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Evidence for a Type III Secretion System in *Aeromonas salmonicida* subsp. *salmonicida*

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***Aeromonas salmonicida* subsp. *salmonicida*, the etiological agent of furunculosis, is an important fish pathogen. We have screened this bacterium with a broad-host-range probe directed against *yscV*, the gene that encodes the archetype of a highly conserved family of inner membrane proteins found in every known type III secretion system. This has led to the identification of seven open reading frames that encode homologues to proteins functioning within the type III secretion systems of *Yersinia* species. Six of these proteins are encoded by genes comprising a *virA* operon. The *A. salmonicida* subsp. *salmonicida yscV* homologue, *ascV*, was inactivated by marker replacement mutagenesis and used to generate an isogenic *ascV* mutant. Comparison of the extracellular protein profiles from the *ascV* mutant and the wild-type strain indicates that *A. salmonicida* subsp. *salmonicida* secretes proteins via a type III secretion system. The recently identified ADP-ribosylating toxin AexT was identified as one such protein. Finally, we have compared the toxicities of the wild-type *A. salmonicida* subsp. *salmonicida* strain and the *ascV* mutant against RTG-2 rainbow trout gonad cells. While infection with the wild-type strain results in significant morphological changes, including cell rounding, infection with the *ascV* mutant has no toxic effect, indicating that the type III secretion system we have identified plays an important role in the virulence of this pathogen.**

Aeromonas salmonicida subsp. *salmonicida*, the causal agent of furunculosis in salmonids, causes large economic losses in the aquaculture of trout and salmon. The disease is characterized by the presence of hemorrhagic and necrotic lesions in the gills, gut, and muscle. Due to the high mortality and contagious nature of the disease, large amounts of antibiotics are often used in closed and open waters for therapy of furunculosis (19). Vaccination has become an important strategy in the control of furunculosis among farmed fish (10); however, current vaccines display considerable variability in efficacy, and epizootics commonly occur in fish farms. In order to develop more effective control measures, a better understanding of the virulence attributes of *A. salmonicida* is needed. To date, several potential virulence factors of *A. salmonicida* have been reported. These include the surface layer protein (9), salmolysin (25), the serine protease AspA (29), and the glycerolipid-cholesterol acyltransferase complexed with lipopolysaccharide (18). However, the roles these factors play in pathogenesis in vivo remain unclear. We have recently reported the identification of an ADP-ribosyltransferase toxin (AexT) in *A. salmonicida* subsp. *salmonicida* that was shown to play a direct role in virulence (6). This toxin has been shown to possess high sequence similarity to *Pseudomonas aeruginosa* exoenzyme S (ExoS), a protein that is secreted by a type III secretion system (TTSS) (31). This information, coupled with observations that secretion of AexT occurs only in contact with fish cells or, alternately, in low-calcium medium, prompted us to speculate that a TTSS is present in *A. salmonicida* subsp. *salmonicida*. To this end, we have screened this bacterium with a broad-host-range probe against *yscV* (formerly designated *lcrD*) of *Yersinia entercolitica*, a gene that encodes an inner membrane component of the type III secretion apparatus (21). The results of this study have led to the identification of several TTSS genes that together comprise an analogue of the *virA* locus, which is central to the TTSS of many gram-negative pathogens. In this communication, we present the findings of these studies and demonstrate that knockout mutagenesis of the *ycsV* homologue in *A. salmonicida* subsp. *salmonicida* prevents secretion of the AexT toxin. Furthermore, we utilize a fish cell infection model to show that inactivation of the *yscV* homologue significantly reduces the bacterium's pathogenicity.

