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Complete Type III Secretion System of a Mesophilic *Aeromonas hydrophila* Strain

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We have investigated the existence and genetic organization of a functional type III secretion system (TTSS) in a mesophilic *Aeromonas* strain by initially using the *Aeromonas hydrophila* strain AH-3. We report for the first time the complete TTSS DNA sequence of an *Aeromonas* strain that comprises 35 genes organized in a similar disposition as that in *Pseudomonas aeruginosa*. Using several gene probes, we also determined the presence of a TTSS in clinical or environmental strains of different *Aeromonas* species: *A. hydrophila*, *A. veronii*, and *A. caviae*. By using one of the TTSS genes (*ascV*), we were able to obtain a defined insertion mutant in strain AH-3 (AH-3AscV), which showed reduced toxicity and virulence in comparison with the wild-type strain. Complementation of the mutant strain with a plasmid vector carrying *ascV* was fully able to restore the wild-type toxicity and virulence.

The genus *Aeromonas* comprises mesophilic and psychrophilic species, both motile and nonmotile. This bacterium is found in both fresh and salt water and in virtually all foods and causes a wide variety of human infections, including septicemia, wound infections, meningitis, pneumonia, and gastroenteritis (7, 12). Out of the 16 reported species within the genus, three of them, *A. veronii*, *A. caviae*, and *A. hydrophila*, represent more than 85% of clinical isolates (11, 13). The pathogenesis of *Aeromonas* has multiple factors, such as O antigens, capsule (16, 23), the S-layer (6), exotoxins such as hemolysins and enterotoxin (4, 9), and a repertoire of exoenzymes which digest cellular components such as proteases, amylases, and lipases (14, 19). These virulence determinants are involved sequentially as the bacteria colonize, gain entry, establish themselves, replicate, cause damage in host tissues, evade the host defense system, and spread, eventually killing the host. The mechanisms of action of most of these virulence factors remain unknown.

Recent studies have shown that the virulence mechanisms of various pathogens are highly similar; one such mechanism is a type III secretion system (TTSS) that plays crucial roles in host-pathogen interactions (5). The TTSS is found in some gram-negative animal and plant pathogens. This system can efficiently deliver antihost virulence determinants into the host cells, directly interfering with and altering host processes. Recently, several reports have attempted to elucidate the existence of the TTSS in *Aeromonas* (1, 2, 3, 21, 22). In this sense a functional TTSS located on a large thermolabile virulence plasmid has been reported for the fish-pathogenic species *A. salmonicida* (1, 2, 21) and in the chromosome of *A. hydrophila* AH-1 (22). However, in both studies the TTSS genes sequenced do not seem to correspond to a complete TTSS compared with the well-known *Yersinia* or *Pseudomonas* TTSS (5). Furthermore, dot blotting and sequencing experiments have provided evidence of the existence of other TTSS genes (*ascF-ascG*) in mesophilic clinical species (3), which reinforces the hypothesis that the *Aeromonas* TTSS sequence organization described recently is incomplete. Therefore, the purpose of the present study was to investigate and describe the complete sequence of genes that constitute the *Aeromonas* TTSS and its preliminary function. In addition we hoped to establish its prevalence in a set of genetically identified clinical and environmental strains of mesophilic species, i.e., *A. veronii*, *A. caviae*, and *A. hydrophila*, more frequently implicated in human infections.

Complete *A. hydrophila* AH-3 TTSS gene cluster. We selected *A. hydrophila* AH-3 for sequencing studies of the TTSS because it is a fish- and mouse-pathogenic strain (17) belonging to serotype O:34, which is the predominant serogroup associated with clinical isolates (15). By using *A. hydrophila* AH-3 genomic DNA and primers (5′-ATGGACGGCGCCATGAAGTT-3′ and 5′-TATTCGCCTTCACCCATCCC-3′) derived from the DNA sequence of *ascV* from *A. salmonicida* (a gene from the type III secretion apparatus which prevents the delivery of the AexT toxin [1, 2, 21]), we generated by PCR a 710-bp fragment which showed high identity (88%) with the *A. salmonicida ascV* gene. We used this DNA fragment labeled with digoxigenin to screen our gene library previously obtained from strain AH-3 (18) by colony Southern blotting. We found several clones, and with two of them we obtained the complete DNA sequence of the TTSS from strain AH-3. DNA sequencing and sequence analysis with various software programs were performed as previously described (10). The *A. hydrophila* AH-3 TTSS DNA sequence (GenBank accession no. AY528667) showed 35 open reading frames, organized as

