESSDLGTDIGAQVVAQPKA
     YscV  (235) ILTVGDGMVSQVPALLIAITAGIIVTRVSSEDSSDLGSDIGKQVVAQPKA
     PcrD  (234) VLTVGDGMVSQVPALLIAITAGIIVTRVSSDESADLGSDIGEQVVAQPKA
Consensus  (251) ILTVGDGMVSQVPALLIAITAGIIVTRVSSEESSDLGSDIG QVVAQPKA
```

(B) Nucleotide alignment

```
     ascV   (501) GCCGGGTAAGCAGATGAGTATCGATGGTGACATGCGCGCCGGGGTGATCG
     yscV   (453) GCCGGGTAAACAGATGAGTATCGATGGCGATATGCGAGCCGGGGTGATCG
     pcrD   (450) GCCAGGCAAGCAGATGAGCATCGACGGCGACATGCGTGCCGGCGTGATCG
Consensus   (501) GCCGGGTAAGCAGATGAGTATCGATGGCGACATGCG GCCGGGGTGATCG

     ascV   (801) CGCCATCACCGCGGGCATTATCGTCACCCGGGTCTCCTCCGAAGAGTCTT
     yscV   (753) AGCTATTACCGCGGGTATTATCGTCACCCGCGTCTCTTCAGAAGATTCAT
     pcrD   (750) CGCCATCACCGCCGGGATCATCGTCACCCGGGTGTCTTCCGACGAATCGG
Consensus   (801) CGCCATCACCGCGGG ATTATCGTCACCCGGGTCTCTTCCGAAGA TC T
```

FIG. V.1. Alignment of LcrD family of proteins among *A. salmonicida* (AscV), *Yersinia* species (YscV) and *P. aeruginosa* (PcrD) at the amino acid (A) and nucleotide sequence levels (B). A pair of degenerated primers (in bold letters) was designed to amplify a 331 bp internal fragment of *asc*V in *A. hydrophila* AH1 with primers *ascV*-F and *ascV*-R.



Homologous type III secretion gene identified in this study

Other type III secretion system gene

Partial open reading frame

Putative transcription terminator

Putative ExsA binding site

FIG. V.2. Genetic organization of TTSS in *A. hydrophila* and other bacteria. Arrows indicate the proposed directions of transcription. The name of each gene is shown underneath the box.

TABLE V.3. *A. hydrophila* AH-1 putative proteins and their homologs in other bacteria

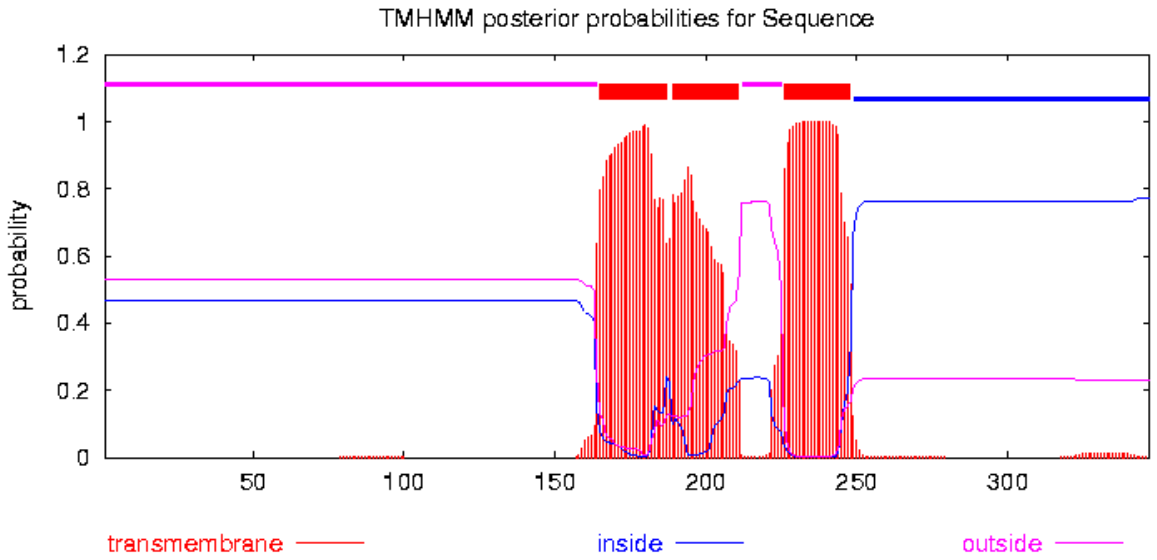
A. hydrophila protein	*A. salmonicida* homolog (I/S)[a]	*P. luminescens* homolog (I/S)	*Yersinia* spp homolog (I/S)	*P. aeruginosa* homolog (I/S)	Putative function
AscU	AscU (93/97)	LscU (75/84)	YscU (69/81)	PscU (68/81)	Regulation of secretion
AscT	AscT (88/94)	LscT (54/63)	YscT (51/62)	PscN (74/88)	Unknown
AscS	AscS (97/98)	LscS (82/90)	YscS (77/89)	PscS (78/90)	Unknown
AscR	AscR (98/99)	LscR (78/87)	YscR (77/84)	PscR (79/87)	Unknown
AscQ	AscQ (74/82)	LscQ (47/58)	YscQ (44/60)	PscQ (47/59)	Unknown
AscP	AscP (52/67)	LscP (36/53)	YscP (39/57)	PscP (34/51)	Regulation of secretion
AscO	AscO (81/870	LscO (40/47)	YscO (37/53)	PscO (42/53)	Regulation of secretion
AscN	AscN (97/97)	LscN (90/90)	YscN (87/90)	PscN (83/90)	ATP synthase
AopN	AopN (86/91)	LopN (66/78)	YopN (51/66)	PopN (59/74)	Regulation of translocation
Acr1	Acr1 (90/94)	LssA (71/86)	TyeA (54/74)	Pcr1 (57/75)	Translocation apparatus
Acr2	Acr2 (81/89)	LssN (63/78)	SycN (60/75)	Pcr2 (46/62)	Chaperone
AscX	AscX (80/89)	LssB (59/73)	YscX (50/70)	Pcr3 (46/61)	Type III secretion apparatus
AscY	AscY (58/62)	LssC (46/52)	YscY (37/43)	Pcr4 (46/54)	Type III secretion apparatus
AscV	AscV (86/88)	LssD (78/84)	LcrD (76/82)	PcrD (74/82)	Type III secretion apparatus
AcrR	AcrR (60/70)	LssR (51/64)	LcrR (58/74)	PcrR (47/61)	Unknown
AcrG	AcrG (43/56)	LssG (43/56)	LcrG (47/63)	PcrG (46/55)	Regulation of low-calcium response
AcrV	AcrV (33/52)	LssV (42/59)	LcrV (41/63)	PcrV (32/48)	Protective antigen, anti-host factor
AcrH	AcrH (62/79)	LssH (58/69)	SycD (61/75)	PcrH (61/73)	Chaperone
AopB	AopB (41/57)	LopB (36/50)	YopB (32/48)	PopB (36/51)	Translocation apparatus
AopD	AopD (47/64)	LopD (49/66)	YopD (45/61)	PopD (47/64)	Translocation apparatus
HscY/ExsC	ExsC (78/85)	LscY (67/80)		ExsC (56/80)	Unknown
AscW/ExsB	ExsB (63/72)	LscW (38/58)	YscW (35/55)	ExsB (33/52)	Regulation of secretion
AscA/ExsA	ExsA (89/94)	LscA (75/82)	VirF (62/76)	ExsA (64/77)	Transcriptional activator
AscZ/ExsD	ExsD (69/81)	LscZ (41/54)		ExsD (33/51)	Anti-activator ExsA
AscB	AscB 76/84)	LscB (54/67)	YscB (45/64)	PscB (44/60)	Unknown
AscC	AscC (90/96)	LscC (72/83)	YscC (74/86)	PscC (67/80)	Outermembrane protein for transporting
AscD	AscD (74/84)	LscD (51/67)	YscD (46/66)	PscD (48/67)	Unknown
AscE	AscE (73/86)	LscE (53/72)	YscE (40/60)	PscE (45/60)	Unknown
AscF	AscF (75/81)	LscF (75/83)	YscF (70/87)	PscF (73/85)	Pore-forming translocator
AscG	AscG (69/76)	LscG (53/60)	YscG (51/62)	PscG (50/61)	Unknown
AscH	AscH (58/73)	LscH (40/53)	YscH (42/61)	PscH (35/51)	Unknown
AscI	AscI (84/91)	LscI (58/80)	YscI (50/71)	PscI (58/73)	Unknown
AscJ	AscJ (90/95)	LscJ (77/88)	YscJ (76/84)	PscJ (75/87)	Intracellular trafficking and secretion
AscK	AscK (70/75)	LscK (49/64)	YscK (48/61)	PscK (48/61)	Unknown
AscL	AscL (84/90)	LscL (75/87)	YscL (70/87)	PscL (62/80)	Intracellular trafficking and secretion

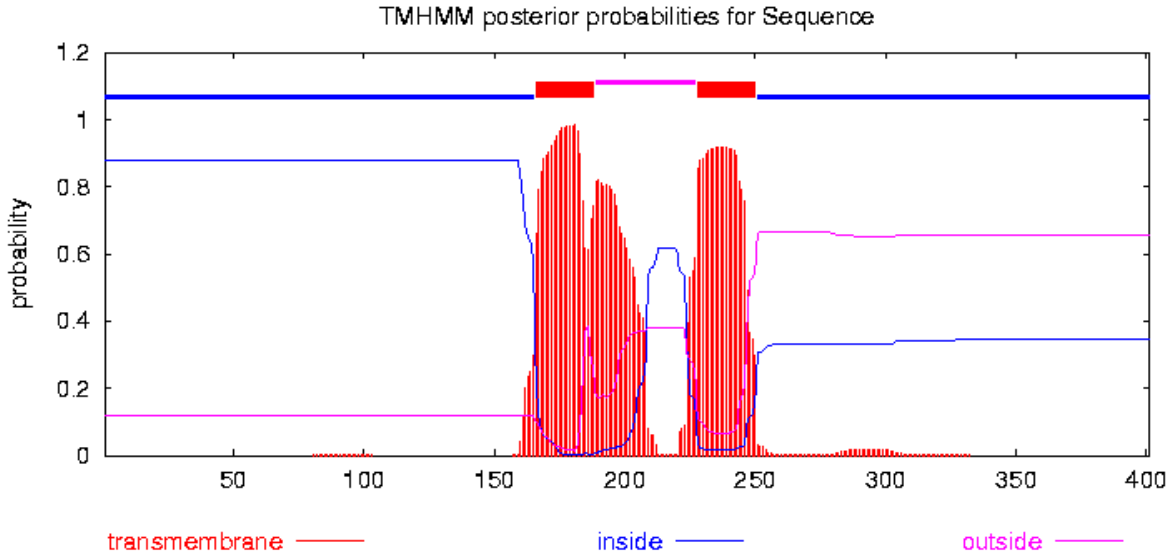
[a]I: % of identity; S: % of similarity.
ORFs AscU to AscB were identified in Chapter V.
ORFs AscC to AscL were identified in Chapter VI.

gene order in *P. luminescens* and *Yersinia* species where the homologous *ascU-aopD* regions are inverted.

Further sequence analysis of this TTSS gene cluster identified five putative promoter regions (Fig.V.2), three of which possess ExsA binding sites (TxAAAAxA) that are similar to those in *A. salmonicida* (Burr *et al.*, 2002) *and P. aeruginosa* (Hovey and Frank, 1995). In *P. aeruginosa*, these consensus sequences are bound by ExsA, a transcriptional activator of exoenzyme S regulon (Hovey and Frank, 1995). These three promoters are located upstream of *aopN*, *acrG* and *hscY*. We also have identified AscA that shows 77% similarity to ExsA (Table V.3), which may regulate the transcription of these operons in AH-1 TTSS cluster. Further work is required to confirm this hypothesis. The other two putative promoters are upstream of *ascN* and *ascZ* without the characteristic ExsA consensus sequences being identified. Terminator analysis showed the presence of three possible transcription terminators. They are downstream of *ascU* (ΔG, -3.30 kcal/mol), *aopD* (ΔG, -18.60 kcal/mol) and *ascA* (ΔG, -6.40 kcal/mol) (Fig.V.2). Based on the above analysis, we speculate that there may be five operons in the AH-1 TTSS gene cluster. They are *ascN* to *ascU*, *aopN* to *aopD*, *acrG* to *aopD*, *hscY* to *ascA* and the last one is from *ascZ* and beyond.