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MATERIALS AND METHODS

Bacterial strains, growth conditions, and cloning vectors. A summary of the bacterial strains and plasmids used in this study is provided in Table 1. *A. salmonicida* strains were cultured on Luria-Bertani (LB) agar plates at 18°C unless otherwise indicated. *Escherichia coli* strains were routinely grown in LB agar or broth at 37°C. Liquid cultures of *A. salmonicida* were grown in Trypticase soy broth (TSB; Becton Dickinson). The media used for selection included sucrose (15% [wt/vol]) in Trypticase soy agar (TSA; Becton Dickinson) and sucrose (15% [wt/vol]) in TSB. When indicated, antibiotics were added to the culture media at the following final concentrations: for *Escherichia coli*, ampicillin, 100 μg/ml; tetracycline, 20 μg/ml; and kanamycin, 50 μg/ml; for *A. salmonicida*, rifampin, 20 μg/ml; and kanamycin, 40 μg/ml.

Screening for the presence of TTSS genes in *A. salmonicida*. *A. salmonicida* subsp. *salmonicida* strain JF2267 was screened for the presence of a TTSS with a probe directed against *yscV*. The *yscV* gene was first amplified from *Y. enterocolitica* strain NZ63-91 with the primers LCRD-L (CCGGAATTCATCCCCATGATCTTGAGT) and LCRD-R (CCGGAATTCTATCGCTACCCAAGTCTG). The presence of *Eco*RI restriction sites in the two primers (underlined) allowed for the subsequent cloning of the PCR product into pBSK. In order to generate a digoxigenin (DIG)-labeled probe, the *Eco*RI fragment was excised from purified plasmid and used as a template for PCR (with primers LCRD-L and LCRD-R) carried out in the presence of 40 μM DIG-11-dUTP (Roche Diag-

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Properties	Source or reference
Strains		
A. salmonicida		
JF2267	Virulent isolate	6
JF2646	Spontaneous Rif^r derivative of JF2267	This study
JF2678	Δ*ascV*::Km	This study
E. coli		
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}ZΔM15 Tn*10* (Tet^r)]	7
S17-1	*thi pro hsdR hsdM*$^+$ *recA* [RP4 2-Tc::Mu-Km::Tn*7* (Tp^r Sm^r) Tra$^+$]	23
Y. enterocolitica NZ63-91	*yscV*$^+$	SNRL[a]
Plasmids		
pBSK	Bluescript II SK(−), cloning and sequencing	Stratagene
pSSVI186	Kanamycin resistance determinant, located on a 1.3-kb *Pst*I fragment from Tn*903*	28
pSUP202sac	Mobilizable suicide vector, confers SucS (Mob$^+$ *sacRB*$^+$ Tet^r)	27

[a] SNRL, Swiss National Reference Laboratory for Foodborne Disease.

nostics, Rotkreuz, Switzerland). Total DNA extracted from *A. salmonicida* subsp *salmonicida* strain JF2267 by the guanidium hydrochloride method (20) was screened by the dot blot technique. The DNA was denatured in a mixture containing 0.4 M NaOH and 10 mM EDTA and applied directly to positively charged nylon membranes. Processing of the membranes and subsequent hybridization with the *yscV* DIG-labeled probe were performed as described previously (17). Southern blot analysis was performed with the *yscV* DIG-labeled probe under low-stringency conditions (2), with total DNA isolated from strain JF2267 and digested with restriction endonucleases *Sac*I and *Sal*I (Roche Diagnostics).

DNA manipulation, cloning, and sequencing. All cloning procedures and genetic methods were carried out according to standard protocols (2). A partial gene library of *A. salmonicida* subsp. *salmonicida* strain JF2267 was constructed from agarose gel-purified *Sac*I-*Sal*I-digested fragments 4 to 6 kb in size cloned into vector pBSK. Recombinant plasmids were transformed into *E. coli* XL1-Blue, and positive clones were screened by colony blotting. Plasmids were prepared from positive clones with the QIA Prep Spin Mini Prep kit (Qiagen) according to the instructions supplied.

For complete sequencing of positive clones, nested deletions were generated from the initial cloned DNA fragment with a double-stranded nested deletion kit (Pharmacia LKB). Primer walking was carried out on total DNA isolated from *A. salmonicida* subsp. *salmonicida* strain JF2267 by using the Vectorette system (Genosys) according to the manufacturer's instructions.

