Identification and Characterization of Putative Virulence Genes and Gene Clusters in *Aeromonas hydrophila* PPD134/91

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Aeromonas hydrophila is a gram-negative opportunistic pathogen of animals and humans. The pathogenesis of *A. hydrophila* is multifactorial. Genomic subtraction and markers of genomic islands (GIs) were used to identify putative virulence genes in *A. hydrophila* PPD134/91. Two rounds of genomic subtraction led to the identification of 22 unique DNA fragments encoding 19 putative virulence factors and seven new open reading frames, which are commonly present in the eight virulence strains examined. In addition, four GIs were found, including O-antigen, capsule, phage-associated, and type III secretion system (TTSS) gene clusters. These putative virulence genes and gene clusters were positioned on a physical map of *A. hydrophila* PPD134/91 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 50% lethal dose to the same level as the TTSS mutation (about 1 log) in a blue gourami infection model. This observation sheds light on the multifactorial 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.

Aeromonas 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) (2, 37). It causes gastrointestinal and extraintestinal infections in humans, including septicemia, wound infections, and gastroenteritis (16). A number of virulence factors have been identified in *A. hydrophila*, namely, pili and adhesins (29, 30), O-antigens and capsules (23, 48, 49), S-layers (10), exotoxins such as hemolysins and enterotoxin (7, 14), and a repertoire of coenzymes which digest cellular components such as proteases, amylases, and lipases (20, 28).

The pathogenesis of *A. hydrophila* is multifactorial. Most studies to date have concentrated on the characterization of a few virulence factors in different animal models by using different strains (10, 34, 42), 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 (22, 47). In this study, we used genomic subtraction to identify 22 unique DNA fragments encoding 19 putative virulence fac-

tors and seven new open reading frames (ORFs), which are frequently present in a group of virulent strains of *A. hydrophila*. In addition, four genomic islands (GIs), which differ from the rest of the genome in G+C content and which carry mobility-associated genes (integrases or transposons) and putative virulence genes, were found. These are genes expressing the O-antigen and capsule (48, 49), a phage-associated gene cluster, and a type III secretion system (TTSS) gene cluster. Subsequently, these putative virulence-associated genes 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 ($\Delta ascN$) significantly affected the 50% lethal doses (LD₅₀s) in a blue gourami infection model. In addition, a triple deletion mutant of S-layer, serine protease and metalloprotease ($\Delta ahsA \Delta serA \Delta mepA$) increased the LD₅₀ to the same level as the *ascN* mutant.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *A. hydrophila* strains were maintained on tryptic soy agar or in tryptic soy broth (Difco/Becton Dickinson) at 25°C. *Escherichia coli*

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TABLE 1. Bacterial strains and vectors used in this study

Strain or plasmid	Genotype and/or relevant property ^a	Source ^b or reference
Strains		
<i>A. hydrophila</i>		
ATCC 7966	O:1, virulent, type strain	ATCC, United States
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
<i>E. coli</i>		
JM109	Col ^s , Amp ^s	Promega
MC1061(λ pir)	(λ pir); <i>thi thr-1 leu6 proA2 his-4 argE2 lacY1 galK2 ara14 xyl5 supE44</i> λ pir	32
S17-1(λ pir)	<i>thi pro hsdR hsdM⁺ recA</i> [RP42-Tc::Mu-Km::Tn7 (Tp ^r Sm ^r)Tra ⁺]	35
Plasmids		
pGEM-T Easy vector	Cloning vector; Ap ^r	Promega
pNEB193	Cloning vector; Ap ^r	New England Biolabs
pRE112	Suicide vector; R6K <i>ori sacB</i> ; Cm ^r	11

^a Virulent strains were defined as having a lower LD₅₀ in blue gourami or rainbow trout (<10^{6.5}) than the avirulent strains (>10^{7.5}).

^b ATCC, American Type Culture Collection; IHB, Institute of Hydrobiology; AVA, Agri-Food and Veterinary Authority; BAU, Bogor Agricultural University; JCM, Japan Collection of Microorganisms; UB, University of Barcelona; UM, University of Montreal.

strains were maintained on L agar or in Luria broth (LB) (Difco/Becton Dickinson) at 37°C. When required, media were supplemented with ampicillin (100 µg/ml) or chloramphenicol (30 µg/ml). The conjugative transfer of plasmids between *A. hydrophila* and *E. coli* strains was carried out by plate mating at 30°C for 24 h.