In addition, transmembrane helices search was carried out only for AopB and AopD, since they were further characterized using insertion mutagenesis. Results showed that they have similar domains to those of the YopB and YopD. Similar transmembrane helices for AopB and YopB are TMH1 (165-211::166-208) and TMH2 (226-248:: 228-250); for AopD and YopD are TMH1 (121-143::129-151) (Parkhill *et al.*, 2001) (Fig. V.3). Coiled-coil domains (Lupas *et al.*, 1991) were found in AopB from amino acids 102-158 and 288-


TMHMM posterior probabilities for Sequence
probability
1.2
1
0.8
0.6
0.4
0.2
0
50
100
150
200
250
300
transmembrane
inside
outside


TMHMM posterior probabilities for Sequence
probability
1.2
1
0.8
0.6
0.4
0.2
0
50
100
150
200
250
300
350
400
transmembrane
inside
outside

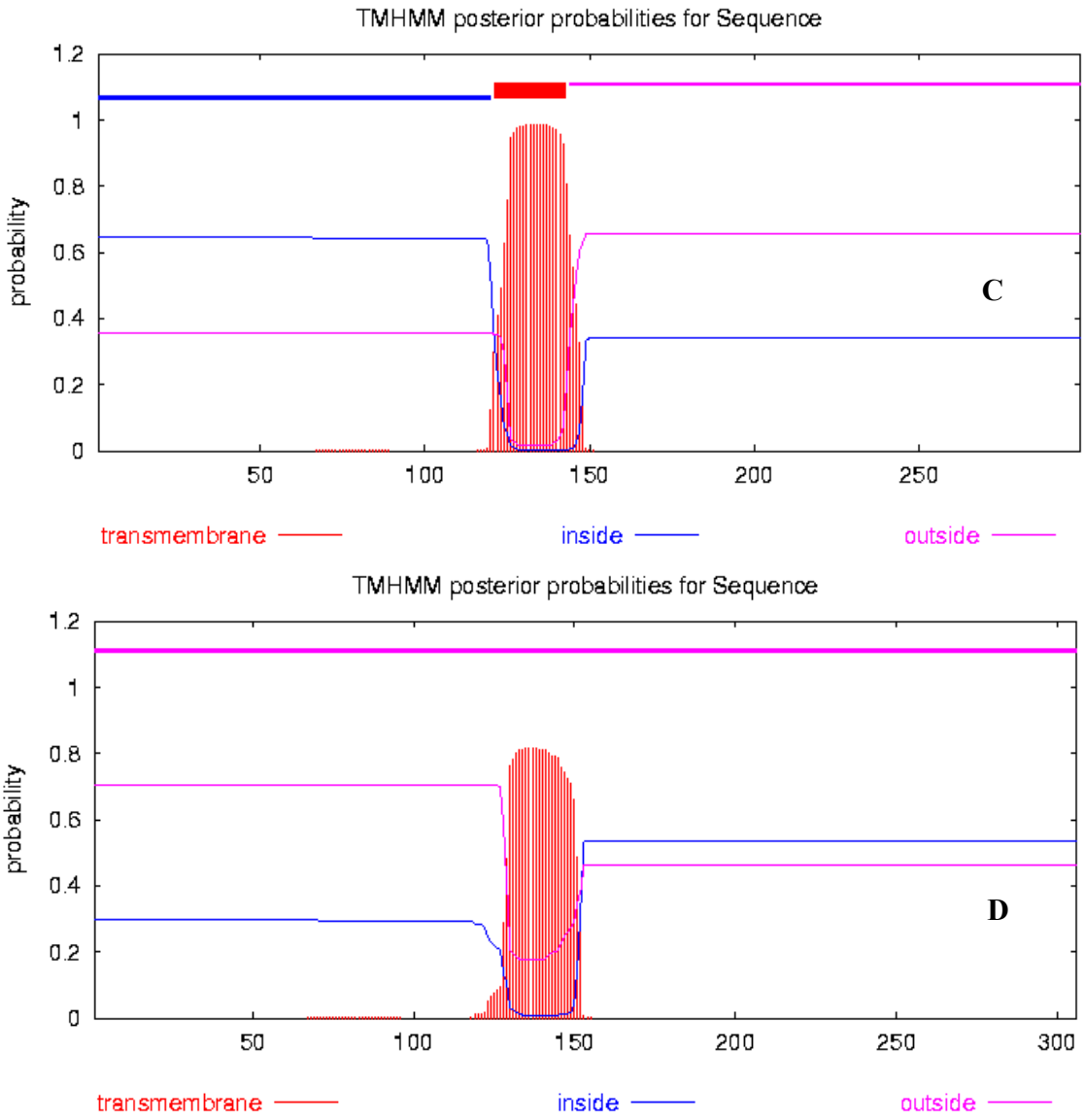


Fig. V.3. Transmembrane helix profiles of AopB (A), YopB (B), AopD (C) and YopD (D). The distribution of transmembrane helixes: 165-211aa and 226-248aa (A); 166-208aa and 228-250aa (B); 121-143aa (C) and 129-151aa (D).

326, and from amino acids 104-331 and 342-386 for YopB. YopB and YopD, consisting of hydrophobic domains, have been shown to function as translocases for the translocation of effector proteins across the eukaryotic cell membrane (Rosqvis *et al.*, 1994; Sory and Cornelis, 1994; Boland *et al.*, 1996; Hakansson *et al.*, 1996a & 1996b).

V.3.2 TTSS is located on the AH-1 chromosome

TTSS gene clusters can be either on a plasmid or on the bacterial chromosome (Plano *et al.*, 2001). PFGE of bacterial DNA of *A. hydrophila* AH-1 was carried out to investigate whether it contained any plasmids. The PFGE results showed that the S1 nuclease cut DNA was brighter than the uncut one (Fig.V.4A). Dodd and Pemberton (1998) also reported that the treatment of *A. hydrophila* JMP636 genomic DNA with S1 nuclease produced a highly intense band compared to the untreated one, indicating the digestion of circular DNA by S1 nuclease. PFGE of S1 nuclease treated *A. hydrophila* AH-1 genomic DNA plug showed only one band as an uncut genomic DNA, indicating that there may not be a large size plasmid in AH-1. We further prepared genomic DNA plugs digested with *Pac*I and carried out Southern blot analysis using the *ascV* probe. Southern results revealed that an *ascV* probe hybridized with S1 nuclease cut genomic DNA, uncut genomic DNA and a 200-kb *Pac*I digested fragment (Figs. V.4B). A 20 kb size plasmid was detected using a plasmid purification kit which was capable of purifying plasmids up to 150 kb. This small plasmid was not detected in PFGE, which could be due to the low copy of this plasmid. The *ascV* probe did not hybridize with this 20 kb plasmid (Fig.V.4C). These results indicate that the TTSS gene cluster is located on the chromosome of *A. hydrophila* AH-1, which is similar to other pathogens such as *P. aeruginosa* (Yahr *et al.*, 1997) and *P. luminescens* (Waterfield *et al.*, 2002). And *A. hydrophila* AH-1 is unlike

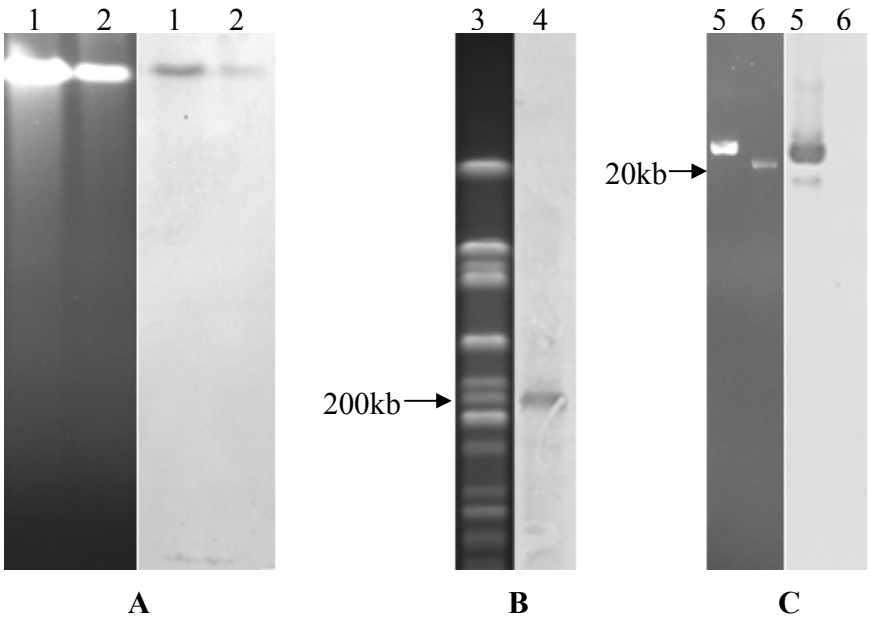


FIG. V.4. Location of *ascV* gene by PFGE and Southern blot analysis. (A) PFGE and Southern blot analysis of S1 nuclease treated (lane 1) and intact genomic plug (lane 2) in Panel A. (B) A gel showing *Pac*I digested genomic DNA plug from AH-1 (lane 3) and Southern blot with *ascV* as probe against gel in lane 3 (lane 4). (C) A gel showing cosmid COS-TTSS (lane 5), an ~20 kb plasmid isolated from AH1 (lane 6) and Southern blot with *ascV* gene as probe against Gel in lanes 5 and 6.

A. salmonicida where TTSS is located on a large thermolabile plasmid of approximately 140 kb (Stuber *et al.*, 2003).

V.3.3 Distribution of TTSS in *A. hydrophila*

In several gram-negative pathogens, TTSSs are encoded by pathogenicity islands which are present in virulent but absent from avirulent strains (Cornelis and Gijsegem, 2000; Winstanley and Hart, 2001). Hence, the distribution of TTSS gene clusters was surveyed among 33 *A. hydrophila* strains isolated from different sources such as fish and human that were from various geographical locations (Table II.1). These isolates represented diverse serotypes with varying levels of virulence. The same pair of degenerate primers (ascV-F and ascV-R) was used to detect the *ascV* gene in all the 33 strains. PCR results showed that a 331-bp DNA fragment was present in all the *A. hydrophila* strains (Appendix I). FlhA, an essential component of the flagellar export apparatus, has also shown some similarity to the LcrD family protein in the TTSS (Hueck, 1998). Hence, DNA sequencing was performed to confirm that these 33 PCR products were highly similar to *ascV* but not to *flhA*. All the sequences showed high identity to *ascV* of *A. hydrophila* AH-1, with amino acid and nucleotide identities ranging from 83% to 100% and from 73% to 99%, respectively. Our result indicates, therefore, that a partial or an entire TTSS gene cluster may be present in all the 33 *A. hydrophila* strains that we examined.

Similarly, the TTSS has also been shown to be ubiquitously present in both clinical and environmental isolates of *P. aeruginosa* (Feltman *et al.*, 2001). Hence, the presence of TTSS genes may not correlate with virulence for some of the bacteria. On the other hand, the TTSS in non-pathogenic strains of *A. hydrophila* may not be functional. Pierson and

Falkow (1990) have reported the presence of non-functional *invA* homologous genes in non-pathogenic *Y. enterocolitica*.