DNA sequencing was performed with the dRhodamine Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's protocol with either the T3 and T7 primers or custom-synthesized internal primers (Microsynth). The details of all oligonucleotide sequences used are available upon request. All sequences were determined on both strands. Reaction products were analyzed on an ABI Prism 310 genetic analyzer (Applied Biosystems).

Sequence alignment and editing were performed with the software Sequencher (Gene Codes Corporation). Comparisons of DNA sequences and their corresponding amino acid sequences with sequences in the EMBL/GenBank and NBRF databases were performed with BLAST (1). The molecular mass and theoretical isoelectric pH (pI) of the TTSS proteins were calculated with ProtParam (13).

Marker replacment mutagenesis. The *A. salmonicida* subsp. *salmonicida* gene *ascV* was inactivated by marker replacement mutagenesis. A 1,061-bp fragment from *ascV* was excised by using the restriction enzymes *Kpn*I and *Spe*I (Roche Diagnostics) and replaced with the kanamycin (Km) cassette from pSSVI186 (28) that had been previously excised on a 1.3-kb *Kpn*I-*Spe*I fragment. The inactivated *ascV* and flanking genes were then cloned into the mobilizable suicide vector pSUP202sac (27). The resulting plasmid was transformed into *E. coli* S17-1 (23) for subsequent conjugation into *A. salmonicida* subsp. *salmonicida*. In order to provide a means for selection against *E. coli*, spontaneous rifampin-resistant (Rif^r) clones of *A. salmonicida* subsp. *salmonicida* strain JF2267 were isolated following growth of the organism on rifampin agar plates (40 μg/ml) for two passages. A single Rif^r clone was selected (strain JF2646) and filter mated (23) with *E. coli* S17-1 carrying the pSUP202sac-Δ*ascV* plasmid for 3 days at 15°C. Double-crossover mutants were selected directly by growth on TSA containing 15% sucrose (wt/vol), 40 μg of kanamycin per ml, and 20 μg of rifampin per ml at 15°C for 7 days. The absence of the wild-type *ascV* gene and insertion of the kanamycin cassette were verified by PCR with the primer pairs AcrD-fwd (GGGAATTCGATGAAGCCCGTTTTGCC) and AcrD-rev (GTGCGGCCGC ACAGGCAGACCCTCCCGAG) and KMTN903-R (CCAATTCTGATTAGA AAAACTC) and KMTN903-L (AAGGGGTGTTATGAGCCATATT).

SDS-PAGE and immunoblot analyzes. Proteins were separated on 12% acrylamide slabs by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (26). Once separated, proteins were either stained with Coomassie brilliant blue or electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked for at least 1 h in 1% milk buffer. In order to detect AexT, the membranes were incubated with rabbit polyclonal anti-AexT immunoglobulin G (IgG) (6) diluted 1:1,000 in milk buffer, followed by incubation with a phosphate-labeled conjugate (goat anti-rabbit IgG heavy and light chains; Kirkegaard & Perry) diluted 1:2,000 in milk buffer. The proteins were then visualized with 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (Sigma).

In vitro cell assay. Rainbow trout (*Oncorhynchus mykiss*) gonad cells (RTG-2, ATCC CCL-55) were grown as described previously (6). RTG-2 cells grown in a monolayer (6×10^5 cells per 2-cm^2 well in 1 ml of medium) were infected with *A. salmonicida* cells suspended in phosphate-buffered saline (PBS) (pH 7.4) at a multiplicity of infection of 20:1 (bacterium/fish cell ratio). The addition of PBS (pH 7.4) to fish cells was used as a negative control. After 6 h of infection at 18°C, the fish cells were photographed under a green-filtered phase-contrast microscope (Zeiss Aixovert 100).

Nucleotide sequence accession number. The nucleotide sequences reported in this communication have been submitted to the EMBL Nucleotide Sequence Database under accession no. AJ458292.