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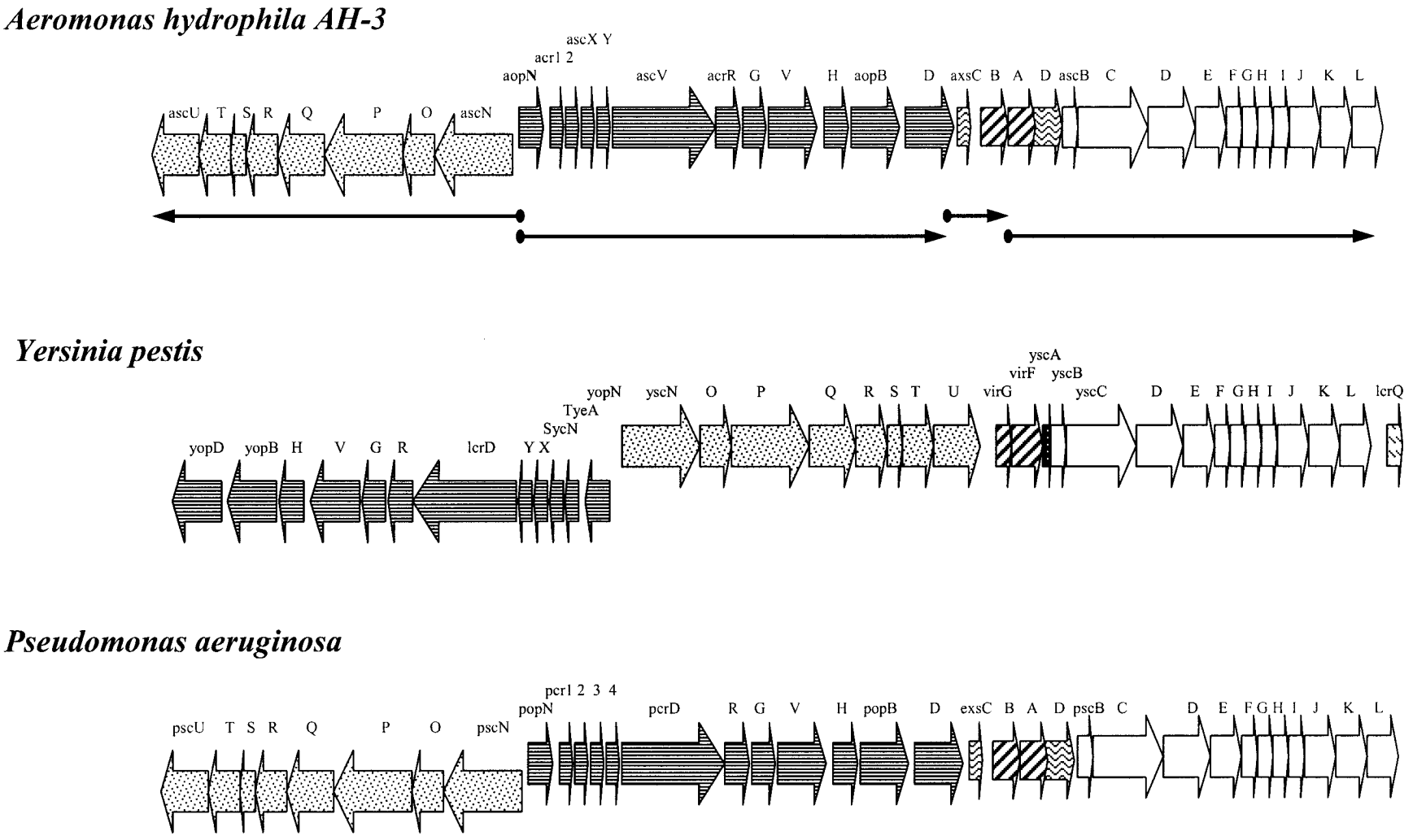