V.3.4 Construction of mutants and LD_{50} studies

To ascertain that the TTSS in *A. hydrophila* AH-1 was functional, we carried out insertional mutagenesis in *aopB* and *aopD*. They are the homologs of *yopB* and *yopD*, respectively in *Y. enterocolitica*, which may form the translocation apparatus for functional type III secretion (Hueck, 1998). Disruptions in *yopB* and/or *yopD* reduced virulence in mice (Hartland *et al.*, 1994 & 1996). In *A. hydrophila* AH-1, both *aopB* and *aopD* mutants showed a similar growth rate in TSB when compared with the wild type (Fig.V.5). LD_{50} for AH-1 by intramuscular injection into blue gourami was estimated as$10^{5.6}$, while the *aopB* and *aopD* mutants were $10^{6.6}$ and $10^{6.4}$, respectively, and it was about 1 log higher than that of the wild type. When blue gourami were inoculated with the same dosage (10^6), most of the fish injected with *aopB* and *aopD* mutants recovered within four days and displayed no marked skin ulceration at the injection site. However, eight out of ten fish injected with the wild type died within three days (Fig.V.6).When fish were infected with bacteria at sub-lethal dosage (1×10^5 CFU), most of the fish infected with the wild type exhibited hemorrhage at the injection site after 3 days (Fig. V.7A). In contrast, all the fish infected with *aopB* or *aopD* mutants recovered within 3 days (Fig.V.7B).The hemorrhage symptom remained even 5 days postinfection most of the fish infected with wild type bacteria (Fig.V.7C). To confirm the stability of the insertional *aopB* and *aopD* mutants genes, bacteria were isolated from live/dead fish inoculated with these mutants, all showing resistance to chloramphenicol. PCR using appropriate primers

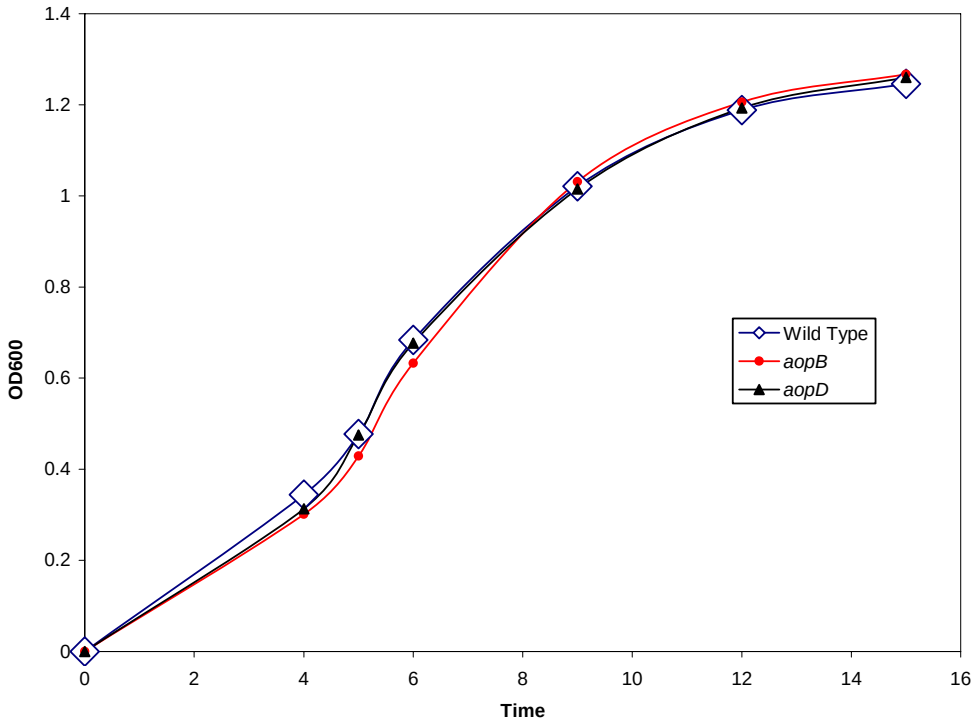


Fig. V.5. Both the *aopB* and *aopD* mutants showed a similar growth rate in TSB when compared with the wild type.

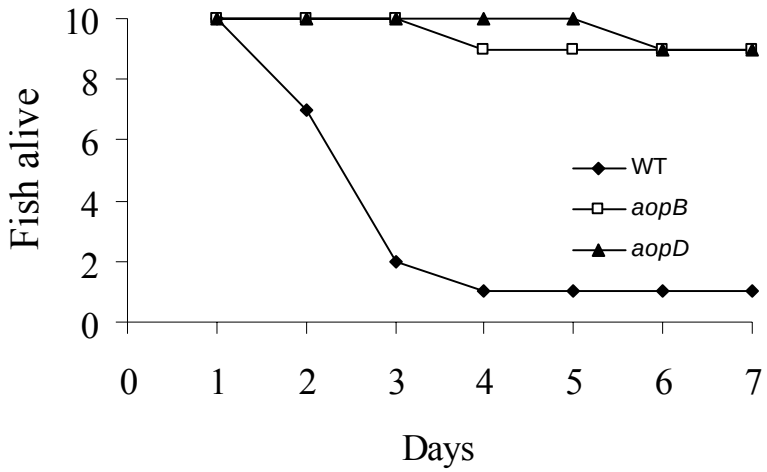
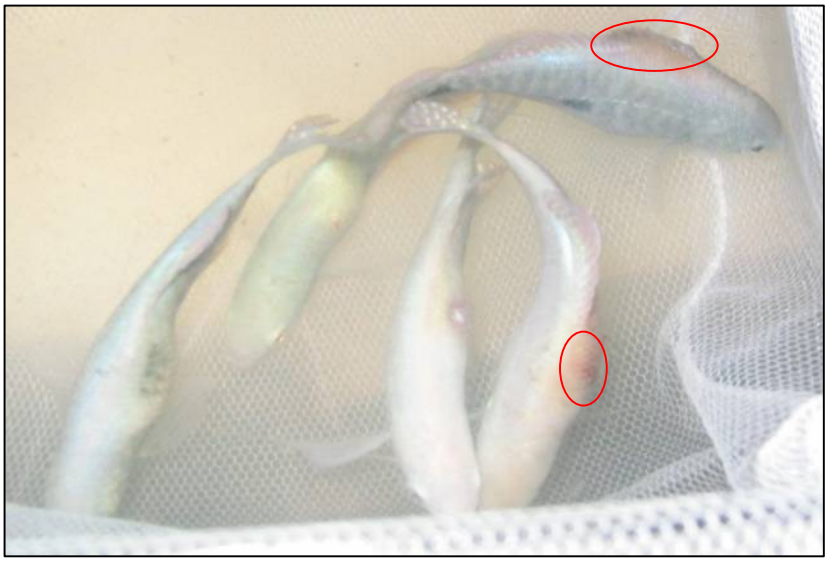


FIG. V.6. *aopB* and *aopD* mutants show a decrease in the pathogenesis of blue gourami infections. The number of fish alive after injected with 10^6 CFU dosage of *A. hydrophila* is plotted against the days when fish died. Blue gourami was monitored for 7 days. WT, wild type AH-1.

A

B



C

Fig. V.7. Blue gourami fish were infected with bacteria at the same sub-lethal dosage (1×10^5 CFU). The fish infected with the wild type (3 days post injection) (A); the fish infected with the *aopB* or *aopD* mutants (3 days post injection) (B); the fish infected with the wild type (5 days post injection) (C). Circled region indicate the hemorrhage portion.

for these mutants also confirmed the stability of these mutants. These results strongly indicate that the TTSS plays an important role in the pathogenesis of *A. hydrophila.*

V.3.5 Delayed cytotoxic effect by *aopB* and *aopD* mutants on EPC

EPC cells were infected with AH-1, *aopB* and *aopD* mutants, respectively. Upon infection with *A. hydrophila* AH-1, the EPC cells underwent a series of cytopathic changes similar to another strain PPD134/91 as described in an earlier study (Tan *et al.*, 1998). The cells infected with AH-1 were progressively detached from one another, elongated to form long spindles, became rounded and eventually detached from one another and also from the well. At 2.5 h post-infection, approximately 50% of the rounded EPC cells remained attached to the tissue culture plate (Fig.V.8A) while EPC cells infected with either *aopB* or *aopD* mutants showed no significant morphological changes (Fig.V.8B) as compared to the uninfected control (Fig.V.8C). An uninfected monolayer of EPC cells appeared as a smooth sheet with the cells adhering tightly to their neighbors. EPC cells infected with *aopB* or *aopD* mutants started to show morphological changes at 3 h post-infection. By 5.5 h post-infection, only 50% of the rounded EPC cells remained. Mutation of *aopB* or *aopD* therefore delayed cytotoxic changes to the EPC cells. There may be other factors mediating cytotoxicity in *A. hydrophila*. Virulence factors such as aerolysin and hemolysin (Wong *et al.*, 1998), and serine protease (Rodriguez *et al.*, 1992) have been reported to be involved in the cytotoxicity for different cultured cells. This may explain the delayed onset of the cytotoxic effect by *aopB* and *aopD* mutants of AH-1.

V.3.6 Phagocytosis assay

Phagocytes are the primary defense barriers in any host, and bacteria have to either avoid or overcome the phagocyte-mediated killing to establish themselves in the host. We

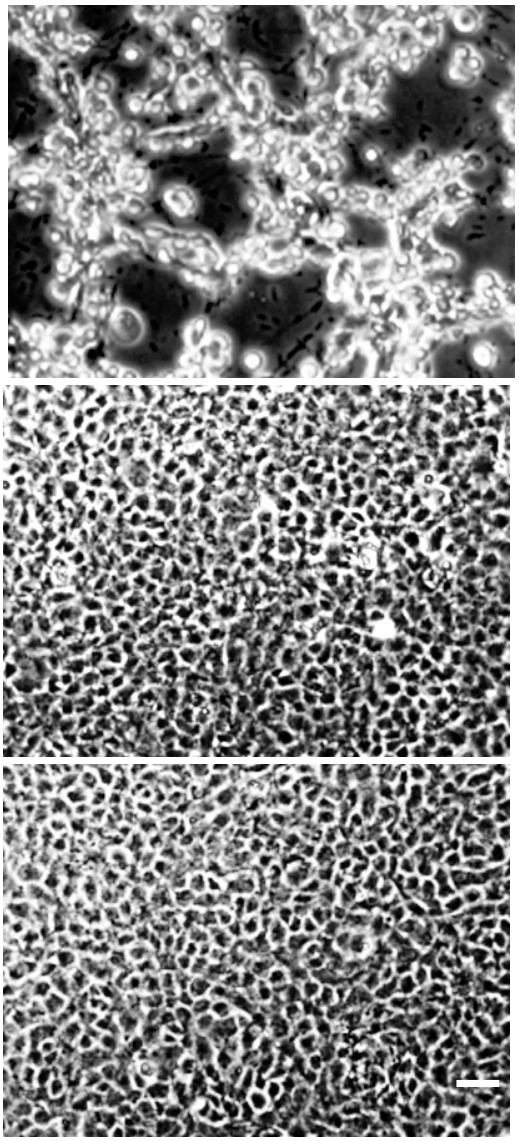


FIG. V.8. Micrographs of carp epithelial cells infected with *A. hydrophila* AH-1 (A), *aopB* mutant (B) and carp epithelial cells inoculated with PBS as a negative control (C). Phase-contrast micrographs of ECP monolayers were infected with *A. hydrophila* strains at 2.5 h post- infection (MOI of 1). Mutants *aopB* and *aopD* had similar results in EPC experiment. Bar inside panel C is 50 μm and corresponds to panel A and B.

carried out a microscopic examination of phagocytes infected with AH-1 and mutants. After 3 h of infection with AH-1, most of the bacteria were outside the phagocytes with a few of them inside (Fig.V.9A). On the other hand, most of the *aopB* and *aopD* mutants were inside the phagocytes (Fig.V.9B). These may indicate that the wild type (AH-1) bacteria may be avoiding or inhibiting phagocytosis but the mutants failed to avoid phagocytosis, thereby getting ingested.

Further to analyze whether the wild type bacteria resists phagocytosis, internalization assay was carried out. After infecting the phagocytes with AH-1, *aopB* and *aopD* mutants for 3 h, the monolayer was treated with gentamicin for 1.5 h to kill all the extracellular bacteria. The results clearly showed that mutants had a four to six times higher ingestion rate compared to the wild type (Fig.V.10). Wild type AH-1 was resistant to phagocytosis, with only 0.24% of bacteria within the phagocytes. However, *aopD* and *aopB* mutants showed increased phagocytosis with 1.1% and 1.5% of bacteria within the phagocytes, respectively. These results indicate that mutations in *aopB* and *aopD* are affecting the phagocytosis and they may be playing an important role in antiphagocytosis.

In *Y. enterocolitica*, YopB and YopD, the homologs of AopB and AopD, have also been shown to be essential for cytotoxicity and antiphagocytosis (Hartland *et al.*, 1994 & 1996). They may form the pore on the host cell membrane to deliver effectors such as YopE and YopH that cause cytotoxicity and antiphagocytosis to the respective target host cells (Rosqvist *et al.*, 1994; Persson *et al.*, 1995; Sory *et al.*, 1995; Håkansson *et al.*, 1996). From the results of cell culture assays, we speculate that AopB and AopD of AH-1 may be acting in a similar manner to those of *Y. enterocolitica*. The TTSS of AH-1 may also use AopB and AopD as translocon components to deliver similar effectors into host cells,

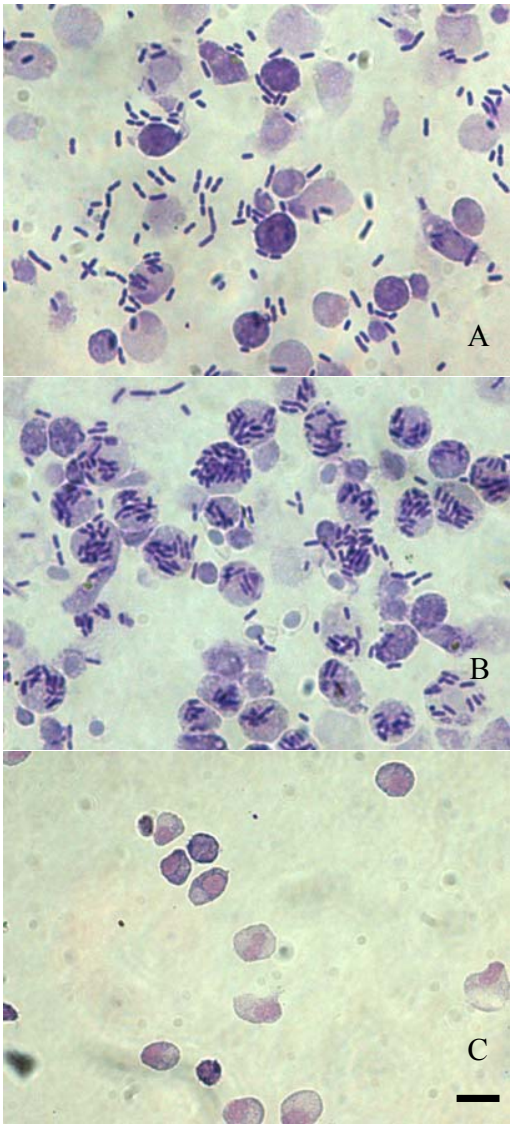


Fig. V.9. Micrographs of blue gourami phagocytes infected with *A. hydrophila* AH-1 (A) and *aopB* mutant (B), and blue gourami phagocytes inoculated with PBS as a negative control (C). Giemsa stained bright-field micrographs of blue gourami phagocytes were infected with *A. hydrophila* strains at 3 h post-infection (MOI of 10). Mutants *aopB* and *aopD* had similar results in phagocytes experiment. Bar inside panel C is 10 µm and corresponds to panel A and B.

