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NOTE

Association of Type III secretion genes with virulence of *Aeromonas salmonicida* subsp. *salmonicida*

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ABSTRACT: *Aeromonas salmonicida* subsp. *salmonicida* possesses a number of potential virulence factors, including a recently identified plasmid-encoded Type III secretion system. A number of field isolates of *A. salmonicida* subsp. *salmonicida* were examined for the presence of Type III secretion genes. Using *in vitro* experiments, it was found that field isolates containing such genes are cytotoxic to fish cell lines, whereas those that lack these genes are not. Using a rainbow trout *in vivo* model, the virulence of a wild type *A. salmonicida* subsp. *salmonicida* strain (Strain JF2267), which possesses Type III secretion genes, was compared to that of a laboratory derivative of the same strain that has lost these genes. While Strain JF2267 was virulent towards rainbow trout, its derivative was not. The *A. salmonicida* subsp. *salmonicida* Type Strain ATCC 33658^T, which also lacks Type III secretion genes, was also found to be avirulent by this challenge model. The findings from both the *in vitro* and *in vivo* experiments suggest that the presence of Type III secretion genes is associated with the virulence of this important fish pathogen.

KEY WORDS: *In vivo* · *In vitro* · Virulence · Furunculosis · Type III protein secretion

INTRODUCTION

Aeromonas salmonicida subsp. *salmonicida* is one of the most extensively studied fish pathogens due to its widespread distribution and its economic impact on aquaculture. This bacterium is the causative agent of furunculosis, a systemic disease primarily affecting salmonids. In spite of the fact that *A. salmonicida* was first identified over a century ago (Emmerich & Weibel 1894), very little is known about its virulence mechanisms. A number of potential virulence factors are produced by *A. salmonicida* subsp. *salmonicida*. These include bacterial surface structures such as the surface layer or A-layer protein (Chu et al. 1991), lipopolysaccharide (Lee & Ellis 1990) and Type IV pili (Masada et al. 2002). The bacterium also secretes a number of extracellular proteins, including haemolysins (Nomura et al. 1988, Hirono & Aoki 1993) and proteases (Whitby et al. 1992). A Type III secretion system (TTSS) has also recently been identified in *A. salmonicida* subsp. *salmonicida* Strain JF2267, a strain originally isolated from an arctic char *Savelinus alpinus* presenting typical furunculosis symptoms (Braun et al. 2002, Burr et al. 2002).

TTSSs are important virulence attributes of several Gram-negative bacteria, including *Salmonella typhimurium*, *Shigella flexneri*, *Escherichia coli* and the pathogenic *Yersinia* species. These secretion systems function by moving effector molecules, produced in the bacterial cytosol, across both the inner and outer bacterial membranes, and directly into the cytosol of target eukaryotic cells. Once in the target cell, the effector molecules are able to disrupt the cytoskeleton and modulate cell-signalling cascades (for a comprehensive review see (Cornelis & Van Gijsegem 2000).

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In *Aeromonas salmonicida* subsp. *salmonicida*, the genes encoding the TTSS are found as a gene cluster on a large plasmid of approximately 140 kb (Stuber et al. 2003a). In this study, we examined field isolates of *A. salmonicida* subsp. *salmonicida* for the presence of these TTSS genes.

MATERIALS AND METHODS

A summary of all *Aeromonas salmonicida* subsp. *salmonicida* strains used in this study is provided in Table 1. Strains were cultured on Luria-Bertani (LB) agar plates at 18°C.

Total DNA was extracted using the guanidium hydrochloride method (Pitcher et al. 1989). Restriction digestion with *Bam*HI and Southern blot hybridization was carried out using standard methods (Ausubel et al. 1999). For the production of a digoxigenin-11-dUTP (DIG)-labeled probe against *ascV*, PCR was carried out in the presence of 40 µM DIG (Roche Diagnostics) using Primers AslcrD-L (GCCCGTTTTGCCTATCAA) and AslcrD-R (GCGCCGATATCGGTACCC) and 20 ng DNA from Strain JF2267 as a template.

Epithelioma papulosum cyprini cells (EPC; ECACC 93120820) and rainbow trout *Oncorhynchus mykiss* gonad cells (RTG-2; ATCC CCL-55) were grown as described previously (Braun et al. 2002). Two days before infection, the cells were trypsinized and seeded into 24-well culture plates. Monolayered cells (8×10^5 EPC cells or 6×10^5 RTG-2 cells per 2 cm^2 well in 1 ml supplemented medium in each well) were infected with *Aeromonas salmonicida* subsp. *salmonicida* suspended in phosphate buffered saline (PBS), pH 7.4, at a multiplicity of infection of 20:1 (bacteria:fish cells). The addition of PBS, pH 7.4, to fish cells was used as a negative control. Following infection at 18°C, cells were monitored under light microscopy for signs of cell rounding and retraction normally induced by the wild type (wt) Strain JF2267.