FIG. 1. Genetic organization of the *A. hydrophila* AH-3 TTSS genes. Open reading frames and their directions of transcription are indicated by black arrows and named according to the *Aeromonas* gene-protein names given by us. We also show the four gene clusters determined by RT-PCRs. The complete TTSSs from *A. hydrophila* AH-3, *Y. pestis*, and *P. aeruginosa* are also shown. Black arrows showing the same drawing correspond to homologous genes among these bacteria.

shown in Fig. 1, that code for 35 proteins. The nomenclature for the genes-proteins newly described in *Aeromonas* follows that employed for *Pseudomonas aeruginosa* (in parentheses), with P for *Pseudomonas* being substituted for A for *Aeromonas*; for the rest we employed the terminology used for *A. salmonicida* (2). The characteristics of individual proteins (amino acid residues, molecular weight, and isoelectric point) and their homologues are shown in Table 1. The first 20 proteins (from AscU to AopD) show high homology with the corresponding proteins of the *A. salmonicida* and *A. hydrophila* TTSSs previously reported (1, 2, 21, 22). However, of the last 15 proteins (from AxsC to AscL), at least eight could be considered novel TTSS proteins for *Aeromonas* because they have not yet been reported for the TTSS of *A. salmonicida*, *A. veronii*, or *A. hydrophila* (2, 22). This may be due to the fact that the genes for these 15 proteins have not been identified or may not be present in the TTSSs of the strains studied (2, 22). However, the fact that 2 and 5 of these 15 genes (*ascF-ascG*) have been sequenced for *A. veronii* (3) and *A. hydrophila* AH-1 (22), respectively, and recognized by dot blotting with other TTSS genes in the type strain of *A. salmonicida* (data not shown) indicates that this strain at least bears this additional pair of genes. Furthermore, none of the TTSSs described for *Aeromonas* spp. have been completely sequenced.

Four putative promoter regions have been identified upstream of the genes (*ascN*, *aopN*, *axsC* [*exsC*], and *axsA* [*exsA*]), and four rho-independent terminator sequences have been identified downstream (*ascU*, *aopD*, *axsB* [*exsB*], and *ascL* [*pscL*]), showing four putative clusters as in Fig. 1. The four clusters have been confirmed using reverse transcription-PCR (RT-PCR) with specific primers derived from the DNA sequence. The total RNA was extracted with Trizol reagent (Invitrogen), and to ensure that the RNA was devoid of contaminating DNA, the preparation was treated with RNase-free RQ1 DNase (Invitrogen) for 1 h. The isolated RNA was used as a template in RT-PCRs, with the use of the SuperScript One-Step RT-PCR system (Invitrogen) according to the manufacturer's instructions. Using DNA sequencing-derived oligonucleotides and RT-PCRs, we found amplification between *ascU* to *ascN*, *aopN* to *aopD*, *axsC* (*exsC*) to *axsB* (*exsB*), and *axsA* (*exsA*) to *ascL*. However, no amplifications were obtained with oligonucleotide pairs from *ascN* to *aopN*, *aopD* to *axsC* (*exsC*), and *axsB* (*exsB*) to *axsA* (*exsA*), thus confirming the four putative clusters of this entire TTSS region (Fig. 1).