RESULTS AND DISCUSSION

Cloning and sequence analysis of the *virA* locus. YscV (formerly called LcrD) is the archetype for a family of inner membrane proteins found in every known TTSS. These proteins are highly conserved, and all members can be aligned over the entire length of their amino acid sequence (12, 21). We therefore chose to utilize the gene encoding this protein for screening a virulent isolate of *A. salmonicida* subsp. *salmonicida*. We utilized a probe directed against the *yscV* gene of *Y. enterocolitica* and screened total DNA from *A. salmonicida* subsp. *salmonicida* strain JF2267, a strain previously isolated from an arctic char (*Savelinus alpinus*) displaying typical furunculosis symptoms (6). The results of dot blot analysis revealed a strong signal with the *yscV* probe, suggesting the gene was present in strain JF2267. Subsequent Southern blot analysis of total DNA isolated from strain JF2267 and digested with restriction endonucleases *Sac*I and *Sal*I revealed a 4.8-kb fragment that hybridized with the *yscV* probe. This fragment was cloned on pBSK, and the nucleotide sequence was determined.

DNA sequence analysis revealed the presence of seven open reading frames (ORFs) encoding homologues to *Yersinia* proteins. In *Yersinia* species, six of these proteins are encoded by genes found within the *virA* locus, specifically *tyeA*, *sycN*, *yscX*, *yscY*, *yscV*, and *lcrR* (3, 15, 16, 21, 22, 26) (Fig. 1). The gene encoding the seventh protein, *lcrG* is found on a separate locus downstream of *lcrR* (5). Because the ORF of the *tyeA* homologue appeared to be incomplete on the initial cloned fragment, we utilized the primer-walking technique to obtain the complete sequence of this gene. In doing this, we also obtained

