Contribution of the type III secretion system (TTSS) to virulence of Aeromonas salmonicida subsp. salmonicida


The recently described type III secretion system (TTSS) of Aeromonas salmonicida subsp. salmonicida has been linked to virulence in salmonids. In this study, three TTSS effector genes, aexT, aopH or aopO, were inactivated by deletion, as was ascC, the gene encoding the outer-membrane pore of the secretion apparatus. Effects on virulence were assayed by live challenge of Atlantic salmon (Salmo salar). The ΔascC mutant strain was avirulent by both intraperitoneal (i.p.) injection and immersion, did not appear to establish a clinically inapparent infection and did not confer protection from subsequent rechallenge with the parental strain. 1H NMR spectroscopy-based metabolite profiling of plasma from all fish showed significant differences in the metabolite profiles between the animals exposed to the parental strain or ΔascC. The experimental infection by immersion with ΔaopO was indistinguishable from that of the parental strain, that of ΔaexT was delayed, whilst the virulence of ΔaopH was reduced significantly but not abolished. By i.p. injection, ΔaexT, ΔaopH and ΔaopO caused an experimental disease indistinguishable from that of the parental strain. These data demonstrate that while the TTSS is absolutely essential for virulence of A. salmonicida subsp. salmonicida in Atlantic salmon, removal of individual effectors has little influence on virulence but has a significant effect on colonization. The ΔascC i.p. injection data also suggest that in addition to host invasion there is a second step in A. salmonicida pathogenesis that requires an active TTSS.

INTRODUCTION

Aeromonas salmonicida subsp. salmonicida is a Gram-negative bacterium in the γ-Proteobacteria group. It is the aetiologic agent of furunculosis, an infectious bacteraemia of salmonid fish. Many fundamental aspects of the host–pathogen relationship between A. salmonicida subsp. salmonicida (hereafter referred to as A. salmonicida) and its salmonid hosts remain poorly understood. Many proteins and systems in A. salmonicida have been implicated in virulence including the S-layer (vapA; Trust et al., 1983), siderophores and their receptors (fST, fSTB, hupA; Ebanks et al., 2004), superoxide dismutase (sodA, sodB; Barnes et al., 1996; Garduno et al., 1997; Dacanay et al., 2003) and extracellular toxins such as glycerophospholipid:cholesterol acetyltransferase (GCAT) and the serine protease AspA (Salte et al., 1992). Despite the presence of multiple virulence systems, until recently no single system appeared to contribute significantly to virulence, as shown by the retention of virulence by strains deficient in any given system (Ellis et al., 1988; Olivier, 1990; Vipond et al., 1998; Fernandez et al., 1998). A type III secretion system (TTSS) in A. salmonicida has been described recently (Burr et al., 2002, 2003a; Stuber et al., 2003) and appears to be the exception to this rule (Burr et al., 2003b, 2005).

The TTSSs of pathogenic Gram-negative bacteria utilize a transmembrane injection apparatus composed of integral membrane proteins and a needle-like structure to translocate a range of effector proteins from the cytosol directly into host cells. The best-characterized TTSS systems are those of the pathogenic yersiniae (Yersinia pestis, Yersinia pseudotuberculosis and Yersinia enterocolitica), which consist of at least six effectors in addition to the inner-, outer- and target cell-transmembrane pores. Secreted effectors act directly upon intracellular signalling pathways by targeting proteins such as Rho or Rac. The downstream effects include modulation of phagocytosis and inhibition of paracrine signalling (Cornelis & Wolf-Hanz, 1997; Hueck, 1998), allowing the bacteria to modulate innate and acquired immune responses. In addition to the pathogenic yersiniae,
the TTSS is a virulence factor for many pathogenic bacteria including *Pseudomonas aeruginosa*, *Shigella flexneri*, *Salmonella enterica* serovar *typhimurium*, enteropathogenic *Escherichia coli* (reviewed by Hueck, 1998) and *Aeromonas hydrophila* AH-1 (Yu et al., 2004). In common with other bacteria, the *A. salmonicida* TTSS consists of bacterial inner- and outer-membrane secretory pores, a host-cell translocation pore and a number of effector molecules. Unlike the yersiniae, where the TTSS is carried on a single 70 kb plasmid (pYV), the various genes of the TTSS of *A. salmonicida* are carried both on plasmids and chromosomally (Burr et al., 2002; Stuber et al., 2003). Two laboratory-derived TTSS-deficient strains of *A. salmonicida* JF2267 have been described as avirulent in a rainbow trout (*Oncorhynchus mykiss*) challenge model. One strain was deficient in the 140 kbp plasmid that carries the TTSS system. The second was a knockout mutant strain in *ascV*, the orthologue of *Yersinia* *yscV*, which forms part of the inner bacterial membrane pore (Burr et al., 2002, 2005).

