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Type III Secretion System Genes in Clinical *Aeromonas* Isolates

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We have identified the genes *ascF* and *ascG*, which encode components of a putative type III secretion system (TTSS) in *Aeromonas*. We investigated the distribution of these and other TTSS genes in 84 clinical isolates and found hybridizing sequences in 50% of the strains, with a higher prevalence in *Aeromonas hydrophila* and *Aeromonas veronii* than in *Aeromonas caviae*.

Aeromonas spp. comprise mesophilic motile and psychrophilic nonmotile gram-negative bacteria. They can be found in both fresh and salt water and are also common in foodstuffs (2). They cause a wide variety of human infections, including septicemia, wound infections, meningitis, pneumonia, and gastroenteritis (10, 17). Three of the 15 species in the genus (i.e., *Aeromonas veronii*, *Aeromonas caviae*, and *Aeromonas hydrophila*) account for more than 85% of the clinical isolates (17).

In addition to several virulence factors' having been investigated in *Aeromonas* (7, 8, 16, 19, 24, 27, 31, 32), genes for a putative type III secretion system (TTSS) were recently identified in this genus (4, 5, 25). The TTSS is common in pathogenic strains of gram-negative bacteria (enteropathogenic *Escherichia coli*, *Salmonella enterica*, *Shigella flexneri*, *Yersinia* spp., and *Pseudomonas aeruginosa*), and the cluster of genes encoding it is frequently included in genomic regions called pathogenicity islands (13, 30). The TTSS plays an essential role in pathogenicity because it facilitates the delivery of toxins directly into the host cells (15, 20, 22, 30). Burr et al. (4, 5) identified, in the fish pathogen *Aeromonas salmonicida* subsp. *salmonicida*, several TTSS genes homologous to those already described for the TTSS of pathogenic *Yersinia* species and identified the toxin AexT, secreted by this system. Inactivation by mutagenesis of two TTSS genes rendered the strain nontoxic for cultured fish cells, indicating that TTSS has an important role in virulence (4, 5).

In the present study, 84 clinical strains of the three most common pathogenic *Aeromonas* species (i.e., *A. veronii*, *A. caviae*, and *A. hydrophila*) were screened for TTSS genes.

The strains were recovered from diarrhea of patients with gastroenteritis ($n = 54$) and from extraintestinal infections (ulcers, $n = 1$; cellulitis and abscesses, $n = 4$; urine, $n = 6$; joint fluids, $n = 5$; and blood, $n = 14$). The strains were isolated from thioglycolate broth, from blood agar supplemented or not with ampicillin, or from cefsulodin-Irgasan-novobiocin agar and were confirmed as belonging to *Aeromonas* by conventional biochemical methods. All strains were cultured on Trypticase soy agar at 30°C and identified to species level by the 16S ribosomal DNA restriction fragment length polymorphism technique (2, 11). The genomic DNA of a blood strain of *A. veronii* (283c) was extracted by the phenol-chloroform method according to general protocols (23). Oligonucleotide primers (ASCV-fwd [5′-ATG GAC GGC GCC ATG AAG TT-3′] and ASCV-rev [5′-TAT TCG CCT TCA CCC ATC CC-3′]) were designed based on the previously reported *A. salmonicida ascV* sequence (5) to amplify a homologous 710-bp region of strain 283c. In addition, a genomic library of this strain was constructed with the SuperCos I cosmid vector kit (Stratagene, La Jolla, Calif.) following the manufacturer's instructions. This library was screened by colony blotting using as a probe the PCR-amplified fragment labeled with digoxigenin by following standard procedures and protocols (6, 23). From a positive clone, the cosmid DNA was isolated and digested with *Eco*RI and *Hin*dIII, and the fragments obtained were probed again by Southern blot hybridization. A 10-kb hybridizing fragment was subcloned into the plasmid vector pBR322, from which a 1,025-bp fragment was sequenced on an ABI-PRISM 310 genetic analyzer (Applied Biosystems, Foster City, Calif.). DNA sequences and their corresponding amino acid sequences were compared with sequences in the EMBL/GenBank databases by using BLAST (1). The molecular weight (MW) and theoretical isoelectric point (pI) of the TTSS proteins were calculated with the ExPASy ProtParam tool (http://us.expasy.org).