DNA manipulations and Southern hybridization. Bacterial genomic DNA was extracted using Genomic DNA isolation/purification kits (QIAGEN). Plasmid DNA was extracted using QIAprep spin miniprep kits. Restriction-digested products of PPD134/91 genomic DNA with PacI and EcoRI were cloned into pNEB193 vector (New England Biolabs), which was also cut by the same two enzymes, transformed into *E. coli* JM109-competent cells, and sequenced. Southern blotting was performed to characterize the distribution of the putative virulence genes of *A. hydrophila* PPD134/91 in other motile aeromonads with the BluGene Non-Radioactive Nucleic Acid Detection system (Gibco-BRL). Transfer of the DNA to a nylon membrane (GeneScreen, NEM Research Products), hybridization conditions, and visualization with streptavidin-alkaline phosphate conjugates were carried out as recommended by the manufacturer.

DNA sequencing and sequence analysis. DNA sequencing was carried out on an Applied Biosystems PRISM 3100 genetic analyzer with an ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems). The sequences were edited using the manufacturer's software. Sequence assembly and further editing were carried out with Vector NTI DNA analysis software (InforMax). 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.

Genome walking and genomic subtraction. Advantage Polymerase 2 (Clontech) was used for genome walking. Genome Walker libraries were constructed by using five restriction enzymes (DraI, EcoRV, PvuII, ScaI, and StuI). The cycling parameters for genome walking were as follows: 7 cycles of 15 s at 94°C, 4 min at 72°C, 32 cycles of 15 s at 94°C, and 3 min at 67°C. The amplified fragments were cloned into the pGEM-T Easy Vector (Promega), transformed into *E. coli* JM109-competent cells, and sequenced. Bacterial genome subtraction was performed as described previously by Zhang and coworkers (47).

Construction of defined insertion mutants and deletion mutants. To obtain defined insertion mutants, oligonucleotides containing flanking XbaI restriction sites (primers sequences available upon request) were used to amplify internal fragments from the targeted genes. The amplified fragment was ligated to pGEM-T Easy vector and transformed into *E. coli* JM109. The internal fragment was recovered by XbaI restriction digestion and ligated to XbaI-digested and dephosphorylated suicide vector pRE112 (11). 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.

Nonpolar deletion mutants were constructed according to the method for suicide plasmid pRE112 (11). Briefly, the targeted gene and at least 300 bp of flanking sequences were amplified with oligonucleotides containing XbaI 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 with XbaI and ligated to XbaI-digested and dephosphorylated pRE112. This construct was transformed into *E. coli* S17-1(λ pir). The single-crossover mutants were obtained by conjugal transfer into *A. hydrophila* AH-1. Double-crossover mutants were obtained by selecting against the presence of the *sacB* gene carried by the vector. Mutants resistant to sucrose were isolated by being plated onto LB-sucrose agar (1% tryptone, 0.5% yeast tract, 1.5% agar, 12% sucrose). The double-crossover mutants were confirmed by PCR.

LD₅₀ studies of fish. Healthy blue gourami (*Trichogaster trichopterus* Pallas) were obtained from a commercial fish farm, maintained in well-aerated dechlorinated water at 25 ± 2°C, and acclimatized to laboratory conditions for at least 15 days. Fish were approximately 13 g each and were about 3 months old. Three groups of 10 fish each were injected intramuscularly with 0.1 ml of phosphate-buffered saline-washed bacterial cells adjusted to the required concentrations. LD₅₀ studies were carried out in triplicate for all of the strains. Fish were monitored for mortality for 7 days, and LD₅₀s were calculated by the method of Reed and Muench (31).

Pulse-field gel electrophoresis. Pulse-field gel electrophoresis was carried out as previously described (46). ProMega-Markers Lambda Ladders (Promega) were used as the size markers. Band sizes were calculated from at least three independent separations of restriction fragments.

Statistical analysis. LD₅₀ data were analyzed using Student's *t* test. *P* values of <0.05 were considered significant.

Nucleotide sequence accession numbers. Thirteen genes of *A. hydrophila* AH-1 or AH-3 for construction of knockouts were assigned the following 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).