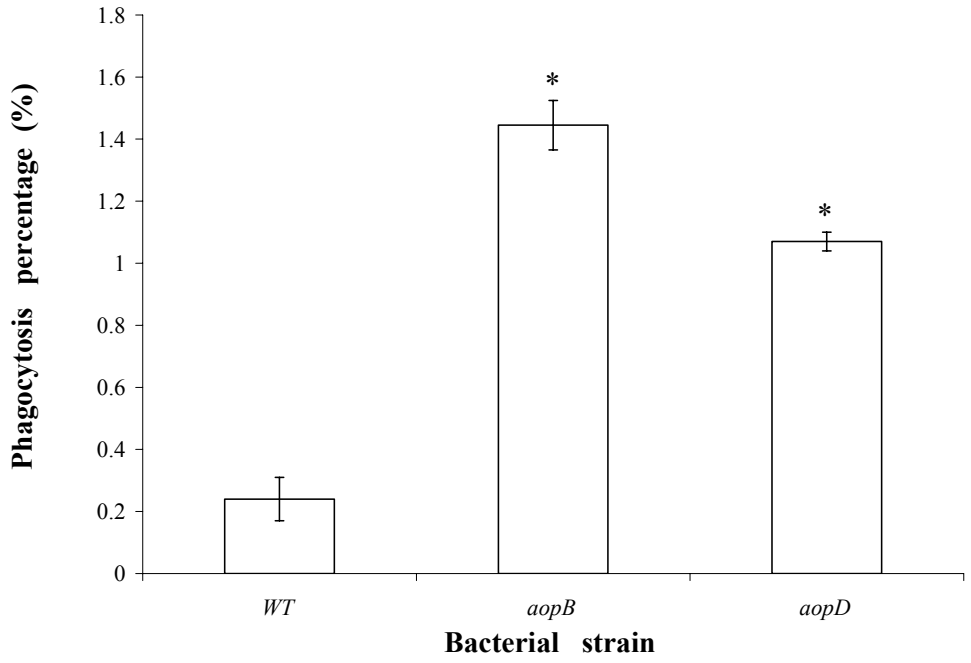


FIG. V.10. Phagocytosis assay. Phagocytosis percentage of gourami phagocytes is calculated after being infected with *A. hydrophila* wild type (AH-1) or mutants (*aopB* and *aopD*). Results are expressed as the representative mean ± SEM from duplicate wells in triplicate experiments. Data presented by bars with asterisks differ significantly from results obtained with *A. hydrophila* AH-1 ($P<0.05$). WT: wild type AH-1.

mediating antiphagocytosis and cytotoxicity. The natures of these effectors produced by AH-1 are being investigated. These results will help in a better understanding of the roles played by the TTSS in *A. hydrophila* pathogenesis.

V.4 Conclusion

This is the first report on sequencing and characterization of a TTSS gene cluster in *A. hydrophila*. Most ORFs of this gene cluster showed high homology to TTSS proteins of other pathogens such as *A. salmonicida*, *P. aeruginosa*, and *Yersinia* species, indicating that they may evolve from a common ancestor. The detection of *ascV* by PCR and sequencing analysis in 33 *A. hydrophila* strains revealed that the TTSS may be present in all the strains we examined, irrespective of their pathogenic or non-pathogenic nature. Ongoing work is being conducted to see whether the functional TTSS is limited to diseased but not environmental isolates of *A. hydrophila*. Insertional inactivation of *aopB* or *aopD* delayed cytotoxic effect in EPC cells and increased uptake by phagocytes significantly. LD_{50} assay of mutants also showed about 1 log increase in lethal dose compared to the wild type. The above mentioned biological activities clearly demonstrate that the TTSS is functional in *A. hydrophila* AH-1. Complementation of *aopB* and *aopD* mutants and creation of mutants in other TTSS genes will help in understanding their roles in cytotoxicity, antiphagocytosis and virulence in fish. The identification of a TTSS in *A. hydrophila* is an important discovery in unlocking the pathogenesis of this bacterium. This will allow us to understand the intimate host-bacteria interactions in order to evolve suitable strategies to overcome diseases caused by *A. hydrophila*.

Chapter VI

Characterization of type III secreted proteins of *Aeromonas hydrophila* AH-1

The results from this chapter are included in the following manuscript:

Yu, H. B., and K.Y. Leung. Characterization of type III secreted proteins of *Aeromonas hydrophila* AH-1. (In preparation)

Abstract

Many Gram-negative bacteria utilize TTSSs to deliver effector proteins (anti-host factors) into eukaryotic cytosols to subvert the host signaling pathway. The TTSS is required for the virulence of *A. hydrophila* AH-1. Inactivation of two negative regulators (AopN and ExsD) allowed us to identify several secreted proteins including structural proteins (AopB, AopD and AcrV), and novel effector proteins, AopE and AopH. All these proteins were further shown to be secreted via a type III secretion pathway. Transfection of AopE or AopH alone into HeLa cells induced cell rounding. The N-terminus of AopE was sufficient for its activity but the function of the C-terminus of AopE remains to be elucidated. Further investigation is required to dissect the functional domains of AopE and AopH. Future work, such as infection of HeLa cells and blue gourami fish with *ΔaopE*, *ΔaopH* and *ΔaopEΔaopH* mutants, will help in revealing the roles these effector proteins play in *A. hydrophila* pathogenesis.

VI.1 Introduction

In the previous chapter, we have shown that a TTSS is required for the virulence of *A. hydrophila* AH-1. Many Gram-negative bacteria were able to utilize the TTSS to deliver anti-host factors into eukaryotic cytosol, interfering with the host signaling pathway (Cornelis and Gijsegem, 2000). These anti-host factors are referred to as effector proteins which are genetically diversified (Cornelis and Gijsegem, 2000).

The effector proteins secreted by TTSSs can be identified by different approaches. Hauser *et al.* (1998) identified the type III secreted effector ExoU by screening mutants defective in cytotoxicity to epithelial cells from a transposon mutant library of *P. aeruginosa* strain. Braun *et al.* (2002) reported a type III secreted effector AexT by screening a phage gene library of *A. salmonicida* with a probe derived from ExoS of *P. aeruginosa*. With the advent of genomes of many bacteria, a genome-wide screen of TTSS effectors is applicable. Guttman *et al.* (2002) identified 13 effectors in *Pseudomonas syringae* based on their secretion and translocation ability. More recently, Panina *et al.* (2005) reported a genome-wide screen of a *Bordetella* type III secretion effector (BteA) and putative effectors in other species based on the common characteristics of class I chaperones and their frequent colocalization with TTSS effectors.

In this study, without the genome sequence of *A. hydrophila* being available yet, we used an alternative strategy to identify potential type III secreted proteins of *A. hydrophila* AH-1. Since the genetic organization of the TTSS of *A. hydrophila* shares great similarity to those of *Yersinia* spp. and *P. aeruginosa* (Chapter V), we hypothesized that the functional similarities may be present among the TTSSs of these bacteria. It has been reported that insertional inactivation of the *yopN* gene resulted in a derepressed transcription of *yop*

genes in *Yersinia* spp. (Forsberg *et al.*, 1991). In *P. aeruginosa*, a *ΔexsD* mutant is competent for type III secretion and translocation of the ExoU cytotoxin to eukaryotic host cells, irrespective of the presence of calcium (McCaw *et al.*, 2002). In *A. hydrophila* AH-1, AopN and AscZ/ExsD are homologous to YopN and ExsD, respectively. Hence, we sought to identify potential type III secreted proteins of *A. hydrophila* AH-1 by comparing the ECP profiles of *ΔaopN*, *ΔexsD* and the wild type. We also described the initial characterization of two effector proteins and discussed their potential roles in *A. hydrophila* pathogenesis.

VI.2 Materials and methods

VI.2.1 Bacterial strains

The bacterial strains and plasmids used in this study are listed in Table VI.1.

VI.2.2 Primers used in this study

Primers used for the construction of deletion mutants and degenerate PCR are listed in Table VI.2.

VI.2.3 Prediction of coiled-coil domains

The coiled-coil domains were predicted using the COILS program (http://www.ch.embnet.org/software/COILS_form.html) (Lupas *et al*., 1991; Pallen *et al*., 1997). The MTIDK scoring matrix was used, and analyses were performed by running scans with a weighting option.

VI.2.3 Protein preparation

A. hydrophila strains were inoculated into 5 ml TSB in a 15 ml falcon tube and grown at 25°C for 18 h, shaking at 200 rpm. The bacteria were then sub-cultured into 13 ml TSB (with or without 5 mM $CaCl_2$) at 1:50 dilution and grown for 6 h. The supernatants of all

Table VI.1. Bacterial strains and plasmids used in this study

Strain	Genotype and/or relevant property	Source
A. hydrophila strains		
ΔaopE	deletion of *aopE* from AH-1S	This study
ΔaopH	deletion of *aopH* from AH-1S	This study
ΔaopNΔascN	deletion of *ascN* from *ΔaopN*	This study
Plasmids		
pEGFP-N1	GFP protein fusion vector, Km^r	Clontech
pEGFP-AopE	Full-length AopE tagged with Flag fused to pEGFP-N1	This study
pEGFP-AopE-N	1-231 aa of AopE tagged with Flag fused to pEGFP-N1	This study
pEGFP-AopE-C	232-513 aa of AopE tagged with Flag fused to pEGFP-N1	This study
pEGFP-AopE-R145K	Point mutation of AopE (R145K) tagged with Flag fused to pEGFP-N1	This study
pEGFP-AopH	Full-length AopH tagged with Flag fused to pEGFP-N1	This study

Table VI.2. Primers used in this study

	Primers used for construction of deletion mutants	
Gene	Primer pairs	Primer sequence (5' to 3')
aopE	DC-AopE-F	GCAGCTCTAGAGCATCCTCTACAACAAGGACAAGCA
	DC-AopE-R	GGAGCTCTAGAGCTTCACCGGTTATGACACCATCCATTA
	Inv-AopE-L	GCCAGTATGTGTTTGAATCTGCATGGTGTT
	Inv-AopE-R	GAGTAATTGGCTCTCTACAAGGAAACTGCATGCAA
aopH	DC-AopH-F	GAAGCTCTAGAGCCACTACCACAAGATGGTGCAA
	DC-AopH-R	GGAGCTCTAGAGCGACCTGTTCGAGCAGTTTAAACAT
	Inv-AopH-L	GTGGATCTGCATTGAACCTCCGAGCGAGTGTA
	Inv-AopH-R	GAGAACAAGGGTCGCTTCAACGTCAGCAACAA
ascN†	DC-opsc-F	GAAGCTCTAGAGCTGTTGCTGTCGCTTAGCTGTA
	DC-opsc-R	GAAGCTCTAGAGCTGGATGACAGGCTGTTGTGATCCA
	Inv-opsc-L	CAGTGGCTCAAACAAGGCACCCATGAA
	Inv-opsc-R	GCCTGCAATGTGGTCGAAGGAAAGATTCAT
	Primers used for pEGFP-N1 derived constructs	
Constructs		
pEGFP-AopE	AopE-F	GCAGAGAAGCTTACCATGCAGATTCAAACACATACT
	AopE-Rflag	GATTAGGATCCTTCTTGTCGTCATCGTCTTTGTAGTCCTCGAGATAACCGGCCTCGA
pEGFP-AopE-N	AopE-F	GCAGAGAAGCTTACCATGCAGATTCAAACACATACT
	AopE-NR	GATTAGGATCCTTCTTGTCGTCATCGTCTTTGTAGTCCGGTTCGCCGGCGACCT
pEGFP-AopE-C	AopE-CF	GAAGAGAAGCTTACCATGCTGCCGGCCACCACCAATGCCGAGGTTGT
	AopE-Rflag	GATTAGGATCCTTCTTGTCGTCATCGTCTTTGTAGTCCTCGAGATAACCGGCCTCGA
pEGFP-AopE-R145K	AopE-F	GCAGAGAAGCTTACCATGCAGATTCAAACACATACT
	R145K-L	TCAGTGAGGTTGCCAGCGATTTCAGGGC
	R145K-R	GCCCTGAAATCGCTGGCAACCTCACTGA
	AopE-Rflag	GATTAGGATCCTTCTTGTCGTCATCGTCTTTGTAGTCCTCGAGATAACCGGCCTCGA
pEGFP-AopH	AopH-F	GAAGAAAGCTTACCATGCAGATCCACGCTCTACAATCACT
	AopH-Rflag	GCTGAGGATCCTTCTTGTCGTCATCGTCTTTGTAGTCTCCCAGATAGTTGTTGCTGACGTT
	Degenerate primers for fishing out *aopE* and *aopH*	
Band 1	B1-deg	CAGGCCGTCGCTCANCACAGTGATGCC
Band 2	B2-deg	GTNAAYCARCARATHAAYCA

† This construct is for deletion of a*scN* in *ΔaopN* background.