For the detection of TTSS genes by dot blotting, 10 μg of genomic DNA of each of the above-mentioned strains and of *A. salmonicida* CECT 894^T was dotted onto three membranes as described earlier (6). Each membrane was hybridized at 50°C with a digoxigenin-PCR-labeled probe, generated by using the DNA of strain 283c. The probes corresponded to the *aexT* gene (a 535-bp fragment obtained with the primers and conditions described by Braun et al. [3]) and to the following TTSS genes: *ascV* (a fragment of 710 bp generated with the primers indicated above) and *ascF-ascG* (a fragment of 900 bp generated with the primers ASCF-G-fwd [5′-ATG AGG TCA TCT GCT CGC GC-3′] and ASCF-G-rev [5′-GGA GCA CAA CCA TGG CTG AT-3′]). The strain *A. salmonicida* CECT 894^T was used as positive control for *ascV* and *aexT*. Fisher's exact test was used to compare the results obtained for different species and for strains of intestinal or extraintestinal origin, using the Statistical Package for Social Sciences (v. 9.0; SPSS

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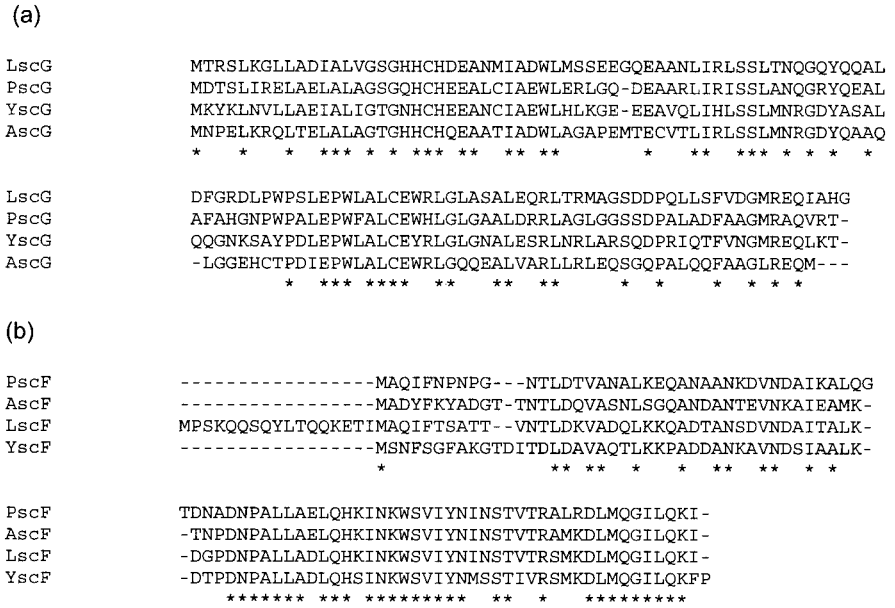

FIG. 1. Protein sequence alignment of *A. veronii* AscG (a) and AscF (b) (AY289105) with related proteins from *Y. enterocolitica* (YscG [NP_783692] and YscF [NP_863538]), *Pseudomonas aeruginosa* (PscG [ZP_00139363] and PscF [NP_250410]), and *Photorhabdus luminescens* (LscG [AAO18030] and LscF [AAO18031]). *, conserved amino acid. Sequences are arranged according to CLUSTAL W similarities.

Inc., Chicago, Ill.). A P value of <0.05 was considered statistically significant.

Two open reading frames encoding two novel TTSS proteins, AscF and AscG, were recognized in the sequenced fragment. The main features of such proteins were 113 amino acids, an MW of 12,500, and a theoretical pI of 5.06 for the former and 85 amino acids, an MW of 9,400, and a pI of 5.63 for the latter. These proteins showed the highest identity (Fig. 1) with those described in the TTSS of *Yersinia enterocolitica* (YscF and YscG), *Photorhabdus luminescens* (LscF and LscG), and *Pseudomonas aeruginosa* (PscF and PscG). In the case of AscF the predicted protein sequence had 72% identity and 80% similarity to LscF of *P. luminescens*, with slighter lower values for PscF of *P. aeruginosa* and YscF of *Y. enterocolitica*. These three proteins were highly homologous at the C-terminal region (Fig. 1). The AscG protein presented identity ranging from 51 to 56% and similarity ranging from 62 to 68% with those of the three above-mentioned microorganisms. In *Yersinia pestis*, the YscG and YscF proteins have a role in secretion and regulation of the high level of expression of low-calcium-response proteins (21). The YscG is a putative chaperone that directly binds to YscE, which is a cytoplasmic component of the TTSS (9).