RESULTS AND DISCUSSION

Identification of putative virulence genes using genomic subtraction. Genomic subtraction was our first approach to identifying common virulence genes, based on screening genetic differences between virulent and avirulent strains of *A. hydrophila*. In our previous study, PPD35/85 was used as a driver and PPD134/91 was used as a tester, and 16 unique DNA fragments (F fragment numbers) (Table 2) derived from 115 subtracted clones were shown to be present in most of the eight virulent strains of *A. hydrophila* (47). However, the first subtraction library was far from complete, considering the presence of only four pairs of identical clones. Therefore, an

TABLE 2. Summary of putative virulence genes identified in *A. hydrophila* PPD134/91

Clone ^b	Size (amino acids)	Position (bp)	Accession no.	Homologies to predicted encoded protein	E value	% Identity (olh ^c)	Homologue accession no.	Location on physical map
F2/F3	344	172–203	AF146597	Outer membrane protein (OmpAI) of <i>Aeromonas salmonicida</i>	e-135	78 (344)	CAA63036	C5
F11	266	993–1790 ^a	AY376445	VsdC protein of <i>S. enterica</i> serovar Typhimurium	e-55	52 (593)	P24419	C3
	266	1790–2587		Type III secretion protein of <i>Erwinia</i> <i>carotovora</i> subsp. <i>atroseptica</i> SCRI 1043	2e-93	62 (265)	YP_050200	
F20	ND ^d	2–712	AY376446	Probable acinetobactin biosynthesis protein of <i>A. baumannii</i>	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 <i>Serratia marcescens</i>	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 <i>Yersinia</i> <i>pestis</i>	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	Yail/Yqx family protein of <i>Shewanella</i> <i>oneidensis</i>	2e-46	64 (146)	NP_718466	C6
F92	ND	73–615	AY378293	Partial ORF of Para- aminobenzoate synthase component I of <i>Vibrio vulnificus</i>	e-31	49 (155)	NP_761116	C7
F93	102	112–417	AY378294	Putative exported protein of <i>Y. pestis</i>	3e-17	49 (81)	NP_404091	C8
F97/PB45	316	8–955	AY378295	Reverse transcriptase like protein of <i>Pirellula</i> sp.	e-38	32 (321)	NP_866340	C18
F99/F106	409	267–1493	AF146029	Transporter transmembrane protein of <i>Ralstonia</i> <i>solanacearum</i>	2e-40	27 (376)	NP_521519	C15
F108	557	2675–4345	AF146599	Hemolysin (HlyA) of <i>A. hydrophila</i>	0	76 (577)	A61372	C16
PA1/PA4/PB38/PB62/ PB78	263	1171–1959	AY378297	Unknown				C8
PA6/PA98	190	449–1018	AY378298	Unknown				C19
PA91	207	5841–6461	AY378300	Putative positive transcription regulator BvgA of <i>B. pertussis</i>	3e-45	44 (209)	NP_880570	C15
	1,252	6467–10222 ^a		Virulence sensory protein BvgS of <i>B. pertussis</i>	e-156	32 (1,238)	P16575	
PB28/PB35	160	96–575	AY378301	Topoisomerase of <i>Pseudomonas</i> <i>aeruginosa</i>	8e-13	57 (56)	AAD20003	C18
				Transmembrane protein of <i>R. solanacearum</i>	3e-07	48 (50)	NP_521535	
PB60	314	54–995	AY378302	Sensory box/GGDEF family protein of <i>Caulobacter crescentus</i>	9e-42	34 (311)	NP_421888	C16
PB80/PA26	488	1128–2591 ^a	AY378303	Aerolysin (AerA) of <i>A. hydrophila</i>	0	84 (488)	AAL04124	C15
	527	2856–4436		Arylsulfotransferase of <i>Eubacterium rectale</i>	e-13	26 (352)	AAK14940	

^a 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.^b 'p' indicates that these subtracted clones overlap with each other.^c olh, overall length of homologous proteins.^d ND, not determined.

TABLE 3. Distribution of PA and PB subtracted clones among virulent and avirulent strains of *A. hydrophila*

Strain	Probe(s) used for Southern analysis ^a					
	PA1/PA4/ PB62	PA6/PA98/ PB78/PB38	PA26/ PB80	PA91	PB28/ PB35	PB60
Virulent						
ATCC 7966	—	+	+	+	—	—
L31	—	—	+	—	+	+
PPD11/90	—	—	+	+	+	+
PPD70/91	+	+	+	—	—	—
PPD134/91	+	+	+	+	+	+
PPD122/91	+	+	+	—	—	—
AH-1	+	+	—	—	—	+
Xs91-4-1	—	+	+	+	+	+
Avirulent						
L15	—	+	—	+	+	—
L36	—	+	—	—	—	—
PPD35/85	—	—	—	—	—	—
PPD45/91	—	—	—	—	—	—
PPD64/90	—	—	—	—	+	+
PPD88/90	—	—	—	—	—	+