the strains were isolated by TCA–acetone precipitation (Shimizu *et al.*, 2002), which can be referred to in Chapter II.5.1.

VI.2.4 Edman N-terminal sequencing

For Edman N-terminal sequencing, proteins were primarily blotted onto polyvinylidene difluoride (PVDF) membrane. The membrane was stained with 0.1% coomassie in 50% (w/v) methanol for 15 to 30 min, followed by destaining with 50% methanol (v/v) until the bands could be observed. Protein spots were excised from the PVDF membranes and loaded onto the amino acid sequencing cartridge. The N-terminal sequencing was performed with a Procise model 494 pulsed-liquid-phase protein sequencer (Applied Biosystems).

VI.2.5 Immunofluorescence microscopy

HeLa cells were seeded on coverslips overnight (1×10^5 cells per ml) in a 24-well tissue culture plate. The HeLa cells were transfected at 70% confluency with 0.8 µg of plasmid. After 24 h of transfection, the cells were washed twice with 1x PBS and fixed for 30 min in 4% formaldehyde. For Flag antibody staining, the fixed cells were permeabilized with 0.1% Triton X-100 for 5 min and washed twice with 1x PBS. 200 µl of primary antibody (anti-Flag, Sigma, clone no. M2) was added to each well at a 1:200 dilution and incubate for 2 h at 37 °C. After washing twice in 1x PBS, 200 µl of anti-mouse IgG R-phycoerythrin (R-PE) (Invitrogen) were added to each well at 1:1000 dilution and the cells were incubated for 45 min at 37°C. The nuclei were stained with Hoechst (Invitrogen) for 10 min and washed twice with 1x PBS. The coverslips were picked up with the help of a needle and forceps. Excess liquid was removed by touching the sides and bottom of the coverslips with clean tissue paper. 10 µl of mounting solution (Sigma) was pipetted to a

glass slide and the coverslips were placed onto the slide with the cells facing down to the mounting solution. The excess liquid was dried with tissue paper by gentle blotting. The coverslips were sealed with nail polish and air dried in a chemical hood for 10 min. The slides were observed on a fluorescence microscope (Olympus, BX60) with 4', 6'-Diamidino-2-phenylindole (DAPI), fluorescein isothiocyanate (FITC) and rhodamine filter sets.

VI.2.6 Nucleotide accession numbers

The DNA sequences of *A. hydrophila* AH-1 TTSS gene cluster, *aopE* and *aopH*, were deposited in GenBank under accession numbers AY394563, # and #, respectively.

VI.3 Results and discussion

VI.3.1 A complete sequence of TTSS

In Chapter V, the sequence of a partial TTSS gene cluster was reported. Further sequencing of the cosmid pLA2917-TTSS (provided by Dr Tomas JM) led to the identification of a complete TTSS in *A. hydrophila* AH-1 (Table V.3). This TTSS cluster is about 26 kb in length and its organization is similar to the TTSS cluster of *A. hydrophila* strains SSU and AH-3 (Fig. VI.1) (Vilches *et al.*, 2004; Sha *et al.*, 2005). The ORFs of TTSS in *A. hydrophila* AH-1 can be classified into regulators, chaperones, secreted proteins and others (secretion apparatus proteins and unknown proteins), based on the characteristics of their homologs in *P. aeruginosa* and/or *Yersinia* spp. (Fig. VI.1). The average G+C content of this cluster is 56.4% which is similar to the average genome G+C content of *A. hydrophila* (57-63%), indicating that this TTSS cluster may have been acquired from species which have similar G+C contents or that it has already adapted to the host genome (Lawrence and Ochman, 1996).

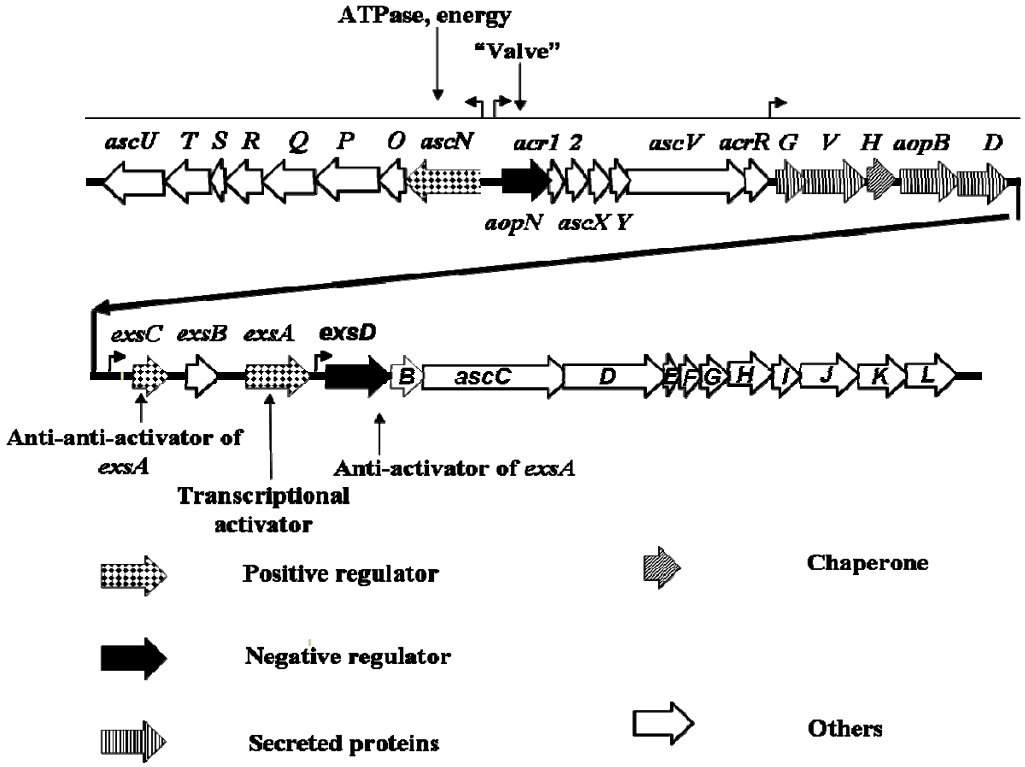


Fig. VI.1. The genetic organization of a complete TTSS gene cluster in *A. hydrophila* AH-1. The TTSS gene cluster consists of regulators, secreted proteins, chaperones and others (secretion apparatus proteins and unknown proteins).

VI.3.2 Identification of type III secreted proteins by MALDI-TOF/TOF and N-terminal sequencing

Both AopN and ExsD function as "negative regulators" of the type III secretion pathway (Chapter IV). When cultured in DMEM, the ECP profiles of *ΔaopN* and *ΔexsD* mutants differed from that of the wild type, and several potential secreted proteins were identified (Chapter IV). To identify other potential type III secreted proteins, *ΔaopN* and *ΔexsD* mutants were cultured in TSB (with or without 5 mM $CaCl_2$) and their ECP profiles were examined.

As shown in Fig. VI.2A, bands 1 to 9 were present in the ECP profiles of *ΔaopN* and/or *ΔexsD* mutants but absent from that of the wild type. Such differences between the supernatants of *ΔaopN*/*ΔexsD* mutants and the wild type became more apparent when cultured in the presence of calcium (Fig. VI.2B). It is possible that high concentrations of calcium are able to down-regulate the secretion or expression of non-type III secreted proteins, but not the type III secretion-dependent proteins in *ΔaopN* and *ΔexsD* mutants. Bands 1 to 9 were subjected to MALDI-TOF/TOF MS, and AopB, AopD, AcrV were identified (Table VI.3). The presence of AopB, AopD and AcrV in the supernatant is not surprising since their homologs (PopB/YopB, PopD/YopD and PcrV/LcrV of *P. aeruginosa*/*Yersinia* spp.) are also secreted into the supernatants (Yahr *et al.*, 1997; Hueck, 1998). Bands 1, 2 and 3 did not match any proteins in the NCBI database and were transferred to the PVDF membrane for N-terminal sequencing. N-terminal sequences of bands 1/3 and 2 showed high similarity to AexT of *A. salmonicida* and YscM1 of *Yersinia* spp., respectively (Table VI.4). We speculate that band 1 has undergone degradation under an *in vitro* condition, resulting in the appearance of band 3. The degenerate primers for

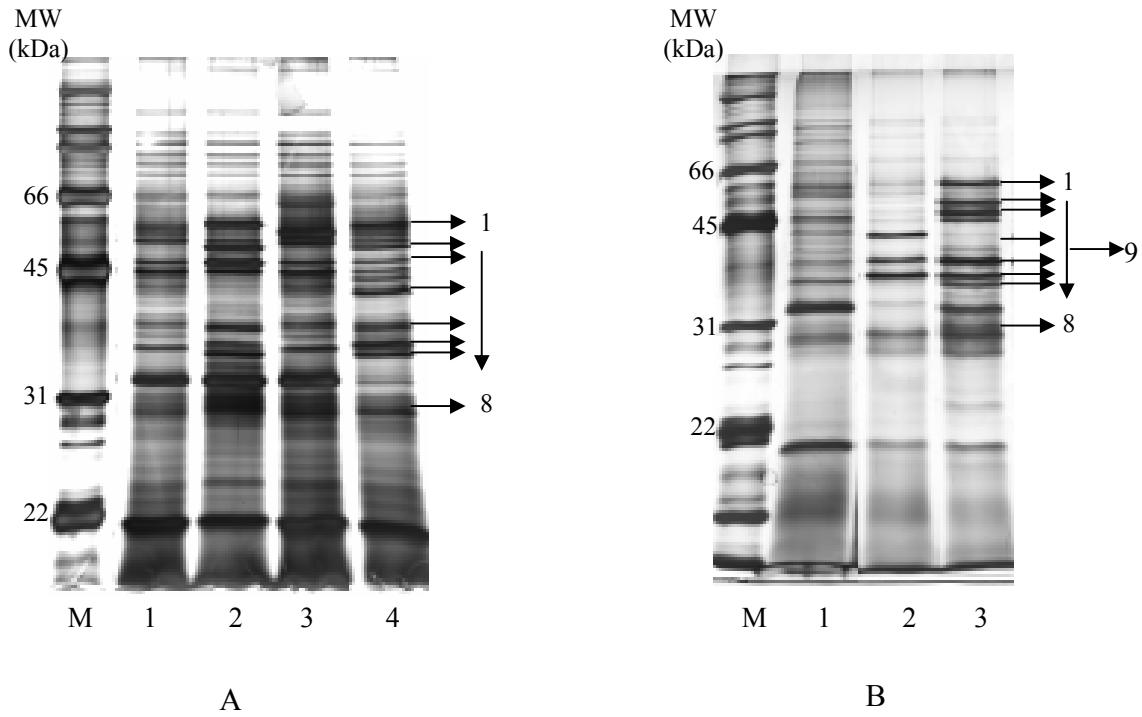


Fig. VI.2. Identification of type III secreted proteins by MALDI-TOF/TOF and N-terminal sequencing. (A) Bacterial strains grown in TSB without adding calcium: M, marker; lanes 1, 2, 3 and 4 are ECPs of AH-1S, *ΔaopN*, *ΔaopNΔascN* and *ΔexsD* mutants, respectively. (B) Bacterial strains were grown in TSB with 5 mM calcium: M, marker; lanes 1, 2 and 3 are ECPs of AH-1S, *ΔexsD* and *ΔaopN* mutants, respectively. Protein bands are labeled 1 to 8 from top to bottom at the right side of the gels.