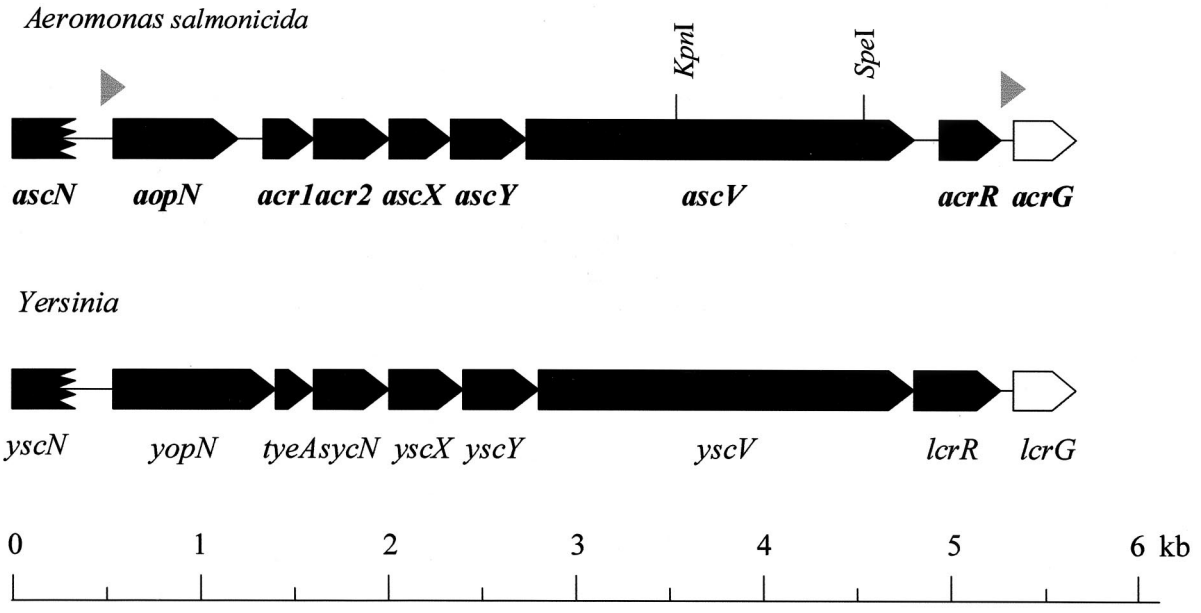

FIG. 1. Genetic organization of the *virA* operon of *A. salmonicida* subsp. *salmonicida* strain JF2267 (and that of the Rif^r derivative, JF2646). The *virA* locus of *Yersinia* species is shown for comparison. Genes that comprise the *virA* locus are represented by black boxes. Flanking genes are shown in white. The jagged box represents an incomplete gene sequence. Potential promoter sequences, represented by gray arrowheads, are found upstream of *aopN* and *acrG*. The *Kpn*I and *Spe*I sites used for the inactivation of the *ascV* gene are indicated.

the sequence of an ORF encoding a YopN homologue (11) and a partial ORF that appears to encode a YscN homologue (4, 30) (Fig. 1).

In keeping with the nomenclature currently used for the designation of TTSS genes in *Yersinia* species, the archetype for these systems, we have given the *yscN*, *yscX*, *yscY*, and *yscV* homologues the designations *ascN*, *ascX*, *ascY*, and *ascV*, respectively, with *asc* representing *Aeromonas* secretion). By the same token, the *lcrR* and *lcrG* homologues have been designated *acrR* and *acrG*; the *yopN* homologue has been designated *aopN*. For simplicity's sake, the *tyeA* homologue (for translocation of Yops into eukaryote cells) and the *sycN* homologue (for specific Yop chaperone) have been termed "*acr1*" and "*acr2*" in analogy to their *P. aeruginosa* counterparts (Fig. 1). All genes have been designated based solely on their sequence similarity to *Yersinia* and *Pseudomonas* TTSS genes. Relevant characteristics of the proteins encoded by these ORFs are summarized in Tables 2 and 3.

Further analysis of the nucleotide sequence of the TTSS genes identified in *A. salmonicida* subsp. *salmonicida* has revealed a potential promoter region upstream of *aopN* (Fig. 1). In addition to −10 (TATAATG) and −35 (TTGGCA) consensus sequences, the promoter also possesses an ExsA consensus element (TAAAAATA) (14), which in *P. aeruginosa* is bound by the transcriptional activator ExsA (14). While we were unable to identify any potential transcription termination sites in the DNA fragment that we have cloned, we did identify another potential promoter region preceding the *acrG* gene (Fig. 1). This promoter also contains −10 (TAGAATA) and −35 (GTGACA) consensus sequences as well as a potential ExsA consensus element (ACAAAAGC). These data suggest that in *A. salmonicida* subsp. *salmonicida*, the genes comprising the *virA* locus may be transcribed by a single operon that in turn may be regulated by an ExsA homologue. We speculate that regulation of the *virA* operon occurs in a manner similar to that seen in *P. aeruginosa* and *Yersinia* species (5, 32).

Secretion of AexT. To determine whether the TTSS genes we have identified in *A. salmonicida* subsp. *salmonicida* are part of a functional secretion system, the *ascV* gene was inactivated by marker replacement mutagenesis with a Km^r cas-

TABLE 2. Comparison between predicted *A. salmonicida* subsp. *salmonicida* proteins and *Yersinia* homologues

A. salmonicida protein	*Yersinia* homologue	Function (reference)	% Identity	% Similarity
AopN	YopN	Regulation of translocation (8, 11, 16)	49	60
Acr1	TyeA	Translocation apparatus, selective translocation of Yops (16)	55	71
Acr2	SycN	Chaperone for YopN (15)	62	77
AscX	YscX	Type III secretion apparatus (15)	54	69
AscY	YscY	Type III secretion apparatus (15)	46	56
AscV	YscV	Type III secretion apparatus (21, 22)	73	79
AcrR	LcrR	Unknown (3, 24)	43	54
AcrG	LcrG	Regulation of low-calcium response (24)	43	64