In this study we created deletion mutant strains in the genes of the outer bacterial transmembrane pore and three TTSS effector genes of *A. salmonicida* strain A449. In addition to conventional methods for assessing effects on virulence of the bacterium in one of its natural hosts, the Atlantic salmon (*Salmo salar*), we also used metabolite profiling (metabonomics) to examine the host response to infection by *A. salmonicida*.

**METHODS**

**Bacterial strains and growth conditions.** Bacteria and plasmids used in this study are listed in Table 1. The parental strain for all knockouts was *Aeromonas salmonicida* strain A449 (hereafter abbreviated to A449). All *A. salmonicida* strains were grown in tryptic soy broth (TSB) or agar (TSA) (Difco) at 17 °C with shaking. *Escherichia coli* strains were grown in Luria–Bertani (LB) broth or agar at 37 °C. Antibiotics were used at the following concentrations: *E. coli*, 100 μg ampicillin ml⁻¹; *A. salmonicida*, 50 μg ampicillin ml⁻¹; 20 μg chloramphenicol ml⁻¹.

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description and origin</th>
<th>Reference(s) source or description</th>
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<tbody>
<tr>
<td><strong>Aeromonas salmonicida</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A449</td>
<td><em>A. salmonicida</em> subsp. <em>salmonicida</em> isolated from a brown trout in Eure, France, <em>Cm</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>W. Kay* (pers. comm.)</td>
</tr>
<tr>
<td>ΔascC</td>
<td>A449 ΔascC, <em>Cm</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>ΔaexT</td>
<td>A449 ΔaexT, <em>Cm</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>ΔaopH</td>
<td>A449 ΔaopH, <em>Cm</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>ΔaopO</td>
<td>A449 ΔaopO, <em>Cm</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<td></td>
</tr>
<tr>
<td>EC100D pir-116</td>
<td>K-12, pir-116</td>
<td>Metcalf <em>et al.</em> (1994)</td>
</tr>
<tr>
<td>BW20767</td>
<td>K-12, pir&lt;sup&gt;+&lt;/sup&gt;, conjugation&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Metcalf <em>et al.</em> (1996)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWM-ascC</td>
<td>pWM91 with <em>ascC</em> flanking regions, <em>Ap</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
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<td>pWM-aexT</td>
<td>pWM91 with <em>aexT</em> flanking regions, <em>Ap</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<td>pWM-aopH</td>
<td>pWM91 with <em>aopH</em> flanking regions, <em>Ap</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
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<td>pWM-aopO</td>
<td>pWM91 with <em>aopO</em> flanking regions, <em>Ap</em>&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
</tbody>
</table>

*Department of Microbiology and Biochemistry, University of Victoria, Victoria, BC, Canada.*
1% body weight per day of a commercially available salmon feed (Signature Salmon Ration, ShurGain). Feeding was suspended for 1 day prior to manipulation and 1 day post-manipulation.

**Challenge.** For the immersion challenge there were two tanks per group with 40 fish per tank. Fish were removed from the resident tank and placed in ~40 l aerated fresh water in a large plastic container to which ~10⁶ c.f.u. ml⁻¹ A449, ΔascC, ΔaexT, ΔaopH or ΔaopO had been added as well as anaesthetic (15 mg tricaine methanesulphonate 1⁻¹, Syndel Laboratories) to sedate the fish during the immersion. Bacterial doses were retrospectively confirmed by direct colony counts on TSA. Control animals were rechallenged with the parental strain. For the i.p. challenge, survivors were rechallenged 43 days after the initial injection with ~10⁶ c.f.u. A449 per animal in 100 μl PBS by injection as before.