As can be observed in Table 1 and Fig. 1, the *A. hydrophila* AH-3 TTSS is similar in gene pair organization to the *P. aeruginosa* TTSS despite individual gene-protein homology sometimes being higher with *Yersinia pestis* TTSS genes-proteins than with those of *P. aeruginosa* (in the last 15 genes not previously described for *A. salmonicida*), thus showing that in this study we have obtained for the first time the complete arrangement of TTSS genes of *Aeromonas*. A comparison of the overall genetic organization between different TTSSs defined three subgroups that match similarities in the order of the large genetic blocks. Subgroup 2 includes *Yersinia* spp. and *P. aeruginosa*, and according to our findings *Aeromonas* TTSS should also be included in this subgroup. The overall G+C content of the *A. hydrophila* AH-3 TTSS region (58.3%) is in the range of the genomic G+C content (57 to 62%) of mesophilic *Aeromonas* strains.

Distribution of the TTSS genes among mesophilic *Aeromonas* strains. Taking advantage of the complete DNA sequence obtained, by PCR we prepared two more digoxigenin-labeled DNA probes from both edges of the TTSS by using AH-3 chromosomal DNA: the first one was 4,815 bp (*ascN* to *ascT*) with oligonucleotides 5′-TATCGAAGCTGATCTGGGGG-3′ and 5′-ATGGCAATAAGCAGCGGG-3′, and the second one was 4,141 bp (*ascC* to *ascJ*, names in *Aeromonas* given by us) with oligonucleotides 5′-GCGCTCTCCATCATCGAC-3′ and 5′-CCACGTCGGATTCTTCAAC-3′. The three labeled DNA probes (*ascV*, *ascN* to *ascT*, and *ascC* to *ascJ*) were used in a dot blot assay to screen genomic DNAs from clinical and environmental *Aeromonas* strains ($n = 182$) to establish their prevalence. The environmental strains ($n = 65$) were isolated mainly from water samples ($n = 54$) and shellfish ($n = 11$) by using ampicillin dextrin agar or Tergitol agar, while the clinical strains ($n = 117$) were isolated from blood agar supplemented or not with ampicillin depending upon their intestinal ($n = 74$) or extraintestinal ($n = 43$) origin. All the strains were identified to the species level by 16S ribosomal DNA restriction fragment length polymorphism analysis (8). The results are summarized in Table 2. As can be observed, all the mesophilic *Aeromonas* strains testing positive or negative for one probe were also positive or negative for the rest of the DNA probes. In addition the TTSS was less prevalent in environmental strains (26%) than in clinical strains (56%). This is to our knowledge the first study that comparatively evaluates the prevalence of TTSS genes in a representative number of genetically identified clinical and environmental *Aeromonas* strains.

Mutant isolation in *ascV* and characterization. We also constructed an *A. hydrophila* AH-3-defined insertion mutation in gene *ascV*. Briefly, we used the previously described oligonucleotides (5′-ATGGACGGCGCATGAAGTTS-3′ and 5′-TATTCGCCTTCACCCATCCC-3′) to amplify an internal *ascV* DNA fragment (710 bp). This DNA fragment was ligated to the vector pGEM-T (Promega) and transformed in *Escherichia coli* DH5α. The DNA fragment was recovered by SalI-NcoI double digestion; blunt ended with Klenow fragment; ligated to EcoRI-digested, blunt-ended, dephosphorylated pSF100 (20); and transformed into *E. coli* MC1061 (λ*pir*) to generate plasmid pFS-AcsV. Plasmid pFS-AcsV was isolated, transformed into *E. coli* SM10 (λ*pir*), and transferred by conjugation from *E. coli* SM10 to an *A. hydrophila* AH-3 rifampin-resistant mutant (from our laboratory collection) as previously described (17, 18). Kanamycin- and rifampin-resistant transconjugants arising from pFS-AcsV integration were obtained and analyzed by Southern blot hybridization with an *ascV* DNA probe to obtain a defined insertion *ascV* mutant (AH-3AscV) as previously described (17, 18). The 50% lethal doses of the mutant and wild-type strains were evaluated using rainbow trout (12 to 20 g) maintained in 20-liter static tanks and albino Swiss female mice (5 to 7 weeks old) in all cases injected intraperitoneally with 0.05 and 0.25 ml of the test samples (approximately 10^9 viable cells), respectively, as previously described (17, 18). Mutant AH-3AscV had decreased virulence as shown by significantly higher 50% lethal doses in rainbow trout ($10^{6.7}$) and mice ($10^{8.5}$) than those of the wild-type strain ($10^{5.3}$ and $10^{7.4}$, respectively). Mutant AH-3AscV showed a clearly reduced cytotoxic effect on different eukary-