Table 1. *Aeromonas salmonicida* subsp. *salmonicida* strains. ATCC: American Type Culture Collection

Strain	Origin	Source or Reference
CC-23	Norway	William Kay[a]
CC-24	Norway	William Kay[a]
CC-27	Norway	William Kay[a]
CC-29	Scotland	William Kay[a]
CC-63	Canada	William Kay[a]
CC-72	Canada	William Kay[a]
SS70.1	Laboratory derivative	Hackett et al. (1984)
JF2267	Switzerland	Braun et al. (2002)
JF2397	Derived from JF2267	Stuber et al. (2003a)
ATCC 33658^T	Type strain	ATCC

[a]Microtek International, Saanichton, British Columbia, Canada

A total of 160 six mo old rainbow trout of 7.5 ± 1.5 cm total length were randomly distributed in groups of 20 fish and stocked in aerated PVC-tanks of 13 l volume. Flow through of tap water was set at 0.6 l min^{-1} and the temperature range during the experiment was 18 ± 0.8°C. The trout were fed a commercial fish diet at 1% body weight d^{-1}. Fish were acclimated to the tanks for 2 wk before the experiment began. *Aeromonas salmonicida* subsp. *salmonicida* Strains JF2267, JF2397 and ATCC 33658^T were grown on LB agar plates for 2 d at 18°C. Bacterial suspensions were then prepared in PBS, pH 7.4, to a bacterial concentration of 1×10^5 colony forming units (cfu) 50 μl^{-1}. The fish (2 tanks per group) were then intraperitoneally injected with 50 µl of the appropriate bacterial suspension. A control group was inoculated with 50 µl PBS only. Mortality was recorded daily, or in cases of high mortality, twice per day, for 3 wk. Dead fish were investigated for the presence of *A. salmonicida* subsp. *salmonicida*, as were 5 of the surviving fish from each tank (where available) at the end of the experiment.

RESULTS

Evidence for Type III secretion genes in field isolates of Aeromonas salmonicida subsp. salmonicida. A number of field strains of *A. salmonicida* subsp. *salmonicida* (Table 1) were examined for the presence of the TTSS gene *ascV* using restriction digestion and Southern blot hybridization of total DNA. Five of the 7 field isolates we examined hybridized with the *ascV* probe (Isolates CC-23, CC-27, CC-29, CC-63 and CC-72; Fig. 1), indicating that they possess this gene and by extension, a TTSS (Stuber et al. 2003). Strain JF2267, which was already known to possess a functional TTSS (Burr et al. 2002), also gave a positive signal. In contrast, the Field Strain CC-24 did not hybridize with the *ascV* probe, nor did Strain SS70.1, suggesting a TTSS is absent in these strains. Likewise, Strains JF2397 (a laboratory strain derived from Strain JF2267 by repeated passaging at 25°C) and ATCC 33658^T (the *A. salmonicida* subsp. *salmonicida* type strain) did not react with the *ascV* probe; this result was expected as these 2 strains have previously been shown to lack the plasmid that codes for the TTSS (Stuber et al. 2003a).

Association of Type III secretion genes with cytotoxicity towards fish cells. A fish-cell infection model was used to determine whether the presence of such secretion genes could be correlated with virulence. *Aeromonas salmonicida* subsp. *salmonicida* strains