**Plasma sampling.** At the termination of the rechallenge, the survivors were killed. At this time blood was drawn from the caudal vein into heparinized containers (Vacutainer, Becton-Dickinson). The erythrocytes were removed by centrifugation at 3000 g after the initial immersion with ~10⁶ c.f.u. A449 ml⁻¹ by immersion as before. For the i.p. challenge, survivors were rechallenged 43 days after the initial injection with ~10⁵ c.f.u. A449 per animal in 100 μl PBS by injection as before.

**Stress test.** Sixty-six days after exposure by immersion and 14 days after the cessation of mortality, half of the surviving animals were assessed for clinically inapparent infections by application of a stress test (adapted from Specker et al., 1994). Briefly, 100 mg cortisol (hydrocortisone, Sigma-Aldrich) in a vegetable oil/vegetable fat emulsion was administered by i.p. injection. This was followed by an increase in water temperature from 14 °C to 18 °C over 2 h, which was maintained for the remainder of the experiment. The remaining animals were left as unstressed controls. All moribund animals were processed as before. The stress test ceased after 10 days, at which time all surviving animals were killed with an overdose of TMS.

**Statistics.** Statistical differences in cumulative morbidity between groups were assessed by the G-test (a modified χ² test). Three indices were used to compare morbidity rates between groups: (a) survival curves were directly compared using the Mantel–Haenszel test; a P value <0·05 indicated the curves were significantly different; (b) calculation of hazard ratios, the ratio of deaths in the test group after the initial immersion with ~10⁶ c.f.u. A449 ml⁻¹ by immersion as before. For the i.p. challenge, survivors were rechallenged 43 days after the initial injection with ~10⁵ c.f.u. A449 per animal in 100 μl PBS by injection as before.

**Table 2. Primers**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence*</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ascC-Co</td>
<td>tgtgctcagCTTGGGTCAAGGATCCTTCTTG</td>
<td>To amplify 5' flanking region for knockout</td>
</tr>
<tr>
<td>ascC-Ni</td>
<td>cccatccactaaacctaaacGCTCAAGGATCCTTCCTTCTTG</td>
<td>To amplify 5' flanking region for knockout, with crossover sequence</td>
</tr>
<tr>
<td>ascC-Ci</td>
<td>tgttaagtttagggCTGACCAGATGACGAAGGC</td>
<td>To amplify 3' flanking region for knockout</td>
</tr>
<tr>
<td>ascC-Co</td>
<td>atatcagatgcgcgcgcACACCTGACGGTAGTTTGGT</td>
<td>To amplify 3' flanking region for knockout</td>
</tr>
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<td>aexT-No</td>
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<td>aexT-Ci</td>
<td>tgttaagtttagggCTGACCAGATGACGAAGGC</td>
<td>To amplify 3' flanking region for knockout</td>
</tr>
<tr>
<td>aexT-Co</td>
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<td>To amplify 3' flanking region for knockout</td>
</tr>
<tr>
<td>aopH-No</td>
<td>tgtgctcagCTTGGGTCAGGATCATTCTTCTTG</td>
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</tr>
<tr>
<td>aopH-Ni</td>
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<td>To amplify 5' flanking region for knockout, with crossover sequence</td>
</tr>
<tr>
<td>aopH-Ci</td>
<td>tgttaagtttagggCTGACCAGATGACGAAGGC</td>
<td>To amplify 3' flanking region for knockout</td>
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<tr>
<td>aopH-Co</td>
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<tr>
<td>aopO-No</td>
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<td>aopO-Ni</td>
<td>cccatccactaaacctaaacGCTGAATGAGTTTCTTCTTCTTG</td>
<td>To amplify 5' flanking region for knockout, with crossover sequence</td>
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<tr>
<td>aopO-Ci</td>
<td>tgttaagtttagggCTGACCAGATGACGAAGGC</td>
<td>To amplify 3' flanking region for knockout</td>
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<tr>
<td>aopO-Co</td>
<td>atatcagatgcgcgcgcACACCTGACGGTAGTTTGGT</td>
<td>To amplify 3' flanking region for knockout</td>
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</table>

*Restriction sites are underlined; non-gene-specific regions are in lower case.
compared to the positive control group; and (c) calculation of the median survival time, the time in days to 50% morbidity in each group. All tests except the G-test, which was calculated manually, were calculated using GraphPad Prism 3.0 (GraphPad Software).