The TTSS is considered a virulent trait that correlates with bacterial pathogenicity, and its presence can be used as a general indicator of virulence (26, 29). The distribution pattern of the TTSS genes (*ascF-ascG* and *ascV*) and of the gene that codes for the AexT toxin in strains of the most common pathogenic species of *Aeromonas*, i.e., *A. veronii*, *A. caviae*, and *A. hydrophila* (Table 1), varied depending on the species and strain origin (intestinal or extraintestinal). Globally, 50% of *Aeromonas* isolates presented these genes. The low incidence of these genes in *A. caviae* is remarkable and significantly different from the incidence in the other species ($P < 0.05$) (Table 1). This agrees with the low virulence reported for this species (12, 14, 18, 28). In contrast, all extraintestinal isolates of *A. veronii* had these genes, which were also present in 86% of the extraintestinal isolates of *A. hydrophila* (Table 1). The TTSS genes were less prevalent in intestinal strains of the latter two species (69 and 78%, respectively). In general, there were no significant differences between the presence of such genes in extraintestinal and intestinal strains ($P = 0.297$). The *ascF-ascG* and *ascV* genes were always found concomitantly, which is not surprising given that they are located in tandem on the TTSSs of other microorganisms (15). The association of *Aeromonas* species with gastrointestinal disease remains unresolved, because only certain strains appear to produce enteric disease (17). However, we have detected the presence of sequences hybridizing putative TTSS genes in 50% of randomly selected clinical strains. This raises the possibility that *Aero-*

TABLE 1. Distribution of TTSS genes within *Aeromonas* species of clinical relevance[a]

Species	No. of strains and origin	No. (%) of strains positive by dot blot assay for:		
		ascF-ascG	*ascV*	*aexT*
A. hydrophila	18; intestinal	14 (78)	14 (78)	14 (78)
	7; extraintestinal	6 (86)	6 (86)	6 (86)
A. veronii	16; intestinal	11 (69)	11 (69)	11 (69)
	9; extraintestinal	9 (100)	9 (100)	9 (100)
A. caviae	20; intestinal	0 (0)	0 (0)	0 (0)
	14; extraintestinal	2 (14)	2 (14)	2 (14)

[a] The presence of TTSS genes in *A. caviae* was significantly different from that in the other species ($P < 0.001$). No significant differences were obtained between intestinal and extraintestinal strains ($P = 0.297$). *A. salmonicida* CECT 894^T was positive for all genes studied.

monas may be a gastrointestinal pathogen with the ability to produce a systemic disease under the proper conditions.

Braun et al. (3) characterized an ADP-ribosylating protein (AexT) derived from *A. salmonicida* subsp. *salmonicida* that showed an in vitro cytotoxic effect on fish cells and was highly similar to the ExoS and ExoT toxins secreted by TTSS in *Pseudomonas aeruginosa*. Later, Burr et al. (4, 5) demonstrated that this protein was secreted by the TTSS. In the present study, we found that the gene that codes for AexT was present in the same strains that possessed the TTSS genes studied (Table 1). Considering the loss of toxicity encountered in mutants with inactivated TTSS genes in *A. salmonicida* (4, 5), the presence of the TTSS in clinical strains suggests that they may possess a similar virulence capacity.

In fact, the discovery of TTSS and toxin genes in such a high proportion of clinical strains could raise these microorganisms to the category of primary human pathogens along with *Y. enterocolitica*, *Salmonella enterica*, enteropathogenic *E. coli*, and *Shigella flexneri*. Further characterization, by sequencing, of the TTSS in *Aeromonas* is ongoing and will enable its full comparison with the TTSS of other microbes. This is key information for a better understanding of the pathogenicity potential and virulence mechanisms of *Aeromonas*.

Nucleotide sequence accession number. The sequence of the genes (*ascF-ascG*) was deposited in GenBank under accession number AY289105.

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ERRATUM

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Volume 42, no. 3, p. 1285–1287, 2004. Page 1287, column 1, line 26: "AY289105" should read "AY289195."