Table VI.3. Identification of type III secreted proteins of *A. hydrophila* AH-1 by MALDI-TOF/TOF MS and N-terminal sequencing

	Methods used for protein identification					
	MALDI-TOF/TOF MS					N-terminal sequencing
Band no.	Protein identity	Sequence coverage (%)	Score	E-value	Calculated MW (kDa)	
1	AopE					√
2	AopH					√
3	AopE					√
4	AcrV	63	1050	2.5e- 099	41.2	
5	AopB	42	401	2e-034	36.4	
6	AopD	65	489	3.2e-043	32.3	
7	Unknown	-	-	-	-	
8	AopD	27	77	1.4	32.3	
9	AopH	39	375	1e-035	51.3	

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Table VI.4. Identification of secreted proteins by N-terminal sequencing

Band no.	N-terminal sequence	Homologs in other bacteria	Accession no.
1	MQIQTHTGGLQAVAQHSDAA	AexT of *A. salmonicida*	CAE17664
2	MQIHALQSLVNQQINQLGH	YscM1 of *Y. enterocolitica*	AAK69245
		YscM of *Y. pestis* CO92	CAB54939
3	VALKEWIGNLLGARPAAPVRSAPP	AexT of *A. salmonicida*	CAE17664

Degenerate primers are designed based on the underlined sequences.

AexT-like and YscM-like proteins were then designed and several rounds of genome walking led to the identification of two putative effector genes (Fig. VI.3, Table VI.4). AexT-like protein and YscM-like protein were renamed AopE (Aeromonas outer protein E) and AopH (Aeromonas outer protein H), respectively. Band 9 was later identified as AopH by searching against a protein database of *A. hydrophila* AH-1 ORFs and it could represent a degraded form of AopH. We are currently trying to identify the unknown protein (band 7).

VI.3.3 Sequence analysis of *aopE* and *aopH* regions

Potential promoter regions upstream of *aopE* and *aopH* were revealed (Fig. VI.3). The promoter region upstream of *aopE* contains -10 (TAGACT) and -35 (GCGATA) consensus sequences as well as a potential ExsA consensus element (TGCAAAAA) which is bound by the transcriptional activator ExsA in *P. aeruginosa* (Hovey and Frank, 1995). Similarly, -10 (TAGAAT), -35 (TAGTGA) and a potential ExsA consensus element (TACAAATT) were also located upstream of *aopH*. Our results suggest that ExsA (a homolog of *P. aeruginosa* ExsA) in *A. hydrophila* may also control the transcription of *aopE* and *aopH* (Table V.3). In addition, both AopE and AopH contain coiled-coil domains which are a major characteristic of secreted proteins (Pallen *et al.*, 1997) (Fig. VI.4).

AopE is a 513 aa protein which is 38 aa longer than its closest homolog, AexT (Table VI.5). The N-terminus (1-240 aa) of AopE shared high similarity to those of AexT (70.2%) of *A. salmonicida*, ExoS (59.3%) and ExoT (63.4%) of *P. aeruginosa* (Table VI.5, Fig. VI.5). It also shared 36.5% similarity to YopE of *Yersinia* spp. (Table VI.5, Fig. VI.5). Although AexT (241-475 aa), ExoS (240-453 aa) and ExoT (241-457 aa) shared 60.4%

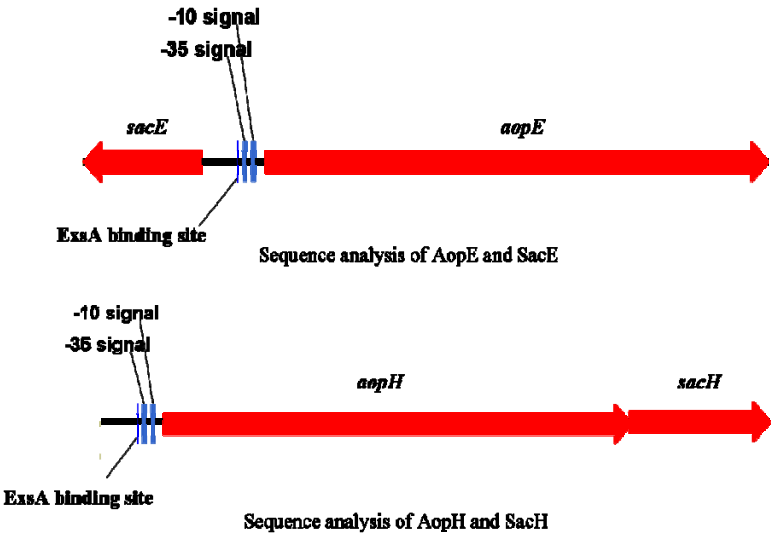

Fig. VI.3. The genetic organization of *aopE* and *aopH* regions. The arrows indicate the direction of transcription of each gene. The name of each gene is shown above the red arrows.

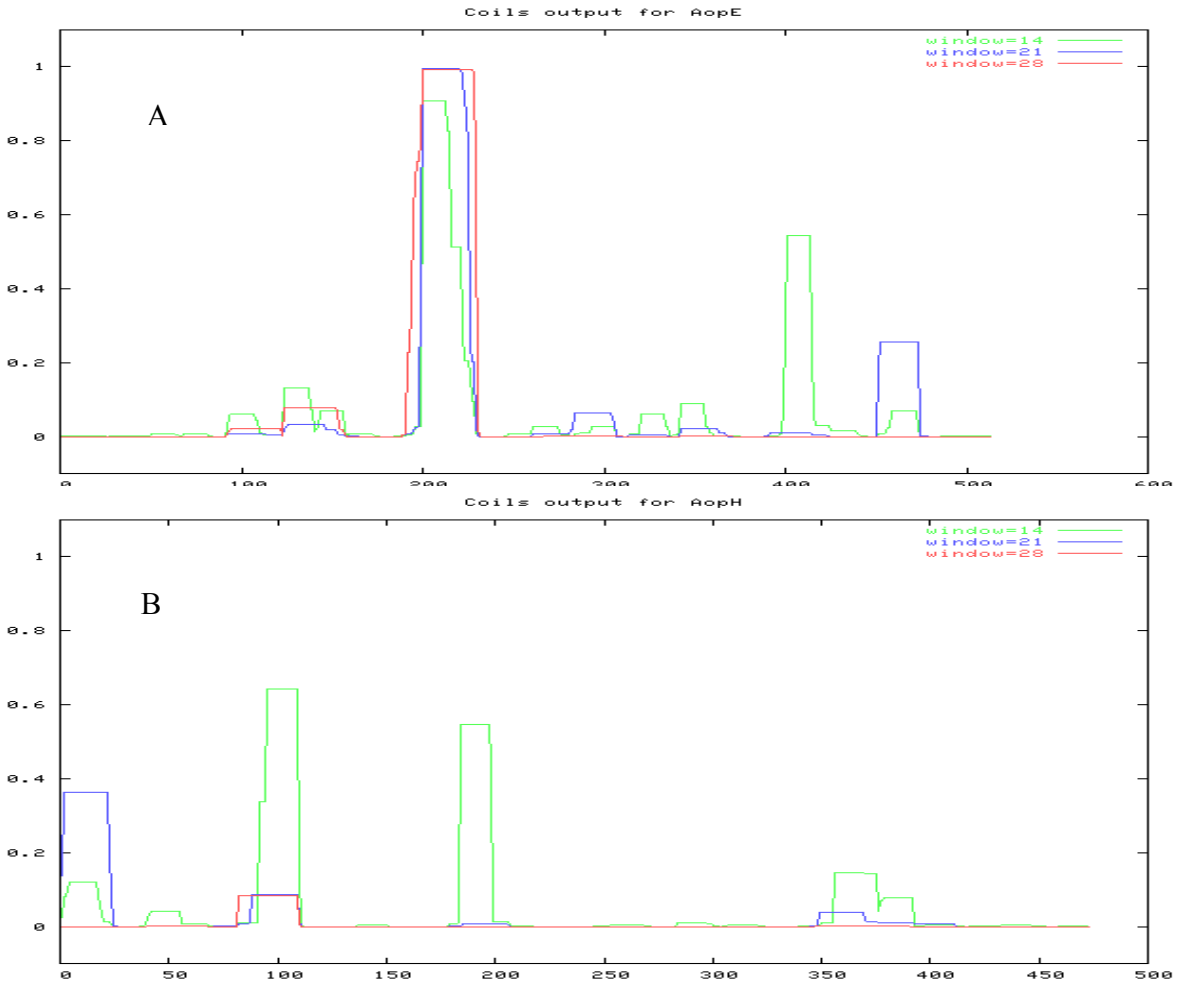

Fig. VI.4. The prediction of coiled-coil domains in AopE (A) and AopH (B) of *A. hydrophila* AH-1. Proteins are predicted by the COILS software and the prediction outputs are presented graphically. Predictions were made using three scanning windows, 14 (green), 21 (blue) and 28 (red) residue windows. Scan ouputs shown were performed using a weighting option. For each protein, at least one coiled-coil regions was predicted with >50% probability using at least one scanning window.

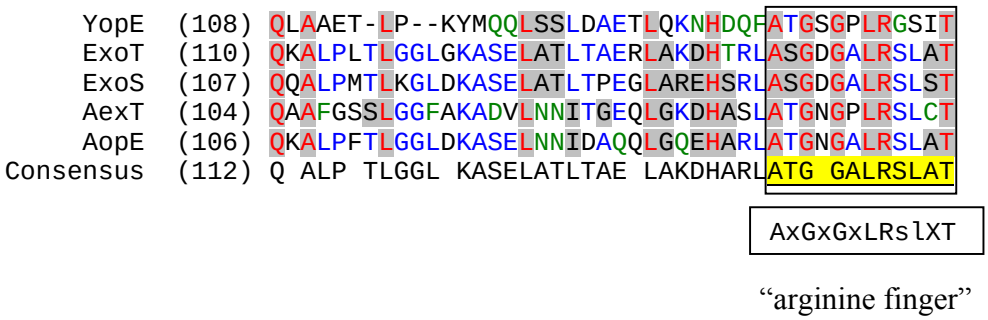

Fig. VI.5. Alignment of AopE with its homologs among *A. salmonicida* (AexT), *Yersinia* spp. (YopE) and *P. aeruginosa* (ExoS/ExoT) at the amino acid level. Boxed region indicates the arginine finger motif. "x" represents any amino acid and lower case symbols denote conserved but not invariant residues.

Table VI.5. Sequence analysis of *aopE* and *aopH* operons

A. hydrophila	*Yersinia* spp (I/S[a])	*P. aeruginosa* (I/S)	*A. salmonicida* (I/S)	Putative characteristics
AopE	YopE (25/45)	ExoT (52/70) ExoS (50/69)	AexT (63/73)	Secreted protein
SacE	SycE (40/55)	ORF1 (66/74) (AAA66490)	Unknown (86/89) (AAK83051)	Chaperone
AopH	YopH (43/59)	-	-	Secreted protein
SacH	SycH (45/63)	-	-	Chaperone

[a]I: % of identity at amino acid level; S: % of similarity at amino acid level; -: no homologs.

identity at their C-termini, the C-terminus of AopE did not show good homology with any proteins in the NCBI database and only shared 7.9% identity with those of AexT, ExoS and ExoT. These data suggest that the N-termini of AopE, AexT, ExoS/ExoT and YopE function in a similar manner but the C-terminus of AopE exhibits quite a different function from those of AexT, ExoS and ExoT.

AopH encodes a 473 aa protein which is similar to the length of its homolog, YopH (468 aa) (Table VI.5). The N-terminus (1-150 aa) of AopH exhibited 70%, 61% and 59% similarity to those of YscM1 (accession no. AAK69245), YscM2 (accession no. AAD16867) and YopH (accession no. AAK69246) of *Y. enterocolitica*, respectively. However, AopH and YopH shared little similarity at their C-termini.

The efficient secretion of YopE and YopH requires chaperones *sycE*/*yerA* and *sycH*, respectively (Forsberg and Wolf-Watz, 1990; Wattiau and Cornelis, 1993; Wattiau *et al.*, 1994). In this study, two chaperone-like proteins, SacE and SacH, were also identified (Fig. VI.3, Table VI.5). In *Yersinia* spp., *sycE*/*yerA* is upstream of *yopE* and transcribed in the opposite orientation to *yopE* (Forsberg and Wolf-Watz, 1990). SacE (specific AopE chaperone), showing 55% similarity to SycE, is located upstream of *aopE* (a *yopE* homolog) and transcribed in the opposite orientation to *aopE* (Fig. VI.3, Table VI.5). SacH (specific AopH chaperone) showed 63% similarity to SycH of *Y. enterocolitica*. SacH is immediately downstream from *aopH* and transcribed in the same direction as *aopH*. This is different from *Y. enterocolitica* where *sycH* is located downstream from *yopH* but transcribed in the opposite direction to *yopH* (Wattiau *et al.*, 1994). In addition, both SacE and SacH possess the characteristics of a TTSS-specific chaperone, including a low molecular mass (13.2 kDa for SacE and 15.3 kDa for SacH) and an acidic isoelectric

point (5.27 for SacE and 5.24 for SacH) (Page and Parsot, 2002; Feldman and Cornelis, 2003). Therefore, we speculate that SacE and SacH function as chaperones for AopE and AopH, respectively.

