First evidence of the existence of Type III secretion system in clinical strains of *Aeromonas*

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ABSTRACT

Two genes (*asc*F and *asc*G) found in the Type III secretion system (TTSS) of diverse gram-negative pathogens are here described for the first time on a clinical isolate of *Aeromonas veronii*. The distribution of those genes and of the *asc*V and *aex*T, genes previously described in a fish pathogen strain of *A. salmonicida*, were investigated by dot blot in 84 clinical isolates of *A. hydrophila*, *A. veronii* and *A. caviae* revealing that over 86% of extraintestinal isolates of the former two species presented those genes.

Aeromonas spp. comprise mesophilic motile and psychrophilic nonmotile gram-negative bacteria. This micro-organism 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 (Figueras et al., 2000a; Janda, 2001). Out of the 15 reported species within the genus, three of them *A. veronii*, *A. caviae* and *A. hydrophila* represents more of the 85% of the clinical isolates (Janda and Abbott, 1998; Joseph and Carnahan, 2000).

It is well established that *Aeromonas* possess several virulence factors (Chopra and Houston, 1999; Janda 2001, Gavin et al., 2002; Chacon et al., 2003) for which its function in pathogenesis have been investigated (Wong et al. 1998; Xu et al., 1998; Vipond et al., 1998; Merino et al., 1999; Sha et al., 2002). However, it has been demonstrated that the pathogenic capacity of numerous gram-negative bacteria (*Escherichia coli*, *Salmonella enterica*, *Shigella flexneri*, *Yersinia* spp. and *Pseudomonas aeruginosa*) is due to large genomic regions called pathogenicity islands (PIs), that frequently include the cluster of genes that codify for the type III secretion systems (TTSS) (Groisman and Ochman, 2000; Winstanley and Hart, 2001). The TTSS plays an essential role in pathogenicity because facilitates the delivery of toxins directly into the host cells (Hueck, 1998; Muller et al., 2001, Winstanley and Hart, 2001; Ramamurthi and Schneewind, 2002).

The PIs have not yet been investigated in *Aeromonas*, however the existence of TTSS in one strain of the fish pathogen *A. salmonicida* subsp. salmonicida has recently been described (Burr et al., 2002; Braun et al. 2002). Burr et al. (2002) have identified 8 TTSS genes homologues to those already described in the TTSS of *Yersinia enterocolitica* and identified one toxin (Aext) secreted by this system. The inactivation by mutagenesis of one of the 8 genes, the *asc*V, rended the strain no toxic on RTG-2 rainbow trout gonad cells, indicating that TTSS has an important role in the virulence of this pathogen (Burr et al., 2002).

Here we report two new genes *asc*F and *asc*G as part of the TTSS in a strain of *Aeromonas* v*eronii* of extraintestinal origin. In addition, we have investigated the distribution of those 2 genes (*asc*F-G), of the gene *asc*V and of the one that codify for the toxin Aext on a group of 84 clinical strains belonging to the most frequently isolated clinical species (*A. veronii*, *A. caviae* and *A. hydrophila*).

A cosmid library was constructed with the SuperCos I Cosmid Vector Kit (Stratagene, California, USA) by using genomic DNA from a strain of *A. veronii* (283c), isolated from blood of a patient. An amplified product of 710 bp from this strain, corresponding to part of the *asc*V gene, was obtained using the following primers: ascV-Forward: $^{5'}$ATG GAC GGC GCC ATG AAG TT$^{3'}$ and ascV-Reverse: $^{5'}$TAT TCG CCT TCA CCC ATC CC$^{3'}$ and the PCR amplifications conditions already described (Chacón et al. 2003) but using an annealing temperature, adjusted to the primers, of 56°C. The library was screened using the *asc*V 710 bp fragment as a probe, which was labelled with digoxigenin by repeating the PCR reaction with 0.5 nmol of Dig-11-dUTP in the total reaction mixture, following procedures and standard protocols previously described (Sambrook et al., 1987; Chacón et al. 2002). From a clone which gave a positive signal, the cosmid DNA was isolated and digested with the enzymes *Eco*RI and *Hind*III. A 10 Kb hybridizing fragment with the *asc*V probe identified by Southern blot analysis (Southern, 1992) was subcloned into the plasmid vector pBR322 and a fragment of 1025 bp was sequenced on an ABI-PRISM^{TM} 310 apparatus following manufacturer's instructions. Comparisons of DNA sequences and their corresponding amino acid sequences in the EMBL/GenBank were performed with BLAST analysis. The molecular mass (MW) and isoelectric point (pI) of the TTSS proteins were calculated with the ExPASy ProtParam Tool (http://us.expasy.org). The nucleotide sequences for the two reported genes in this communication (*asc*F-G) have been submitted to the GenBank database under the following accession number AY289105.