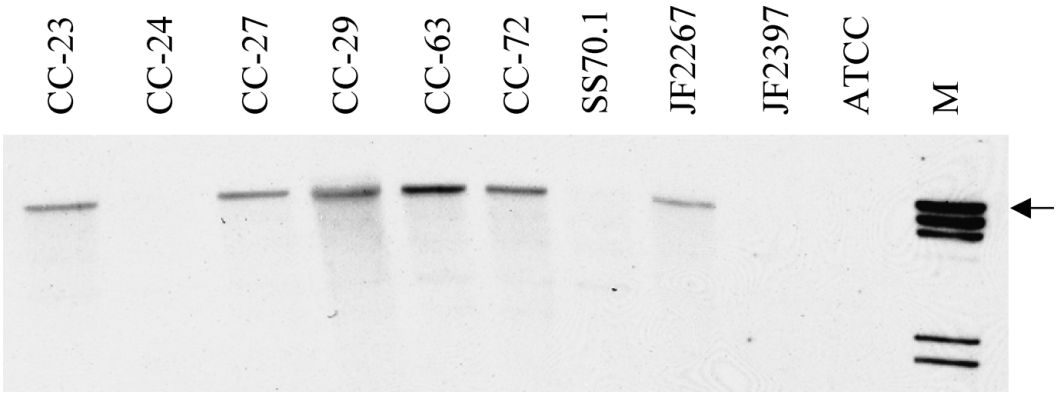

Fig. 1. *Aeromonas salmonicida* subsp. *salmonicida*. Evidence for Type III secretion genes in isolates. Total DNA extracted from *A. salmonicida* subsp. *salmonicida* strains and digested with *Bam*HI was analyzed by Southern blot using a digoxigenin-11-dUTP (DIG)-labeled probe against the Type III secretion gene *ascV*. Strain names are indicated; ATCC refers to Type Strain ATCC 33658^T. Lane M contains lamda phage DNA digested with *Hind*III; fragment sizes (from top to bottom) are 23.1 (marked by arrow), 9.4, 6.5, 2.3, and 2.0 kb

were tested for cytotoxicity against EPC and RTG-2 fish cell lines; the results are summarized in Table 2. The Field Strains CC-23, CC-27, CC-29, CC-63 and CC-72 were toxic for both the EPC and RTG-2 cell lines, as evidenced by induction of cell rounding and detachment from the plastic support. These morphological changes could be detected as little as 2 h following inoculation, and were also apparent in the Control Strain JF2267. In contrast, infection with Strains CC-24 and SS70.1 did not result in any morphological changes; 6 h following infection, fish cells inoculated with these strains looked similar in appearance to the control cells that were inoculated with PBS only.

Table 2. *Aeromonas salmonicida* subsp. *salmonicida* infecting *Epithelioma papulosum cyprini* cells (EPC) or rainbow trout *Oncorhynchus mykiss* gonad cells (RTG-2). PBS was used as a negative control; cells were inoculated with PBS only. +: infection resulted in cell-rounding and retraction; –: infection resulted in no morphological changes; NA: not applicable

Strain	Type III Secretion genes	Toxicity EPC	Toxicity RTG-2
CC-23	+	+	+
CC-24	–	–	–
CC-27	+	+	+
CC-29	+	+	+
CC-63	+	+	+
CC-72	+	+	+
SS70.1	–	–	–
JF2267	+	+	+
JF2397	–	–	–
ATCC 33658^T	–	–	–
PBS	NA	–	–

Strains JF2397 and ATCC 33658^T were also non-toxic towards both cell lines. In each case, the strains that displayed toxicity towards the EPC and RTG-2 cells were those that were found to possess TTSS genes. Those strains that did not have a toxic effect towards the fish cell lines lacked these genes. This result suggests that the presence of TTSS genes in *A. salmonicida* subsp. *salmonicida* is associated with cytotoxicity towards fish cells.

Association of Type III secretion genes with virulence towards rainbow trout. To assess the possibility that the presence of TTSS genes is associated with virulence *in vivo*, we utilized a rainbow trout infection model to compare the virulence of the wt isolate (JF2267), which possesses TTSS genes, to its derivative strain (JF2397), which lost the plasmid that encodes the TTSS genes following laboratory cultivation at 25°C. Strain ATCC 33658^T was also included in the assay.

The results, displayed in Fig. 2, indicate that the onset of mortality in fish infected with the wt Strain JF2267 began 2 d following infection. Within 3 d, all fish (in both tanks) that had been injected with this strain had died. The dead fish showed only minor macroscopic alterations, such as slight haemorrhages in the perivisceral fat and the intestines, a swollen vent and protruding anus. These symptoms correspond to those described by Hiney & Olivier (1999) for acute furunculosis. *Aeromonas salmonicida* subsp. *salmonicida* could be cultured from all of these dead fish. In contrast, no mortalities occurred in any of the fish injected with the laboratory derivative JF2397 or Strain ATCC 33658^T (Fig. 2). Furthermore, *A. salmonicida* subsp. *salmonicida* could not be isolated from any

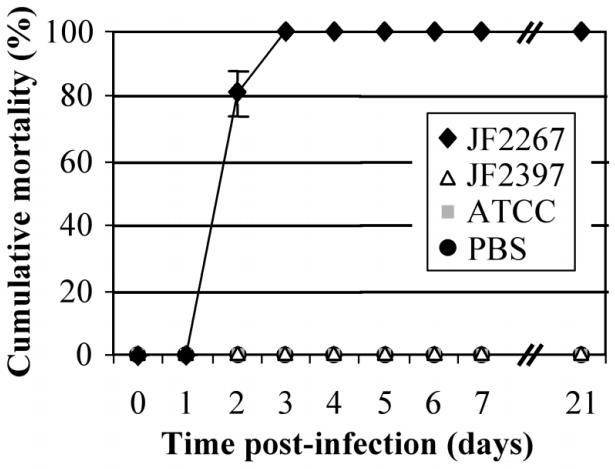

Fig. 2. Cumulative mortality resulting from challenge of rainbow trout *Oncorhynchus mykiss* with isolates of *Aeromonas salmonicida* subsp. *salmonicida*. Strains used are indicated. Strain JF2267 contains Type III secretion system (TTSS) genes, whereas strains JF2397 and ATCC 33658^T do not

of the surviving fish in these groups. As expected, there were no mortalities observed in the control group that was injected with PBS only.