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

Protein	Putative function	No. of amino acids	Mol wt (thousands)	pI	Protein homologues (% identity/% similarity)
AscU	Regulation of secretion	352	39.36	8.51	AscU, *Aeromonas salmonicida* (87/90) AscU, *Aeromonas hydrophila* AH-1 (86/89) LscU, *Photorhabdus luminescens* (72/82)
AscT	Type III secretion apparatus	262	28.29	6.54	AscT, *Aeromonas salmonicida* (72/74) AscT, *Aeromonas hydrophila* AH-1 (69/74) PscT, *Pseudomonas aeruginosa* (56/69)
AscS	Type III secretion apparatus	88	9.66	5.30	AscS, *Aeromonas hydrophila* AH-1 (98/100) AscS, *Aeromonas salmonicida* (96/98) LscS, *Photorhabdus luminescens* (82/90)
AscR	Type III secretion apparatus	217	24.17	6.11	AscR, *Aeromonas salmonicida* (91/90) AscR, *Aeromonas hydrophila* AH-1 (89/90) PscR, *Pseudomonas aeruginosa* (79/88)
AscQ	Unknown	308	33.45	4.92	AscQ, *Aeromonas salmonicida* (74/77) AscQ, *Aeromonas hydrophila* AH-1 (63/70) YscQ, *Yersinia* spp. (43/57)
AscP	Regulation of secretion	408	44.99	5.07	AscP, *Aeromonas salmonicida* (65/75) AscP, *Aeromonas hydrophila* AH-1 (54/68) LscP, *Photorhabdus luminescens* (37/53)
AscO	Regulation of secretion	153	18.56	7.92	AscO, *Aeromonas salmonicida* (49/51) AscU, *Aeromonas hydrophila* AH-1 (43749) PscO, *Pseudomonas aeruginosa* (41/48)
AscN	ATP synthase	440	47.80	6.17	AscN, *Aeromonas salmonicida* (95/96) AscN, *Aeromonas hydrophila* AH-1 (95/96) LscN, *Photorhabdus luminescens* (90/94)
AopN	Regulation of translocation	292	32.03	5.54	AopN, *Aeromonas salmonicida* (86/87) AopN, *Aeromonas hydrophila* AH-1 (83/85) LopN, *Photorhabdus luminescens* (60/63)
Acr1	Translocation apparatus	93	10.52	4.29	Acr1, *Aeromonas salmonicida* (93/95) Acr1, *Aeromonas hydrophila* AH-1 (88/96) LssA, *Photorhabdus luminescens* (70/86)
Acr2	Chaperone	123	13.74	5.57	Acr2, *Aeromonas salmonicida* (92/96) Acr2, *Aeromonas hydrophila* AH-1 (83/87) LssN, *Photorhabdus luminescens* (63/77)
AscX	Type III secretion apparatus	121	13.63	5.84	AscX, *Aeromonas salmonicida* (96/97) AscX, *Aeromonas hydrophila* AH-1 (81/90) LssB, *Photorhabdus luminescens* (61/75)
AscY	Type III secretion apparatus	116	12.90	5.23	AscY, *Aeromonas salmonicida* (77/79) AscY, *Aeromonas hydrophila* AH-1 (67/69) LssC, *Photorhabdus luminescens* (57/66)
AscV	Type III secretion apparatus	721	79.26	6.09	AscV, *Aeromonas salmonicida* (88/89) AscV, *Aeromonas hydrophila* AH-1 (85/87) LssD, *Photorhabdus luminescens* (77/82)
AcrR	Unknown	151	16.89	9.22	AcrR, *Aeromonas salmonicida* (89/93) LcrR, *Yersinia* spp. (56/68) AcrR, *Aeromonas hydrophila* AH-1 (53/66)
AcrG	Regulation of low-calcium response	94	10.52	5.87	AcrG, *Aeromonas salmonicida* (90/94) PcrG, *Pseudomonas aeruginosa* (47/63) AcrG, *Aeromonas hydrophila* AH-1 (44/59)
AcrV	Protective antigen, anti-host factor	361	40.14	5.22	AcrV, *Aeromonas salmonicida* (74/81) LssV, *Photorhabdus luminescens* (41/53) AcrV, *Aeromonas hydrophila* AH-1 (38/60)
AcrH	Chaperone	167	18.53	4.32	AcrH, *Aeromonas salmonicida* (86/89) LssH, *Photorhabdus luminescens* (63/76) AcrH, *Aeromonas hydrophila* AH-1 (57/72)
AopB	Translocation apparatus	390	40.24	9.17	AopB, *Aeromonas salmonicida* (63/73) LopB, *Photorhabdus luminescens* (38/56) AopB, *Aeromonas hydrophila* AH-1 (32/45)
AopD	Translocation apparatus	299	32.17	6.21	AopD, *Aeromonas salmonicida* (57/69) AopD, *Aeromonas hydrophila* AH-1 (52/71) YopD, *Yersinia* spp. (40/59)
AxsC (ExsC)	Unknown	147	16.72	4.59	HscY, *Aeromonas hydrophila* AH-1 (79/87) LscY, *Photorhabdus luminescens* (70/80) ExsC, *Pseudomonas aeruginosa* (60/84)
AxsB (ExsB)	Regulation of secretion	133	14.86	9.22	AscX, *Aeromonas hydrophila* AH-1 (55/67) LscW, *Photorhabdus luminescens* (36/56) VirG, *Yersinia* spp. (32/51) ExsB, *Pseudomonas aeruginosa* (24/41)