VI.3.4 AopE and AopH are secreted via the TTSS

To demonstrate that AopE and AopH are secreted via a TTSS in *A. hydrophila* AH-1, a *ΔaopNΔascN* mutant was created by deletion of *ascN* in *ΔaopN* background, since disruption of *ascN* (a putative energizer for a TTSS) can render the whole TTSS non-functional (Chapter III). As expected, bands 1 to 2 and 4 to 8 were present in the supernatant of the *ΔaopN* mutant but absent in that of the *ΔaopNΔascN* mutant (Fig. VI.2A). The intensity of band 3 was also significantly reduced, but not missing in the supernatant of the *ΔaopNΔascN* mutant when compared to that of the *ΔaopN* mutant. The presence of other ECPs may account for this result. Hence, we conclude that AopE and AopH are truly secreted proteins via the type III secretion pathway.

In addition, a comparison of the ECP profiles of *ΔaopN*, *ΔaopNΔaopE* and *ΔaopNΔaopH* mutants revealed that the deletion of either *aopE* or *aopH* did not affect the secretion of other proteins but only led to the loss of AopE and AopH, respectively (Fig. VI.6). This suggests that AopE and AopH are not parts of the type III secretion machinery. Our preliminary data have also shown that AopE and AopH can be translocated into HeLa cells by fusing the N-termini of AopE and AopH to a reporter gene (β-lactamase) (Charpentier and Oswald, 2004) (data not shown).

VI.3.5 Full-length or N-terminus of AopE elicits cell rounding in HeLa cells

Homologs of AopE, namely, ExoS, ExoT and YopE, have all been shown to induce a rounded cell morphology involving disruption of actin microfilaments (Rosqvist *et al.*,

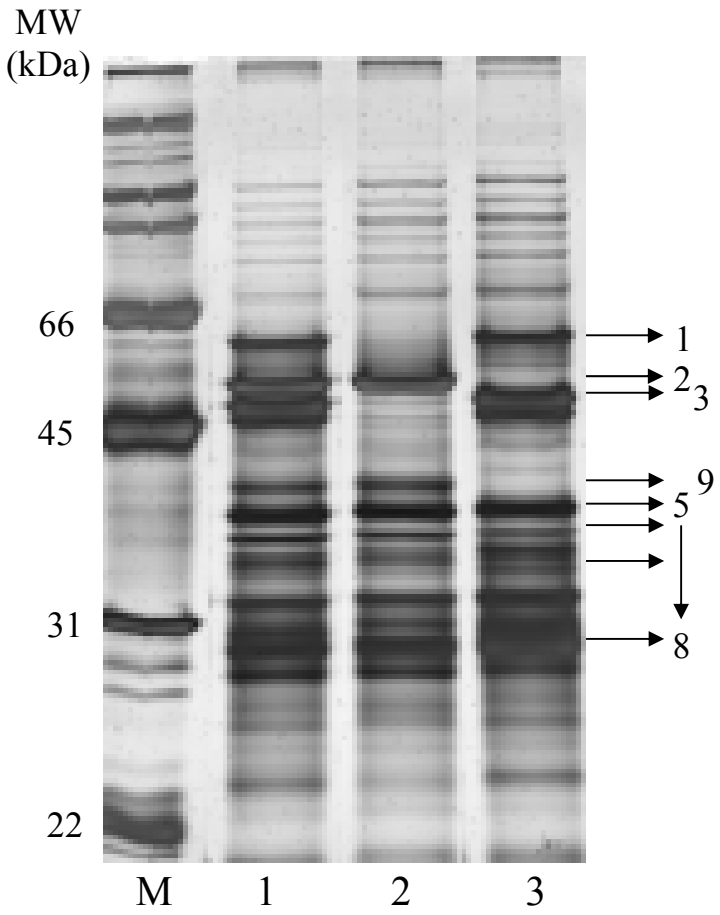

Fig. VI.6. ECP profiles of *ΔaopNΔaopE* and *ΔaopNΔaopH* mutants. Bacterial strains were grown in TSB with 5 mM calcium: M, marker; lanes 1, 2 and 3 are extracellular proteins of *ΔaopN*, *ΔaopNΔaopE* and *ΔaopNΔaopH* mutants, respectively. Protein bands are labeled at the right side of the gels.

1991; Frithz-Lindsten *et al.*, 1997; Goehring *et al.*, 1999; Von Pawel-Rammingen *et al.*, 2000; Sundin *et al.*, 2001). To elucidate the function of AopE inside host cells, full-length AopE tagged with Flag was fused to a mammalian expression vector pEGFP-N1 (the construct was termed pEGFP-AopE), allowing the simultaneous expressions of AopE-Flag and green fluorescent protein (GFP). Plasmids pEGFP-AopE and pEGFP-N1 (empty vector expressing GFP as a negative control) were transfected into HeLa cells. After 24 h of transfection, the HeLa cells were fixed and subjected to immunofluorescence microscopy analysis. The transfected cells exhibited green color by GFP signal and the expression of AopE-Flag was detected with anti-Flag antibody (Fig. VI.7). The cells transfected with pEGFP-N1 (empty vector) remained flat and the GFP signal was present throughout the HeLa cells including the nuclei (Fig. VI.7E). In sharp contrast, the cells transfected with pEGFP-AopE exhibited a rounded morphology and the GFP/RFP signals were only present in membrane and/or cytosol (Fig. VI.7A). These results indicate that AopE is cytotoxic to HeLa cells and it may specifically interact with membrane and/or cytosol components. Sub-fractionation of HeLa cells transfected with pEGFP-AopE will allow us to localize AopE accurately. AopE may exhibit similar characteristics as compared to ExoS, since the full-length ExoS elicits cell rounding and localizes to membranes and is processed to a soluble form in CHO cells (Pederson *et al.*, 2000). Currently, we are staining the actin with phalloidin-rhodamine to investigate whether AopE affects the cytoskeleton structure of HeLa cells as ExoS (Pederson *et al.*, 1999).

In addition, the N-terminus (1-231 aa) and C-terminus (232-513 aa) of AopE tagged with Flag were fused to pEGFP-N1 to create constructs pEGFP-AopE-N and pEGFP-AopE-C, respectively. HeLa cells were transfected with these two plasmids individually and

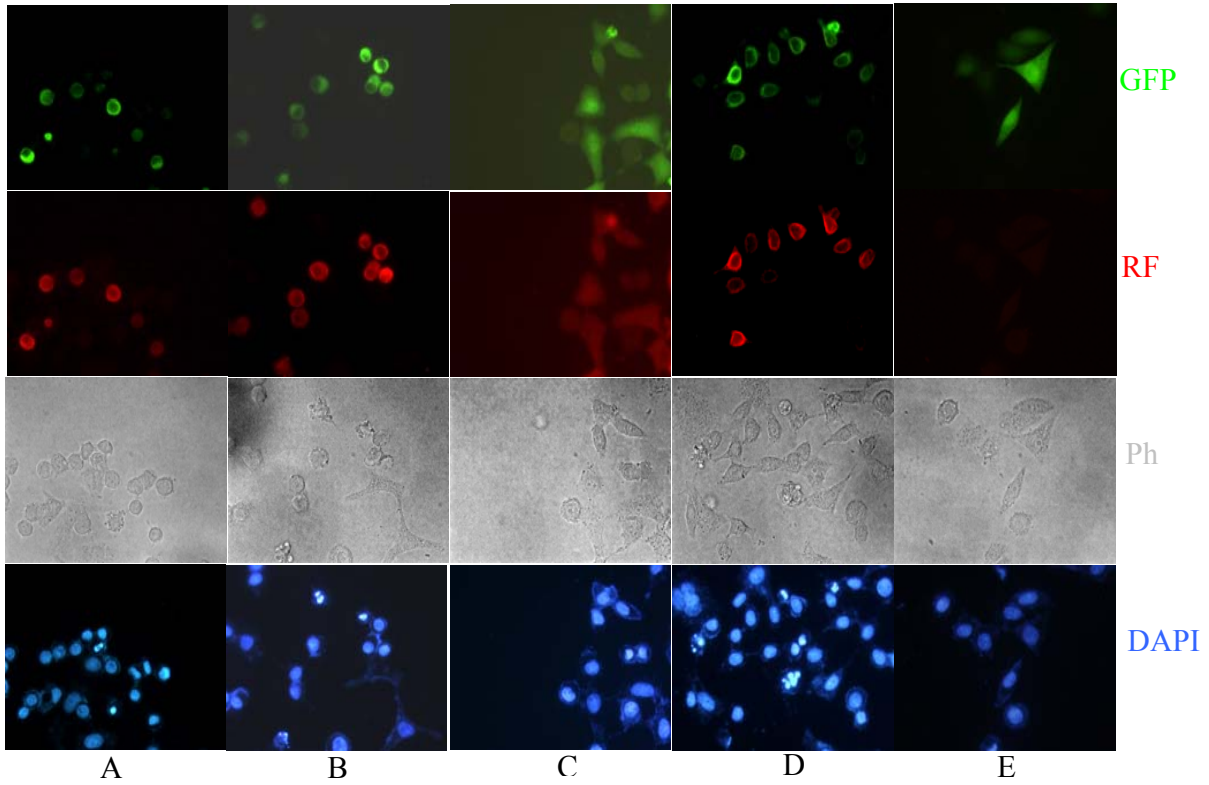

Fig. VI.7. HeLa cells were transfected with pEGFP-AopE (A), pEGFP-AopE-N (B), pEGFP-AopE-C (C), pEGFP-AopE-R145K (D), or pEGFP-N1 (E) for 24 h. All the samples were imaged directly on a fluorescence microscope (Olympus, BX60) with FITC set (green) or Phase-contrast microscopy (grey). The samples were also stained with anti-Flag and anti-mouse IgG R-phycoerythrin (red) to identify cells expressing AopE, and DAPI (blue) to visualize nuclei. In columns A and B, cells expressing AopE became rounded; cells transfected with the C-terminus of AopE (C), point mutation (R145K) of AopE (D), or empty vector (E) remained flat and normal.

examined by the immunofluorescence microscope. The cells transfected with pEGFP-AopE-N showed the same phenotype as cells transfected with pEGFP-AopE (Figs. VI.7A and B), indicating that the N-terminus of AopE is sufficient for the cytotoxicity of AopE *in vitro*. However, HeLa cells transfected with pEGFP-AopE-C remained flat and the GFP/RFP signals were distributed evenly throughout the cells (Fig. VI.7C). These results suggest that the C-terminus of AopE is not required for cell rounding and the membrane and/or cytosol localization signal of AopE is present in the N-terminus of AopE. Future work will be carried out to decipher the function of the C-terminus of AopE.

A comparison of the amino acid sequences of AopE, YopE, ExoS, ExoT and AexT revealed a conserved region, AxGxGxLRslXT (Fig. VI.5). This region includes an invariant arginine residue and is referred to as an arginine finger domain showing homology with other GTPase-activating proteins (GAPs) (Scheffzek *et al.*, 1998). It has been reported that ExoS, ExoT and YopE function as GTPase-activating proteins (GAPs) for small GTPases, such as Rho, Rac and Cdc42 (Goehring *et al.*, 1999; Pederson *et al.*, 1999; Black and Bliska, 2000; Kazmierczak and Engel, 2002). The GAP activity toward small GTPases is required for these three effectors to induce cell rounding and disrupt actin microfilaments.

Mutation of the invariant arginine within the arginine finger motif eliminated the GAP activities of YopE, ExoS and ExoT (Black and Bliska, 2000; Pederson *et al.*, 2000; Kazmierczak and Engel, 2002). As a result, the point mutants of these effector proteins were unable to induce cell rounding or result in the loss of stress fibers. To determine whether the arginine finger motif is important for the cytotoxicity of AopE, the arginine 145 in AopE was changed to a lysine residue (pEGFP-AopE-R145K) and the effect of this

mutation (R145K) on the activity of AopE was examined. As shown in Fig. VI.7D, cells transfected with pEGFP-AopE-R145K did not stimulate cell rounding and remained flat as cells transfected with pEGFP-N1. In addition, AopE (R145K) localized to the perinuclear region of the HeLa cells (Fig. VI.7D). Pederson *et al.* (2000) also showed that a mutant form of ExoS [ExoS(1±234R146K)] lost the ability to cause cell rounding and localized to the perinuclear region with a punctate staining pattern in CHO cells. Such a great similarity between AopE (R145K) and ExoS (R146K) indicates that AopE may also function as GAPs and the invariant arginine residue is crucial for its activity. Future work will be carried out to investigate whether AopE exhibits GAP activity and to determine which GTPases are involved in this process.