DISCUSSION

Although *Aeromonas salmonicida* subsp. *salmonicida* is an important and economically significant fish pathogen, surprisingly little is known about its pathogenicity. While the bacterium produces many potential virulence factors, inactivation of several of these factors does not have a marked effect on virulence (Vipond et al. 1998, Masada et al. 2002). The role of the well characterized surface-layer protein (A-layer) is also unclear. While certain A-layer-deficient strains are avirulent (Noonan & Trust 1995), others are in fact virulent (Adams et al. 1988, Ellis et al. 1988, Bernoth 1990). There have also been reports of A-layer-positive strains that are avirulent (Adams et al. 1988). Strain SS70.1, examined in this study, is one such strain. This strain is positive for a number of suspected virulence factors: A-layer and lipopolysaccharide, as well as protease, elastase, lecithinase, and haemolysin activity (Olivier et al. 1992). However, this strain lacks the TTSS, as shown by the absence of the *ascV* gene by Southern blot analysis, and was not cytotoxic in our cell assays. It has also previously been shown to be avirulent to brook trout *Salvelinus fontinalis* when injected intraperitoneally (Olivier 1990).

In this study, we have used a probe against the TTSS gene *ascV* to determine whether TTSSs are present in field isolates of *Aeromonas salmonicida* subsp. *salmonicida*. This gene encodes AscV, an inner membrane component of the Type III secretion apparatus. AscV and its homologues are members of a highly conserved family of proteins found in every known TTSS; all members can be aligned over the entire length of their amino acid sequence (Galan et al. 1992, Plano et al. 1991). Because of this, this family of genes can be used as an effective method for screening bacteria for the presence of TTSSs (Stuber et al. 2003). We have shown that 5 field isolates that possess *ascV* (indicating they contain a TTSS) were all cytotoxic for both EPC and RTG-2 cells *in vitro*. In contrast, the remaining field isolate (CC-24) lacked *ascV* and was avirulent towards the same cell lines. The *A. salmonicida* subsp. *salmonicida* Type Strain ATCC 33658^T and Strain SS70.1, which also lack TTSS genes, were also noncytotoxic towards the fish cells. These findings suggest that the TTSS is an important virulence factor.

More importantly, an association between TTSS genes and virulence was found under *in vivo* conditions. Strain JF2267, which possesses a functional TTSS, was found to be highly virulent towards rainbow trout. In contrast, Strain JF2397 (a derivative of Strain JF2267) lacks the plasmid that codes for the TTSS genes, and was avirulent towards rainbow trout. Strain ATCC 33658^T, which also lacks TTSS genes, was also avirulent by our challenge method. These results strengthen the argument that the TTSS plays a role in *Aeromonas salmonicida* subsp. *salmonicida* pathogenicity. Furthermore, they indicate that laboratory cultivation of *A. salmonicida* subsp. *salmonicida* can have a dramatic effect on the virulence of the organism.

Loss of virulence as a result of laboratory subculture of *Aeromonas salmonicida* has been previously described. It has long been known that growth of *A. salmonicida* at elevated temperatures (above 27°C) results in the loss of A-layer (Ishiguro et al. 1981). In fact, serial passaging of A-layer-positive strains at 28°C has been used to generate A-layer-minus strains in order to study the role of the A-layer in virulence (Olivier 1990). However, this method likely also results in the loss of TTSS genes, as these genes are located on a thermosensitive plasmid that is lost at temperatures above 20°C (Stuber et al. 2003a); therefore it cannot be used as a reliable method to draw conclusions as to the role that A-layer protein alone plays in virulence.

The results of this study indicate that the presence of TTSS genes is associated with pathogenicity of *Aeromonas salmonicida* subsp. *salmonicida* towards rainbow trout (and potentially other *Salmonidae* spp.), and that virulence of *A. salmonicida* subsp. *salmonicida* isolates can be predicated by the presence or absence of these TTSS genes. Furthermore, the correlation we have found between the *in vitro* and the *in vivo* exper-

iments suggests that assaying *A. salmonicida* subsp. *salmonicida* virulence in cell culture is a valuable model which can replace *in vivo* experiments.

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