1H NMR spectral acquisition. Plasma samples from the rechallenge survivors were thawed. A 100 μl aliquot of each sample was mixed with 50 μl D2O for analysis in a 2.5 mm outer diameter (o.d.) tapered Wilmad 520-1A NMR tube. Proton nuclear magnetic resonance (1H NMR) spectra were acquired at 4°C on a Bruker Avance-DRX 500 MHz spectrometer operating at 500-13 MHz using a 5 mm Bruker triple-axis gradient, triple-band inverse (TBI) probe. Three types of 1D 1H spectra with different pulse sequences were acquired for each sample, as for previous studies with salmon plasma (Solanky et al., 2005). The sequences were: presaturation (PS), WATERGATE (WG) and Carr–Purcell–Meiboom–Gill (CPMG). Conditions for data acquisition, processing and analysis were also as described by Solanky et al. (2005), apart from the use of 2.5 mm o.d. NMR tubes allowing spectra from smaller volumes of plasma (100 μl) to be acquired.

RESULTS

Identification of TTSS genes

In the process of sequencing the genome of A. salmonicida strain A449, a gene encoding the TTSS outer transmembrane pore protein AscC was identified, with 100% amino acid identity to a previously identified AscC from A. salmonicida strain JF2267 (Burr et al., 2005). Three TTSS effectors were also identified. One, AexT, was chromosomally located, had 100% amino acid identity to a previously characterized translocated ADP-ribosylating cytotoxin from A. salmonicida strain JF2267 and had similarity to exoT of P. aeruginosa (Braun et al., 2002, 2003b). The other two, located on plasmid pAsa5, were termed aop (Aeromonas outer protein) H and aopO, with similarity to Versinia yopH and yopO/ypkA, respectively (Table 3). Neither aopH nor aopO are located within a cluster of genes encoding the TTSS apparatus, which is also located on pAsa5 (Table 3).

Knockouts

To investigate the contribution of the TTSS and its effector proteins to A. salmonicida virulence, unmarked isogenic deletion mutant strains were created with each of ascC, aopH, aopO or aexT deleted. The knockouts were confirmed by PCR from total DNA using primer sets both internal to and flanking the relevant gene. Generation time and cell density for all four mutant strains when cultured in TSB at 17°C were comparable to those of the parental strain (data not shown).

Immersion challenge

Immersion with ~10⁶ c.f.u. ml⁻¹ A. salmonicida strain A449 or the isogenic mutant strains ΔaexT, ΔaopH and ΔaopO caused an experimental infection in Atlantic salmon that started at 7 days after immersion for ΔaopO, 14 days for ΔaopH and A449 and 15 days for ΔaexT. Morbidity ceased 60 days after challenge (data not shown). A449 or the appropriate isogenic mutant strain was isolated from the posterior kidney of all moribund and dead animals. Cumulative morbidity, hazard ratio and median survival time data for the immersion challenge are reported in Table 4. There was no A. salmonicida-related morbidity in groups exposed to ΔascC or PBS.

Groups exposed to the parental strain experienced high morbidity (60%). There were no significant differences in cumulative morbidity or survival curves between ΔaopO and the parental strain. The median survival time for animals exposed to ΔaopO was 29 days, 2 days longer than for the parental strain. The hazard ratio was 0.99, indicating that the morbidity rates between the two strains were essentially the same. Similarly, there were no significant differences in cumulative morbidity between the parental strain and ΔaexT (47.5%; G-test, P=0.0559) and the survival curve was not significantly different from that of the parental strain (Mantel–Haenszel test; P=0.14). However, the median survival time was 39 days, 12 days longer than that for the parental strain and the hazard ratio was 1.37, indicating that the morbidity rate for the parental strain was 1.4 times that of ΔaexT. There was significantly lower morbidity with ΔaopH (35.0%; G-test, P=0.0008) compared to the parental strain. The survival curve was also significantly different (Mantel–Haenszel; P=0.02). This

Table 3. Location and predicted physicochemical and functional characteristics of the TTSS pore protein AscC and effector proteins AexT, AopH and AopO