^a Hybridization results: +, strong signal; —, no signal.

additional round of genomic subtraction was carried out using PPD35/85 or PPD64/90 as the driver and using PPD134/91 again as the tester. The restriction enzyme EcoRV (which cuts less frequently) rather than RsaI was used to generate longer subtracted DNA fragments. Subtracted clones using PPD64/90 or PPD35/85 as the driver were named PA or PB, respectively. Southern hybridization was then performed to survey the distribution of these subtracted fragments among 8 virulent and 6 avirulent strains of *A. hydrophila* (the first 14 strains listed in Table 1). The results showed that seven unique DNA fragments (derived from about 100 subtracted clones) (Table 2) from the PA and PB libraries were present in most of the eight virulent strains (≥ 4) but absent in most of the six avirulent strains (≤ 2) (Table 3). Altogether, 22 unique DNA fragments (F97 and PB45 are in the same DNA fragment) representing about 200 subtracted clones were obtained from two rounds of genomic subtraction and were commonly present in the eight virulent strains (≥ 4) (Table 2) (47). These 22 DNA fragments were assumed to encode potential virulence genes and were subjected to further analysis.

Sequence analysis of the 22 unique DNA fragments. Genome walking was carried out to obtain complete ORFs, as well as upstream and downstream sequences, of these 22 DNA fragments to predict their genetic organizations and functions. Fifteen of the DNA fragments encode 19 proteins with homologues in other bacteria. Eleven of these homologues showed significant homology to virulence-associated factors (Table 2). These included known virulence factors of *Aeromonas* species such as hemolysin (HlyA and F108) and aerolysin (AerA and 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 and F52), oligopeptidase A (OpdA and F88/F109/F61/F72), outer membrane protein (OmpAI and 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 *Salmonella enterica* serovar Dublin (18). 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* (25). In *Bordetella pertussis*, the *bvgA* and *bvgS* two-component system controls the expression of many virulence genes, including those encoding pili (26) and adenylate cyclase (12). 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 (43, 45).

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), a YaiI/YqxI family protein (F89), *para*-aminobenzoate synthase (F92), a putative exported protein (F93), a transporter 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, and PA6/PA98/PB38/PB78), the ORFs encoded had 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.

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 they are present in most of the pathogenic bacteria (13). 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 (8) and serves as the insertion site for acquired sequences such as the cryptic phage CP4-57 in *E. coli* and PAIs in *Vibrio cholerae* (17) and *Dichelobacter nodosus* (3).

Primers *ssrA*-F (5-CAAACGACGAAACTACGC-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 using these primers. Subsequently, a series of genome walking experiments 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 to 63%), which is a common characteristic of GIs (13). It also shows a mosaic distribution of G+C content (Table 4; Fig. 1). Furthermore, two ORFs (ORF1 and ORF2) (Table 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 4) did not differ significantly from that of *A. hydrophila*, indicating that they might have been acquired

FIG. 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.

from species with G+C content similar to that of *A. hydrophila* or that the base composition of these acquired DNA has gradually adapted to the host genome (19).

Many putative virulence factors are bacteriophage encoded (4), and like insertion elements, bacteriophages are often associated with GIs and are involved in the transfer of virulence determinants between bacterial species (15). This island carries several homologues to bacteriophage-encoded proteins (Table 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 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 24 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 a phage-associated island.