Continued on following page

TABLE 1—*Continued*

Protein	Putative function	No. of amino acids	Mol wt (thousands)	pI	Protein homologues (% identity/% similarity)
AxsA (ExsA)	Transcriptional activator	271	30.82	6.18	AscA, *Aeromonas hydrophila* AH-1 (87/93) LscA, *Photorhabdus luminescens* (74/83) ExsA, *Pseudomonas aeruginosa* (65/76)
AxsD (ExsD)	Putative regulator	271	31.53	5.57	AscZ, *Aeromonas hydrophila* AH-1 (68/81) LscZ, *Photorhabdus luminescens* (44/60) ExsD, *Pseudomonas aeruginosa* (36/51)
AscB (PscB)	Putative chaperone	141	15.72	5.43	AscB, *Aeromonas hydrophila* AH-1 (76/80) LscB, *Photorhabdus luminescens* (56/68) YscB, *Yersinia* spp. (45/61) PscB, *Pseudomonas aeruginosa* (41/56)
AscC (PscC)	Secretin	618	67.83	5.07	YscC, *Yersinia* spp. (73/85) LscC, *Photorhabdus luminescens* (67/79) PscC, *Pseudomonas aeruginosa* (66/79)
AscD (PscD)	Type III secretion apparatus	433	48	6.15	LscD, *Photorhabdus luminescens* (50/65) PscD, *Pseudomonas aeruginosa* (46/64) YscD, *Yersinia* spp. (45/63)
AscE (PscE)	Translocation apparatus	67	7.48	4.93	SctE, *Photorhabdus luminescens* (47/70) PscE, *Pseudomonas aeruginosa* (48/64) YscE, *Yersinia* spp. (35/60)
AscF (PscF)	Translocation apparatus (needle)	81	9.02	6.55	AscF, *Aeromonas veronii* (82790) PscF, *Pseudomonas aeruginosa* (79/80) LscF, *Photorhabdus luminescens* (70/79)
AscG (PscG)	Chaperone	117	12.94	5.04	AscG, *Aeromonas veronii* (75/77) VP1693, *Vibrio parahaemolyticus* (49/60) YscG, *Yersinia enterocolitica* (49/58)
AscH (PscH)	Unknown	183	20.69	5.71	PscH, *Pseudomonas aeruginosa* (50/68) YscH, (yopR), *Yersinia* spp. (38/57) LscH, *Photorhabdus luminescens* (37/54)
AscI (PscI)	Chaperone	112	12.04	4.48	LscI, *Photorhabdus luminescens* (56/72) PscI, *Pseudomonas aeruginosa* (61/72) YscI, *Yersinia* spp. (46/60)
AscJ (PscJ)	Type III secretion apparatus	246	27	7.01	LscJ, *Photorhabdus luminescens* (73/84) YscJ, *Yersinia* spp. (73/80) PscJ, *Pseudomonas aeruginosa* (70/82)
AscK (PscK)	Unknown	207	22.61	6.59	LscK, *Photorhabdus luminescens* (47/57) YscK, *Yersinia* spp. (46/59) PscK, *Pseudomonas aeruginosa* (42/54)
AscL (PscL)	Unknown	221	24.71	5.20	LscL, *Photorhabdus luminescens* (73/86) YscL, *Yersinia* spp. (68/84) PscL, *Pseudomonas aeruginosa* (61/77)