<table>
<thead>
<tr>
<th>Protein</th>
<th>Predicted function</th>
<th>Mol. mass (Da)*</th>
<th>Closest orthologue†</th>
<th>Identity (%)‡</th>
<th>Location</th>
<th>Accession no.§</th>
</tr>
</thead>
<tbody>
<tr>
<td>AscC</td>
<td>Outer-membrane secretin</td>
<td>67401</td>
<td>YE YscC</td>
<td>70</td>
<td>Plasmid</td>
<td>DQ386863</td>
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<tr>
<td>AexT</td>
<td>Secreted toxin, ADP-ribosyltransferase</td>
<td>50102</td>
<td>PA ExoT</td>
<td>58</td>
<td>Chromosome</td>
<td>DQ386860</td>
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<tr>
<td>AopH</td>
<td>Secreted toxin, tyrosine-phosphatase</td>
<td>50411</td>
<td>YE YopH</td>
<td>56</td>
<td>Plasmid</td>
<td>DQ386861</td>
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<td>AopO</td>
<td>Secreted toxin, serine/threonine protein kinase</td>
<td>79565</td>
<td>YE YopO</td>
<td>65</td>
<td>Plasmid</td>
<td>DQ386862</td>
</tr>
</tbody>
</table>

*Predicted, before processing.
†YE, Versinia enterocolitica; YP, Versinia pseudotuberculosis; PA, Pseudomonas aeruginosa.
§For strain A449.
difference was reflected in both a median survival time of 49 days, 22 days longer than the parental strain, and a hazard ratio of 1·68, showing that the morbidity rate for the parental strain was 1·68 times greater than for ΔaopH.

**Intraperitoneal challenge**

Intraperitoneal (i.p.) injection with \( \sim 10^5 \) c.f.u. per animal of either A449 or the isogenic strains ΔaexT, ΔaopH or ΔaopO caused an experimental infection that started for all strains 3 days after injection and ceased after 21 days (data not shown). A449 or the appropriate isogenic mutant strain was isolated from the posterior kidney of all morbid and dead animals. Cumulative morbidity, hazard ratio and median survival for the i.p. challenge are shown in Table 4. Again there was no *A. salmonicida*-related morbidity in groups exposed to ΔascC or PBS.

There was high morbidity in the group injected with the parental strain (76·3 % cumulative morbidity). There were no significant differences in morbidity (G-test; \( P > 0·05 \)) between the parental strain and any of the TTSS effector mutant strains: ΔaexT (71·0 % cumulative morbidity), ΔaopH (67·2 %) and ΔaopO (84·9 %). The survival curves were not significantly different (Mantel–Haenszel test; \( P > 0·05 \)). The hazard ratio was \( \sim 0·9 \) for all three mutant strains when compared to A449 and the median survival time was equal at 5 days post-challenge.

**Stress test**

Clinically inapparent (covert) *A. salmonicida* infection levels were assessed by a stress test: the application of the twin stressors of an increase in water temperature from 14 °C to 18 °C and injection of 100 mg cortisol per animal (Specker *et al.*, 1994). In a parallel experiment with the immersion challenge, Atlantic salmon were exposed by immersion to A449, PBS or ΔascC and stress tested 66 days after the initial exposure. Ten days after the stress test started there was no *A. salmonicida*-related morbidity in either the PBS- or ΔascC-exposed groups. There was 85 % *A. salmonicida*-related morbidity in the A449 group and the median survival was 7 days.

**Rechallenge**

We tested whether prior exposure to the avirulent ΔascC strain conferred protection from subsequent challenge with the virulent parental strain. Survivors from both the i.p. and immersion challenges were rechallenged with A449; data from these rechallenges are shown in Table 5. When rechallenged with A449 by immersion 85 days after an immersion exposure to A449, ΔascC or PBS, the latter two groups experienced high morbidity; PBS (57·5 % cumulative morbidity) and ΔascC (57·9 %). Median survival was 15 days for both groups. Morbidity in the group initially exposed to A449 (4·2 % cumulative morbidity) was significantly lower than in either the PBS or ΔascC group (G-test; \( P < 0·0001 \)).

Again, animals initially exposed to PBS or ΔascC by injection showed high morbidity upon rechallenge with the parental strain. Animals initially exposed to the parental strain showed some protection from rechallenge, as cumulative morbidity was significantly reduced compared to that of the PBS group (28·6 %; G-test; \( P = 0·005 \)).