The distribution of this putative GI among 14 *A. hydrophila*

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

ORF	Function or expression	Homology	G+C %	E value	% Identity (olh ^a)	Homologue accession no.
		<i>ssrA</i> gene (<i>A. salmonicida</i>)	52.1	e-144		AF440330
1	Transposase	IS1650 orf B (<i>Shigella flexneri</i>)	54.3	e-29	46 (171)	AAK18513
2	Transposase	IS1650 orf A (<i>S. flexneri</i>)	55.2	4e-25	46 (149)	AAK18514
3	Hypothetical protein	Hypothetical protein (Bacteriophage VT2-Sa)	59.7	0.03	25 (2,806)	NP_050570
4	Putative phage nin-region protein	Putative phage nin-region protein (<i>Y. pestis</i> KIM)	59.9	7e-37	54 (148)	NP_993273
5	Hypothetical protein	Hypothetical protein (<i>Nostoc punctiforme</i>)	49.5	e-05	37 (216)	ZP_00109981
6	Hypothetical protein	Hypothetical protein (<i>Thermotoga maritima</i>)	33.6	3e-04	22 (718)	NP_228994
7	No good homology		31.3			
8	Unknown protein	Unknown (<i>Zymomonas mobilis</i>)	56.7	2e-30	43 (318)	AAD19409
9	No good homology		33.6			
10	Putative phage protein	Putative phage protein (<i>S. enterica</i> serovar 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 protein (<i>Shigella flexneri</i> bacteriophage V)	59.3	e-31	55 (272)	AAL89441
15	Hypothetical protein	Gp12 (<i>Burkholderia cenocepacia</i> phage Bcep1)	59.8	e-26	36 (181)	NP_944320
16	Putative phage repressor	Putative phage repressor (<i>Bordetella bronchiseptica</i> RB50)	51.5	4e-05	34 (227)	NP_888223
17	No good homology		60.8			
18	No good homology		50.9			
19	Transcriptional protein	Predicted transcriptional regulators (<i>A. pleuropneumoniae</i> 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 (<i>S. enterica</i> serovar Typhimurium LT2)	55.9	3e-38	31 (975)	T03004
23	Hypothetical protein	Hypothetical protein (<i>S. enterica</i> subsp. <i>enterica</i> serovar Typhi)	55.7	2e-61	62 (350)	NP_456435
24	Hypothetical protein	Hypothetical protein (<i>Bacillus cereus</i> ZK)	46.2	0.003	27 (613)	YP_082425

^a olh, overall length of homologous protein.

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

Strain	Probe used for Southern analysis ^a			
	I	II	III	IV
Virulent				
ATCC 7966	+	+	+	+
L31	±	—	±	±
PPD11/90	±	±	—	—
PPD70/91	±	±	±	—
PPD134/91	+	+	+	+
PPD122/91	±	±	—	±
AH-1	±	±	—	±
Xs91-4-1	+	+	+	+
Avirulent				
L15	—	—	—	—
L36	+	+	+	+
PPD35/86	—	—	—	—
PPD45/91	—	—	—	—
PPD64/90	±	—	—	—
PPD88/90	—	—	—	—

^a Hybridization results: +, strong signal; ±, weak signal; —, no signal.

strains was surveyed by Southern analysis using probes from four regions of the DNA (Fig. 1; Table 4). The four probes hybridized with most of the eight virulent strains but only hybridized with one out of the six avirulent strains tested (Table 5). The hybridization signals were strong in virulent strains

ATCC 7966, 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 (39). 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 insertion elements and bacteriophages, conferred the greatest defect in virulence (8). This phage-associated island may also contribute to differences in host specificity or disease manifestation.

Identification of a TTSS gene cluster. TTSSs have been reported in many gram-negative animal and plant pathogens, including *A. hydrophila* (6, 9, 46). As reported previously (46), an *ascV* homologue is present in *A. hydrophila* PPD134/91. Furthermore, an *ascU* homologue, near one end of the TTSS in *A. hydrophila* AH-1, was also identified in *A. hydrophila* PPD134/91 by PCR (*ascU*-F, 5-TGGTGATCGCCATCGCCG A-3; *ascU*-R, 5-GACGGCGCTTGCTCTTGAT-3). The identification of these two TTSS homologues strongly indicated that 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. 2). The complete TTSS sequence of

FIG. 2. Physical map of *A. hydrophila* PPD134/91. The locations of virulence genes and clusters were determined with respect to PacI fragments.

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 (15) 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 (accession no. AY763611) (38), which further suggests that a complete TTSS gene cluster may be also present in PPD134/91.

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 to provide insight into the mechanisms of gene regulation and the molecular basis of pathogenicity of this bacterium. A physical map of *A. hydrophila* was therefore constructed. We chose the AT-rich recognition site endonuclease *PacI* to digest the PPD134/91 genomic DNA, based on the high G+C content (57 to 63%) of *Aeromonas* species (21). This yielded 19 fragments (named C1 to C19), with sizes of 11 to 733 kb. To link these fragments, PPD134/91 genomic DNA was digested with *PacI* and *EcoRI* and cloned into the pNEB193 vector. More than 100 clones were obtained, 65 of which contained sequences flanked by *PacI* sites. The clones whose sequences were homologous to the different portions of the same gene were considered joined to each other. For those clones which could not be joined together according to the above rule, genome walking was carried out to link them by searching for the shared *PacI* sites. The two half sites of *PacI* were located in two different clones ("clone pairs"). All of the clones (totaling 38 of the *PacI*- and *EcoRI*-digested fragments, i.e., 19 clone pairs) were prepared as probes and hybridized with *PacI*-digested fragments of genomic DNA from *A. hydrophila* PPD134/91. The two digested fragments which hybridized with the "clone pairs" were thus linked to each other. In this way, all of the fragments generated by *PacI* digestion of PPD134/91 were linked, and a physical map was formed (Fig. 2).