TABLE 3. Properties of *A. salmonicida* subsp. *salmonicida* proteins

A. salmonicida protein	No. of residues	Theoretical pI	Molecular mass (kDa)
AopN	229	5.6	25.0
Acr1	93	4.3	10.6
Acr2	123	5.5	13.7
AscX	121	5.4	13.6
AscY	116	5.2	12.9
AscV	721	6.0	79.3
AcrR	93	9.5	10.4
AcrG	94	5.5	10.5

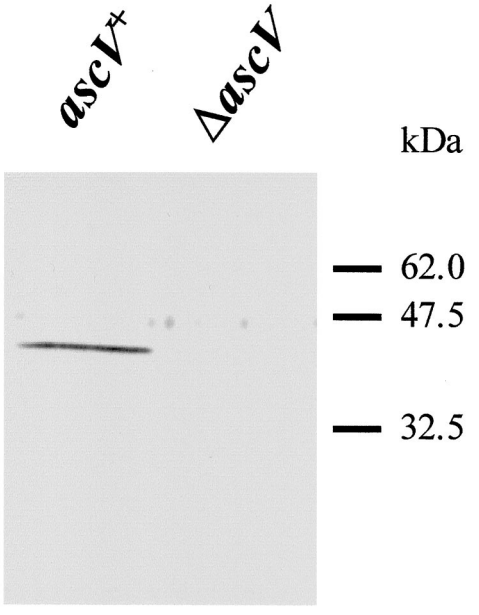

FIG. 2. Secretion of AexT under low-calcium conditions. Bacterial cultures were grown overnight in TSB medium. Left lane, *A. salmonicida* subsp. *salmonicida* strain JF2646 (*ascV*$^+$); right lane, *A. salmonicida* subsp. *salmonicida* Δ*ascV* mutant JF2678. Horizontal lines mark the positions of molecular mass standards.

sette. We then examined the extracellular protein profiles of *A. salmonicida* subsp. *salmonicida* strain JF2646 (a rifampin-resistant derivative of strain JF2267 used in the construction of the Δ*ascV* mutant [see Materials and Methods]) and the Δ*ascV* mutant strain JF2678, under low-calcium conditions. The bacteria were grown overnight in TSB, and the culture supernatant was analyzed by SDS-PAGE. The results revealed a number of protein bands present in the culture supernatant of strain JF2646 that were not seen in that of strain JF2678 (results not shown), indicating that the type III secretion genes we have identified are part of a functional system. Because *A. salmonicida* subsp. *salmonicida* has previously been shown to secrete the ADP-ribosylating toxin AexT, when grown under low-calcium conditions, we speculated that AexT is secreted in a type III-dependent manner. If this were true, then AexT should be detected in the culture supernatant of strain JF2646, but not that of strain JF2678 (Δ*ascV*). To confirm this hypothesis, we performed an immunoblot on culture supernatants from both strains by using anti-*aexT* antibodies. The results can be seen in Fig. 2. AexT is found in the culture supernatant of JF2646 (*ascV*$^+$) cells, but not in the supernatant from JF2678 (Δ*ascV*) cultures, indicating this toxin is secreted into the external environment via the TTSS we have identified.

Toxicity of the *ascV* mutant. Finally, we were interested to determine whether the TTSS in *A. salmonicida* subsp. *salmonicida* plays a role in the virulence of this organism. We assayed the toxicity of *A. salmonicida* subsp. *salmonicida* strain JF2646 and that of its Δ*ascV* mutant derivative, strain JF2678, by infection of cultured rainbow trout (*Oncorhynchus mykiss*) gonad cells (RTG-2 cells). Six hours following inoculation of RTG-2 cells with the bacteria, the cells that had been infected with the *ascV*$^+$ cells (JF2646) displayed characteristic cell rounding and had become detached from the plastic support (Fig. 3A). In contrast, RTG-2 cells infected with the isogenic *ascV* deletion mutant displayed no marked morphological changes in spite of the high numbers of bacterial cells in the cultures (Fig. 3B). RTG-2 fish cells that were inoculated with PBS were used as a negative control (Fig. 3C), and as expected, they displayed no morphological changes.