**Principal components analysis (PCA) of \(^{1}H\) NMR spectra of plasma**

Metabolite profiles from plasma samples drawn from survivors of the immersion and rechallenge were compared by \(^{1}H\) NMR and PCA. Samples were (I) exposure to PBS/re-exposure to PBS (naïve controls, \( n = 29 \)); (II) immersion challenge with A449/survivors rechallenged by immersion with A449 (\( n = 18 \)); (III) exposure to PBS/rechallenge by
DISCUSSION

The TTSS of pathogenic Gram-negative bacteria delivers effector molecules directly to the cytosol of host cells, where they interact with intracellular signalling pathways. The downstream effect is a modulation of the host immune system in a manner beneficial to the bacterium. Although the TTSS has apparently spread through the prokaryotes by horizontal gene transfer to perform this singular function (Gophna et al., 2003), the roles performed by individual effectors or the TTSS itself are not necessarily conserved (Hueck, 1998). To investigate the contribution of the TTSS in the virulence of A. salmonicida, deletion mutations were created in the outer-membrane pore gene, ascC, and three effectors aexT, aopH and aopO. Atlantic salmon, one of the natural hosts of this bacterium, were exposed to these strains by either immersion or i.p. injection. Animals that survived the initial infection were later assessed for a clinically inapparent (covert) infection or for protection from a subsequent challenge with the parental strain.

In accordance with the reports of others (Burr et al., 2002, 2005; Yu et al., 2004), the outer-membrane pore of the secretion apparatus was clearly required for A. salmonicida virulence. The ΔascC strain caused no morbidity when administered either i.p. or by immersion (Table 4). This was likely due to the inability of this strain to release effectors, since AscC is critical for secretory apparatus assembly and yscC knockouts completely block TTSS secretion in Y. enterocolitica (Koster et al., 1997) and Y. pestis (Plano & Straley, 1995). The avirulence of ΔascC may reflect the inability of this strain to resist phagocytosis; secretion-apparatus-deficient mutants are reported to be more readily phagocytosed than their respective parental strains (Burr et al., 2005; Yu et al., 2004).

The precise site of infection during the clinically inapparent infection state remains unknown but most workers recognize that it is due to the colonization of an outer surface of the fish by A. salmonicida (Hiney et al., 1997). The stress test apparently showed ΔascC was not capable of establishing such an infection. Whereas this should be prima facie evidence for a significant role for the TTSS of A. salmonicida in colonization, the inability of ΔascC to cause clinical disease even by direct injection suggests that it would have been equally unable to cause disease during the stress test. Therefore as overt disease is the ‘positive’ outcome for the stress test, these data cannot be used as evidence that the TTSS is required for host colonization.

Prior exposure to ΔascC by either immersion or injection did not confer protection from rechallenge. In an attempt to understand why, we assayed plasma from the rechallenge survivors. Analysis of specific anti-A. salmonicida immunoglobulin by ELISA was inconclusive (data not shown).
Metabonomic analysis was also performed on these plasma samples. Metabonomics uses PCA of $^1$H NMR spectra to qualitatively and quantitatively compare metabolites in biofluids from, in this case, control and challenged individuals. The data, when presented as scores plots (Fig. 1a–f), where each spectrum is represented by a single point (for more details see Eriksson et al., 1999; Solanky et al., 2005), show that similar spectra cluster together whilst dissimilar spectra do not. We have shown recently that metabonomic analysis of plasma can discriminate between infected and uninfected Atlantic salmon following exposure to *A. salmonicida* (Solanky et al., 2005). The use of three different spectroscopic conditions provided three independent datasets for each sample. PS and WG use different means to suppress the H$_2$O signal whilst CPMG suppresses broad signals from high-molecular-mass compounds in order to highlight otherwise superimposed sharp signals from low-molecular-mass metabolites. The plasma metabolite profiles correlated with a protective immune response. The A449/ A449 group showed clear protection during the rechallenge and its metabolic response clustered distinctly from the naïve group (PBS/PBS). The metabolic responses of the other challenged groups (groups III, IV and V) were not distinguishable from the naïve group nor were any of these groups protected from rechallenge. Thus neither the rechallenge nor metabonomic data shows evidence of acquired immunity in Atlantic salmon in response to exposure to $\Delta$ascC.