The 22 unique DNA fragments from the two rounds of genomic subtraction as well as the O-antigen and capsule clusters (48, 49), the phage-associated island, and the TTSS gene cluster have been positioned on the physical map by Southern hybridization (Fig. 2). The results showed O-antigen and capsule gene clusters were located at different regions of the chromosome (Fig. 2), while these two clusters were located near each other in *E. coli* (33). 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 (15).

Ten subtracted clones consisting of seven unique DNA fragments were found to be located in the same *PacI*-digested

fragment C14 (Fig. 2) of strain PPD134/91. However, there is as yet no indication that these virulence genes are located in a PAI. Most of these genes were not clustered in the same *PacI*-digested fragments in the genomes of seven other virulent strains by Southern analysis (data not shown).

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 (24, 46) were used as the hosts for the construction of mutants. Fifteen genes in *A. hydrophila* 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 LD₅₀s of insertion mutants increased by at least 0.5 log, 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 $\Delta ompAI$, $\Delta ascN$, and $\Delta bvgA$ to confirm that there were no differences in the LD₅₀s 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 is ongoing and will shed light on their roles in PPD134/91 pathogenesis.

The genes used for construction of mutants can be classified into three groups. Group I includes genes expressing hemolysin (*hlyA*) and aerolysin (*aerA*), which have been reported to be associated with the virulence of *A. hydrophila* (1, 42). Group II includes genes expressing histone-like protein (*hup1* for AH-1 and *hup3* for AH-3) (36); *opdA*, expressing ligopeptidase A (27); *bvgA*, *bvgS*, and *ascN* (46); and *ompAI*, expressing an outer membrane protein (40). These gene products are homologous to known virulence factors of other pathogens. Group III includes genes expressing 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), which appears to be involved in biofilm formation (H. B. Yu and K. Y. Leung, unpublished data).

Our results showed that the LD₅₀s of all the mutants except *ascN* were comparable to that of the wild-type strains by using blue gourami fish as the infection model. The LD₅₀s of the wild types AH-1 and AH-3 were 10^{5.3} and 10^{6.5}, respectively. LD₅₀s of both insertion and deletion mutants of *ascN* in AH-1 were determined to be 10^{6.3}. Wong and coworkers (42) 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. In all the mutants tested, only *ascN* exhibited an LD₅₀ 1 log higher than that of the wild type-strain AH-1. *AscN*, a homologue of the *YscN* of *Yersinia* species (41), 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 nonfunctional, leading to the increase in LD₅₀s and consistent with our previous study (46). The fact that most of the virulence genes did not attenuate the mutants suggested that the pathogenesis of *A. hydrophila* is multifactorial 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 LD₅₀s. 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 an ~300-fold increase in LD₅₀s by intraperitoneal injection in Swiss-Webster mice (44); a single mutation in elastase of *A. hydrophila* AG2 resulted in an ~100-fold increase in LD₅₀s by intraperitoneal challenge in rainbow trout (5).

In addition, double deletions mutations were made in strain AH-1, such as S-layer and metalloprotease (Δ *ahsA* Δ *mepA*; LD₅₀ = 10^{5.7}), S-layer and serine protease (Δ *ahsA* Δ *serA*; LD₅₀ = 10^{5.7}), and a triple-deletion mutant of S-layer, metalloprotease, and serine protease (Δ *ahsA* Δ *serA* Δ *mepA*; LD₅₀ = 10^{6.5}). Different proteases have been shown to be involved in *A. hydrophila* virulence (5). The double-deletion mutants resulted in about half of a log increase in LD₅₀s, but the attenuation was not statistically significant ($P > 0.05$). However, fish infected with these double mutants survived 1 to 2 days longer than those infected with the wild type at the same lethal dosage. Statistically significant attenuation was observed for the triple mutant, where the LD₅₀ increased about 1 log. 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.

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 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 with *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 same infection 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 under way 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 pathogen-

esis 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.

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