For the dot blot experiments we selected 84 *Aeromonas* of the species: *A. hydrophila* (n=25), *A. veronii* (n=25) and *A. caviae* (n=34), all identified by 16S rDNA-RFLP method (Borrell et al. 1997; Figueras et al., 2000b) with the following origins: stools of patients with diarrhoea (n=54), wounds and ulcers (n=4), cellulites (n=1), abscesses (n=2), urine (n=4), articulate liquid (n=2) and blood (n=15). Ten μg of DNA of each strain was dotted onto 3 different nylon membranes, which were hybridized and detected as previously described (Chacón et al., 2002) with digoxigenin-PCR labelled probes of: *asc*V gene (710 bp), *asc*F-G genes (a fragment of 900 bp generated with the primers ascF-G Forward: $^{5'}$ATG AGG TCA TCT GCT CGC GC$^{3'}$ and AscF-G Reverse: $^{5'}$ GGA GCA CAA CCA TGG CTG AT$^{3'}$), and with *aex*T gene (a 535 bp fragment obtained with the primers and conditions already described by Braun et al. 2002).

A BLASTX analysis of the 1025 bp Open Reading Frame sequence revealed the presence of 2 putative proteins named AscF and AscG, following the nomenclature used for the designation of the TTSS in *A. salmonicida* subsp. salmonicida (Burr et al., 2002). These proteins showed the best homologies to those described in the TTSS of *Yersinia enterocolitica*, *Protorhabdus luminescens* and *Pseudomonas aeruginosa* (Table 1). For AscF, *P. luminescens* LscF protein had the closest identity and similarity, 72 and 80% respectively, followed by PscF of *P. aeruginosa* and YscF of *Y. enterocolitica*. The three proteins exhibited strong homologies at the C-terminal region (Figure 1). The AscF protein has 85 aminoacids with a MW of 9.4 kDa and a theoretical pI of 5.63 (Table 2). The AscG protein presented lower identity and similarity among the above 3 mentioned closest micro-organism ranging from 51 to 56% of identity and 62-68% of similarity (Table 1). The AscG protein has 113 amionoacids with 12.5 kDa of MW and pI of 5.6. The characteristics of these proteins such pI and MW is very similar to their homologous suggesting that the role of those putative proteins in *Aeromonas* may be similar that in those micro-organims. In *Y. pestis*, both proteins, YscG and YscF, have been postulated to have a role in secretion and regulation of high level of expression of low calcium response proteins (Plano and Straley, 1995). Recent data supports that in *Y. pestis*, the YscG is a Syc-like chaperone that directly binds to YscE, that is a cytoplasmatic component of the TTSS (Day et al., 2000).

TTSS is a virulent trait that correlate with bacterial pathogenicity and their presence can be used as a general indicator of virulence (Winstanley and Hart, 2000; Stuber et al., 2003). The detection by dot blot hybridisation experiments of 3 genes that are part of the TTSS in 84 isolates belonging to the 3 clinical *Aeromonas* most relevant

species is shown in Table 3. *Aeromonas caviae* intestinal isolates had no presence of TTSS genes and only 14% of the extraintestinal isolates were positive for the genes tested. The low incidence of the TTSS genes in strains of *A. caviae* is in concordance to the lowest virulence associated to this species in the literature (Granum y cols., 1998; Kingombe et al, 1999; Heuzenroeder et al., 1999; Wang et al., 2003). All extraintestinal isolates of *A. veronii* presented the genes tested versus 86% of *A. hydrophila* isolates. Intestinal strains of *A. hydrophila* and *A. veronii* were more variable showing less prevalence of the TTSS genes; while the *asc*F-G and *asc*V were present in similar percentages within the same species. This is not rare because these genes have been located in tandem on the genome (Hueck 1998).

One of the major goals in further characterising TTSS in *Aeromonas* is to identify potential secreted toxins by this system. Braun et al. (2002) have characterised an ADP-ribosylating protein (AexT) derived from *A. salmonicida* subsp. salmonicida which has a significant similarity to the ExoS and the related ExoT toxins secreted by TTSS in *Pseudomonas aeruginosa*. It has been demonstrated that AexT is secreted via the TTSS and that it has an important role in virulence. In the present study we have demonstrated that the gene that codify for this protein, the *aex*T, was present in clinical isolates of *Aeromonas* in similar proportion than the TTSS genes studied (Table 3). These data demonstrated, for the first time, the presence of TTSS associated genes in clinical isolates of *Aeromonas*. The evidence of the existence of TTSS in clinical strains of *Aeromonas* is important, since raises this micro-organism to the same category of other classical pathogens, i.e. *Y. enterocolitica*, *S. enterica*, *E. coli* and *S. flexneri*. Further characterisation of the TTSS in *Aeromonas* is now ongoing. The complete sequencing of the TTSS in *Aeromonas* will be important for the generation of mutants that can be tested on lethal dose experiments, this information will provide the real implication of TTSS in the virulence of *Aeromonas* and will help to find new toxins secreted by the TTSS. This information will be important to get a better understanding of the virulence mechanisms of this pathogen.