otic cell lines (epithelioma papillosum of carp [EPC] or HEp-2 cells) in comparison with the wild-type strain. At 2 h postinfection with the wild-type strain (AH-3 or the rifampin-resistant mutant), approximately 50% of the eukaryotic cells from the monolayer (EPC or HEp-2 cells) became rounded and were detached from the well. At the same time after infection no morphological changes in the eukaryotic cells (EPC or HEp-2 cells) were observed when inoculated with mutant AH-3AscV; however, some morphological changes and detachment (<50%) were observed after 4 h postinfection. No complementation with the plasmid vector alone (pACYC184) was achieved, while a full complementation (restoring the same lethal doses and cytotoxic effects as those of the wild-type strain) was obtained with the plasmid vector with the complete *ascV* gene. Data were analyzed by a one-way analysis of variance; P values of <0.05 were considered significant.

TABLE 2. Distributions by dot blotting of TTSS genes in clinical and environmental mesophilic *Aeromonas* strains

Species	Strain type	No. of positive strains/no. of strains tested		
		ascN to *ascT*	*ascV*	*ascC* to *ascJ*
A. hydrophila	Clinical	28/35	28/35	28/35
	Environmental	4/25	4/25	4/25
A. veronii	Clinical	32/40	32/40	32/40
	Environmental	9/20	9/20	9/20
A. caviae	Clinical	5/40	5/40	5/40
	Environmental	4/20	4/20	4/20
A. jandaei	Clinical	1/2	1/2	1/2

This is the first report of the complete TTSS DNA sequence of an *Aeromonas* strain (*A. hydrophila* AH-3) that comprises 35 genes. From the results obtained using the different DNA probes, which cover the entire TTSS region, it seems logical to conclude that the mesophilic *Aeromonas* strains that possess TTSS genes have a complete TTSS. Due to the fact that some of the mesophilic *Aeromonas* strains tested do not show any plasmid DNA and that the G+C content of the TTSS is in the range of the genomic G+C content of the genus, we suggest

that the TTSS on mesophilic *Aeromonas* strains is located in the chromosome and not on a plasmid as in *A. salmonicida* (1, 2, 21), as with the *A. hydrophila* AH-1 TTSS (22). As in the clinical strains with other pathogenic features, this characteristic virulence trait (the presence of TTSS) is more frequent in *A. veronii* and *A. hydrophila* (80% of strains) than in *A. caviae* (13% of strains) (Table 2). Finally, to judge by the results obtained with mutant AH-3AscV, the *A. hydrophila* AH-3 TTSS is required for the virulence of this strain. Since the bacterium's virulence is known to be multifactorial in many cases, the presence of TTSS in *Aeromonas* strains seems to be an important factor related to their pathogenicity.

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