The inability of the *ascV* mutant to cause damage to the RTG-2 cells indicates that this gene is required for the toxicity of *A. salmonicida* subsp. *salmonicida* against fish cells in vitro. Because AscV displays such high sequence homology to corresponding genes in other TTSSs (e.g., 73% identity and 80% similarity to YscV from *Yersinia* species and 72% identity and 78% similarity to PcrD from *P. aeruginosa*), we can expect it is an integral component of the TTSS apparatus. Therefore, AscV in itself is not likely to be directly responsible for the toxic effect of strain JF2646 toward RTG-2 cells. Rather, it can

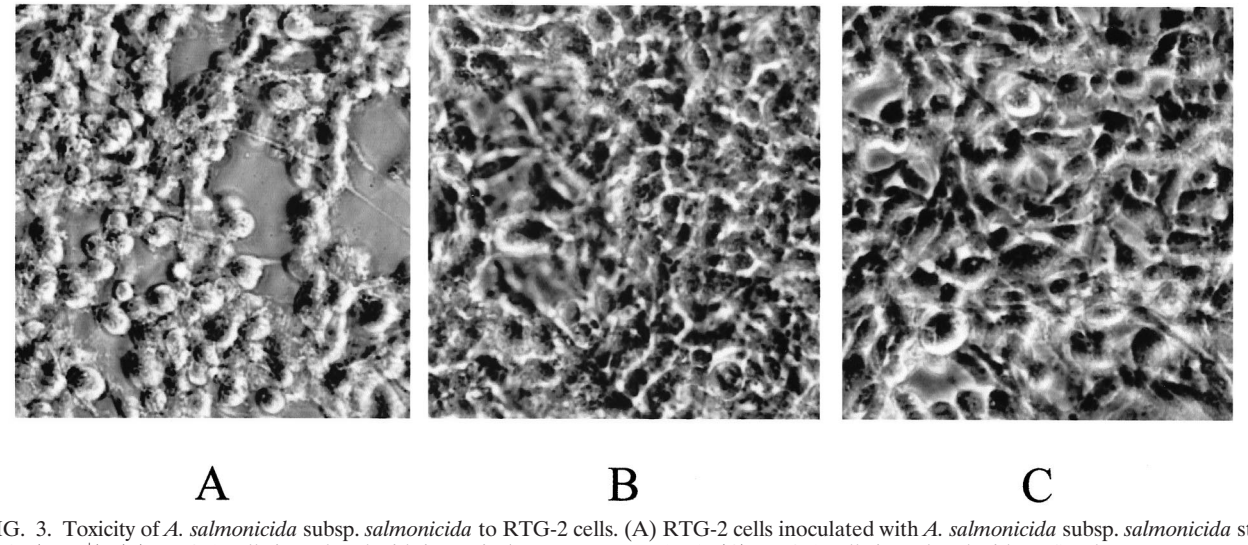

FIG. 3. Toxicity of *A. salmonicida* subsp. *salmonicida* to RTG-2 cells. (A) RTG-2 cells inoculated with *A. salmonicida* subsp. *salmonicida* strain JF2646 (*ascV*$^+$). (B) RTG-2 cells inoculated with isogenic Δ*ascV* mutant JF2678. (C) RTG-2 cells inoculated with PBS only.

be expected to play a role in the secretion or translocation of toxins, including AexT, or other virulence factors into the external environment or into the cytosol of target cells.

While the TTSS of *A. salmonicida* subsp. *salmonicida* certainly appears to play a role in the pathogenesis of this organism in vitro, its role in the disease process in vivo has yet to be established. However, the identification of a TTSS in *A. salmonicida* subsp. *salmonicida* is clearly an important step toward a better understanding of the virulence mechanisms of this pathogen.

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