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(a)

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LscG            MTRSLKGLLADIALVGSGHHCHDEANMIADWLMSSEEGQEAANLIRLSSLTNQGQYQQAL
PscG            MDTSLIRELAELALAGSGQHCHEEALCIAEWLERLGQ-DEAARLIRISSLANQGRYQEAL
YscG            MKYKLNVLLAEIALIGTGNHCHEEANCIAEWLHLKGE-EEAVQLIHLSSLMNRGDYASAL
AscG            MNPELKRQLTELALAGTGHHCHQEAATIADWLAGAPEMTECVTLIRLSSLMNRGDYQAAQ
                *   *   *  *** * * *** **  ** **       *   **  *** * * *  *

LscG            DFGRDLPWPSLEPWLALCEWRLGLASALEQRLTRMAGSDDPQLLSFVDGMREQIAHG
PscG            AFAHGNPWPALEPWFALCEWHLGLGAALDRRLAGLGGSSDPALADFAAGMRAQVRT-
YscG            QQGNKSAYPDLEPWLALCEYRLGLGNALESRLNRLARSQDPRIQTFVNGMREQLKT-
AscG            -LGGEHCTPDIEPWLALCEWRLGQQEALVARLLRLEQSGQPALQQFAAGLREQM---
                        *  *** ****  **   **  **     *  *    *  * * *
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(b)

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PscF            -----------------MAQIFNPNPG---NTLDTVANALKEQANAANKDVNDAIKALQG
AscF            -----------------MADYFKYADGT-TNTLDQVASNLSGQANDANTEVNKAIEAMK-
LscF            MPSKQQSQYLTQQKETIMAQIFTSATT--VNTLDKVADQLKKQADTANSDVNDAITALK-
YscF            -----------------MSNFSGFAKGTDITDLDAVAQTLKKPADDANKAVNDSIAALK-
                                 *             ** **  *   *  **  **  * *

PscF            TDNADNPALLAELQHKINKWSVIYNINSTVTRALRDLMQGILQKI-
AscF            -TNPDNPALLAELQHKINKWSVIYNINSTVTRAMKDLMQGILQKI-
LscF            -DGPDNPALLADLQHKINKWSVIYNINSTVTRSMKDLMQGILQKI-
YscF            -DTPDNPALLADLQHSINKWSVIYNMSSTIVRSMKDLMQGILQKFP
                    ******* *** *********  **  *   *********
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Figure 1. Protein sequence alignment of *Aeromonas veronii* AsG (a) and AscF (b) sequences with related proteins from *Yersinia enterocolitica*, *Pseudomonas aeruginosa and Protorhabdus luminescens*. *Conserved aminoacid

Table 1. Comparison between predicted Type III *A. veronii* proteins with homologues from other miro-organisms

A. veronii protein	Homologue (micro-organism)	Predicted location/properties	% Identity	% Similarity
AscG	YscG (*Yersinia enterocolitica*)	Cytoplasmatic or peripheral membrane proteins /Syc-like chaperone (Day et al. 2000)	56	68
	LscG (*Protorhabdus luminescens*)	Unknown	53	62
	PscG (*Pseudomonas aeruginosa*)	Unknown	51	62
AscF	LscF(*Protorhabdus luminescens*)	Unknown	72	80
	PscF (*Pseudomonas aeruginosa*)	Unknown	70	78
	YscF(*Yersinia enterocolitica*)	Cytoplasmatic (Huek, 1997)/Unknown	62	76

Table 2. Properties of *A. veronii* proteins

A. veronii protein	No of residues	Isoelectric point	Molecular mass(kDa)
AscG	113	5.06	12.5
AscF	85	5.63	9.4

Table 3. Distribution of TTSS genes within *Aeromonas* species of clinical relevance

	No of strains and origin	Dot blot assay		
		*asc*F-G	*asc*V	*aex*T
A. hydrophila	18 intestinal	14 (77%)	14 (77%)	12 (66%)
	7 extraintestinal	6 (86%)	6 (86%)	6 (86%)
A. veronii	16 intestinal	11 (69%)	11 (69%)	10 (63%)
	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%)