VI.3.6 Full-length AopH elicits cell rounding in HeLa cells

As a homologue of AopH, YopH functions as a protein tyrosine phosphatase to disrupt focal adhesion complexes and actin filaments (Black and Bliska, 1997; Persson *et al.*, 1997; Hamid *et al.*, 1999). To investigate the function of AopH, a full-length AopH tagged with Flag was fused to a mammalian expression vector pEGFP-N1 (the construct was termed pEGFP-AopH) and the plasmid pEGFP-AopH was transfected into HeLa cells. As shown in Fig. VI.8B, the cells transfected with pEGFP-AopH became elliptical which is different from cells transfected with pEGFP-N1 or cells transfected with pEGFP-AopE (rounded cells) at 24 h post-transfection. This indicates that AopH is also cytotoxic to HeLa cells via a yet unknown mechanism. Of note, the GFP/RFP signals appeared to be mostly present in the membrane as shown in the merged picture in Fig. VI.8C. Confocal microscopy and sub-fractionation analysis will be further applied to define the localization of AopH inside HeLa cells.

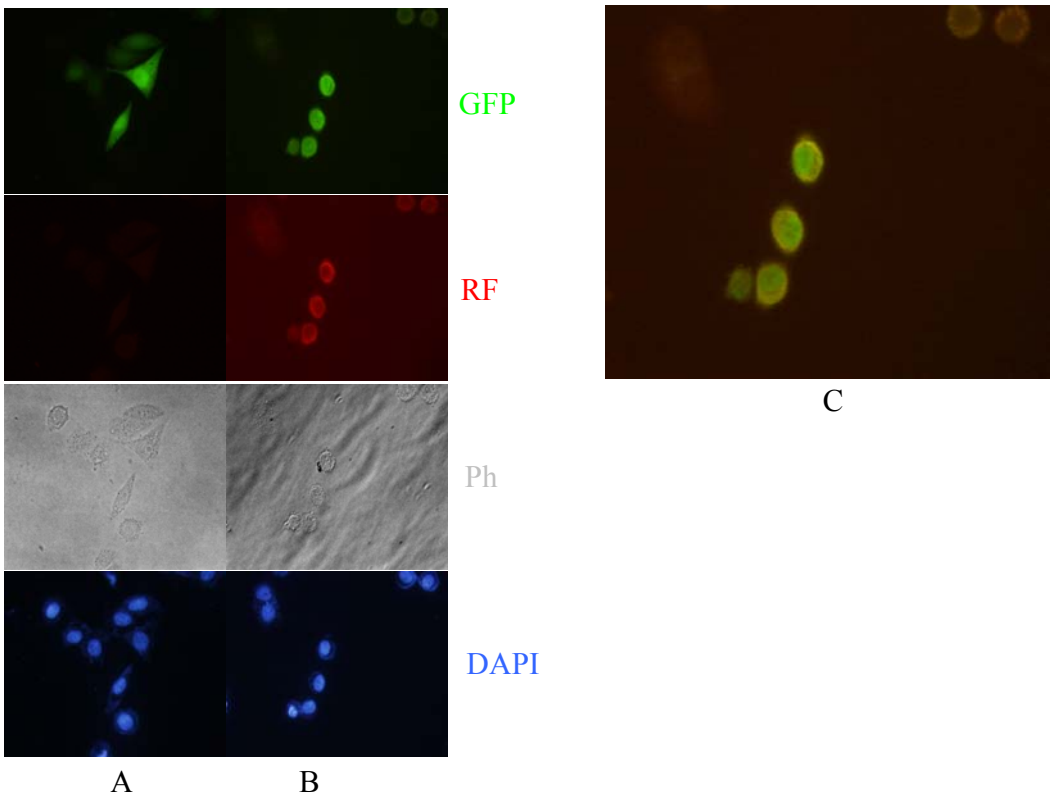

Fig. VI.8. HeLa cells were transfected with pEGFP-N1 (A) and pEGFP-AopH (B) for 24 h. The samples were imaged on a fluorescence microscope (Olympus, BX60) with FITC set (green) or Phase-contrast microscopy (grey). The samples were also stained with anti-Flag and anti-mouse IgG R-phycoerythrin (red) to identify cells expressing AopH, and DAPI (blue) to visualize nuclei. In column B, cells expressing AopH became elliptical. The merge between green and red fluorescence is shown in C.

In conclusion, we have demonstrated that AopB, AopD, AcrV and an unknown protein are secreted proteins via the TTSS of *A. hydrophila* AH-1. Two novel effector proteins, AopE and AopH, have also been identified. These two proteins can be secreted and translocated into host cells via the type III secretion pathway. Expression of AopE or AopH in HeLa cells can induce cell rounding. The N-terminus of AopE is required for the induction of cell rounding, but the function of the C-terminus of AopE remains to be elucidated. Further investigation into dissecting the functional domains of AopE and AopH is being carried out. Future work on infection of HeLa cells and blue gourami fish with *ΔaopE*, *ΔaopH* and *ΔaopEΔaopH* mutants will help in revealing the roles these effector proteins play in *A. hydrophila* pathogenesis.

Chapter VII. General conclusions and future directions

VII.1 General conclusions

In the present study, we initially identified a phage-associated genomic island and a TTSS gene cluster from *A. hydrophila* PPD134/91 by using a forward genetic approach. Concurrently, genome walking was carried out to obtain complete ORFs as well as upstream and downstream sequences of 22 unique DNA fragments (Zhang *et al.*, unpublished data) encoding 19 putative virulence factors and seven ORFs. These 22 DNA fragments are commonly present in the eight virulent *A. hydrophila* strains examined (Zhang *et al.*, unpublished data). Fourteen of these virulence-associated factors (hemolysin, aerolysin, *bvgA*, *bvgS*, *ompAI*, *flhA*, *f85*, *ascN*, *hup*, *opdA*, *ahsA*, *ahyR*, *serA* and *mepA*) were studied in a comparative manner. Mutants were constructed for these genes and tested for their virulence in a blue gourami fish model. Results showed that the TTSS may be one of the most important virulence factors in *A. hydrophila* pathogenesis. However, it is also possible that genes other than the TTSS gene cluster may be important in the pathogenicity of *A. hydrophila*, which needs to be studied further. Statistically significant attenuation was observed for a triple mutant (Δ*ahsA*Δ*serA*Δ*mepA*) but not for the two double mutants (Δ*ahsA*Δ*mepA* and Δ*ahsA*Δ*serA*), which further supports the notion that the pathogenicity of *A. hydrophila* is multifactorial.

Many virulence factors can be secreted extracellularly and these ECPs play a major role in the pathogenesis of bacteria. The major extracellular virulence factors were therefore characterized by both proteomic and transcriptional studies. In this study, an extracellular proteome map of *A. hydrophila* was established. Twelve spots in the reference map did not match any proteins in the NCBI database, suggesting that they may encode novel

proteins. The major ECPs identified in this proteome map included S-layer, hemolysin, GCAT, polar and lateral flagellins, serine protease and metalloprotease. The major extracellular virulence factors were subsequently characterized by comparing the extracellular proteomes of mutants and the wild type. Results suggest that a serine protease was involved in the processing of secreted enzymes such as hemolysin, GCAT and metalloprotease. We also demonstrated that the expression of polar and lateral flagellins was under the control of temperature and other flagellar regulatory proteins. Most interestingly, a cross-talk between a lateral flagellar secretion system and the TTSS was discovered. The TTSS appears to control the expression of lateral flagella via a TTSS central regulator (ExsA) as a deletion of *exsA* can abolish the regulatory effect of TTSS on the lateral flagellar secretion system.

In this study, the sequence of a complete TTSS gene cluster in *A. hydrophila* AH-1 was also obtained by a series of genome walking and cosmid sequencing. Most ORFs of this gene cluster showed high homology to TTSS proteins of other pathogens such as *A. salmonicida*, *P. aeruginosa*, and *Yersinia* species. The detection of *ascV* by PCR and sequencing analysis in 33 *A. hydrophila* strains showed that the TTSS may be present in all the strains examined. Insertional inactivation of *aopB* or *aopD* led to decreased cytotoxicity in carp epithelial cells, increased phagocytosis and reduced virulence in blue gourami fish, indicating that a TTSS is required for *A. hydrophila* pathogenesis. Thus, the identification of a TTSS has undoubtedly opened a new avenue for exploring the virulence determinants of *A. hydrophila*.

Subsequently, inactivation of two negative regulators (AopN and ExsD) led to the identification of several type III secreted proteins including AopB, AopD, AcrV, and a

few unknown effector proteins, AopE and AopH. All these secreted proteins were shown to be secreted via the TTSS by comparing the ECP profiles of *ΔaopN* and *ΔaopNΔascN* mutants. AopE and AopH are cytotoxic to HeLa cells, since expression of either AopE or AopH in HeLa cells was able to induce cell rounding. The N-terminus of AopE was sufficient for its cytotoxicity but the function of the C-terminus of AopE remains to be elucidated. How AopE and AopH interact with host components awaits further study.

Future investigations of the interactions between TTSS effectors and hosts may help in understanding the process of *A. hydrophila* infection and may even reveal novel mechanisms shared by other pathogens. The virulence-associated genes identified and characterized in this study will be helpful for data-mining for the development of effective vaccines, and diagnostic and novel therapeutics against animal and human infections caused by motile aeromonads. Therefore, the results presented in this study will definitely contribute to the understanding of pathogenesis of *A. hydrophila*.

VII.2 Future directions

A. hydrophila is a waterborne bacterium and causes diseases in reptiles, amphibians, fish and humans. The present study characterizes the virulence-associated factors involved in the pathogenesis of *A. hydrophila* using both the proteomic and molecular approaches. We have identified a TTSS cluster and several novel effector proteins in *A. hydrophila*. We have also demonstrated the multifactorial pathogenicity of *A. hydrophila* and revealed a cross-talk between the TTSS and a lateral flagellar secretion system. However, how these virulence-associated factors are involved in the pathogenesis is far from understood. Hence, future work will be carried out as follows:

1. Define the importance of phage-associated island genes and F11 in virulence. This will be carried out after solving the genetic problem of *A. hydrophila* PPD134/91 using a different conjugative plasmid or a helper plasmid.
2. In-depth characterization of the virulence-associated factors identified from the two rounds of genomic subtractions. The construction of multiple mutants, infections of different animal models, and the inclusion of other functional assays (such as adherence, invasion, biofilm formation and motility) will help to elucidate the involvements of these putative virulence factors in pathogenesis.
3. Investigate the relationship between quorum sensing systems and the TTSS in *A. hydrophila* AH-1. A comparison of the transcriptional or expression levels of AopD or AopE in both the wild type and the *ΔahyR* mutant under different culture conditions (*i. e.* different concentrations of calcium, magnesium or salt; different pH or media) will allow us to understand such a relationship. Alternatively, other quorum sensing related genes (such as *ahyI*) can be mutated to examine the transcription or secretion of TTSS secreted proteins.
4. Examine whether ExsA controls the expression of lateral flagellins directly or indirectly by an overexpression of ExsA in a Δ*exsA* mutant and by electrophoretic mobility shift assay.
5. Identify the intermediate gene(s) involved in the interaction of TTSS and the lateral flagellar secretion system. Construction of a transposon library in a Δ*exsD* background, followed by a screening for mutants whose swarming motility restores to the wild type level, may help to answer this question.
6. Confirm the translocation of AopE and AopH into HeLa cells by fusing the N-termini of AopE and AopH to a reporter gene (β-lactamase).

7. Examine whether AopE exhibits GAP activity and determine which GTPases are involved in this process.
8. Investigate whether the expression of AopE affects the cytoskeleton structure of HeLa cells by staining the actin with phalloidin-rhodamine.
9. Study the localization of AopE and AopH inside HeLa cells by confocal microscopy and sub-fractionation analysis.
10. Reveal how AopE and AopH are involved in the pathogenesis of *A. hydrophila* by tissue culture models or *in vivo* infection with *A. hydrophila* strains lacking *aopE* and/or *aopH*.
11. Identify AopE and AopH interaction partners inside host cells by an yeast two-hybrid assay.
12. Identify the unknown proteins secreted by the *ΔaopN* mutant.
13. Identify other unknown effector proteins secreted via the TTSS. The construction of an *A. hydrophila* mini-Tn5 library, and followed by the screening of mutants that are defective in cytotoxicity on fish epithelial cells, may help in revealing many novel effector proteins for further studies.

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Appendix I

The partial sequences of *ascV* in *A. hydrophila* strains

Aer-19
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Aer-27
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Aer-184
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