This is believed to be the first study that has investigated the *in vivo* behaviour of *A. salmonicida* TTSS effectors by conducting animal challenges with mutants deleted in three effector genes. *A. salmonicida* TTSS effectors are poorly characterized compared with those of other systems; only the functionality of AexT has been studied in any detail (Braun et al., 2002, 2003b, 2005). The *in vivo* behaviour of the three effector mutant strains mimicked the behaviour of a suite of effector mutant strains created in *Y. enterocolitica O*:8 (Trülzsch et al., 2004). Both $\Delta$yoPO and $\Delta$yoPE mutant strains of *Y. enterocolitica O*:8 were capable of colonizing mice and causing an overt disease following oral challenge; $\Delta$yoPO behaved as wild-type and was lethal whereas $\Delta$yoPE was able to colonize mice but did not cause an overt disease.

![Fig. 1. (a–d) PCA scores plots (PC1 vs PC2) based on $^1$H NMR PS spectral data of Atlantic salmon plasma collected following immersion challenge with a virulent strain of *A. salmonicida* (A449) or the avirulent mutant strain $\Delta$ascC. The scores present the relationship between $^1$H NMR spectral profiles of group I (PBS/PBS control) (●) with group II (A449/A449, a) (□); with group III (PBS/ A449, b) (△); with group IV (ΔascC/A449, c) (○); and with group V (ΔascC only, d) (◇). Group II forms a distinct cluster from group I, whereas groups III, IV and V form clusters that are indistinguishable from group I. Panels (e) and (f) show similar clustering results for group II versus group I, obtained with WG and CPMG spectral data respectively. Each symbol represents a single individual. All panels: abscissa, PC1; ordinate, PC2.](http://mic.sgmjournals.org)
and was eventually cleared. Virulence was abolished in the ΔyopH mutant strain. Similar findings are reported for Y. pseudotuberculosis TTSS effectors (Logsdon & Mecasas, 2003). In this study both ΔaexT and ΔaopO behaved as the parental strain; there was only a subtle reduction in the virulence of ΔaexT. Whereas virulence was significantly reduced in ΔaopH, it was not abolished.

YopE and YopO share several intracellular targets, none of which are shared by YopH (Aepfelbacher, 2004). If the target ranges of the A. salmonicida effectors are similar, then the differing effects on virulence of the TTSS effector mutant strains may be explained. The absent/subtle effects on virulence in ΔaexT and ΔaopO in contrast to a more obvious effect on virulence in ΔaopH suggest that AopO and AexT either share intracellular targets or target the same process(es); thus in ΔaexT and ΔaopO the presence of one effector complemented the absence of the other. Further study is required to confidently ascribe definitive targets to A. salmonicida TTSS effectors.

Unlike deletion of secretory apparatus genes, deletion of individual TTSS effector genes in A449 lessened, but did not abolish, virulence. This may be due to the presence of other effectors in A449. A. salmonicida strain JF2267 is reported to carry a fourth effector, AopP/J, not present in A449 and neither strain appears to carry YopT or YopM orthologues (Burr et al., 2002, 2003a; M. Reith, unpublished data). Even though the full genomic sequence of A449 is available, the presence of other effectors in this strain cannot be discounted.

Previous reports on the effects of the TTSS on aeromonad virulence in two species of fish assessed the virulence of TTSS-deficient mutant strains by injection (Burr et al., 2002, 2003; Yu et al., 2004). As the TTSS mediates host invasion in some species of bacteria, injection may not accurately assess the role of the TTSS in virulence. In this study bacterial virulence was assessed as the ability of the bacteria to cause disease when administered by either immersion or i.p. injection.

Attempts to quantify invasion of A449 and its isogenic TTSS mutant strains by serial sampling of tissues from apparently healthy animals after immersion were unsuccessful (data not shown). However, the challenge data suggest strongly that the TTSS of A. salmonicida is required for host invasion. By immersion, a route of administration that requires host invasion to establish an overt disease state (Cardella & Eimers, 1990; Nordmo & Ramsted, 1997), an inactive secretory complex completely abolished virulence. This may be due to the presence of other effectors in this strain. Even though the full genomic sequence of A449 is available, the presence of other effectors in this strain cannot be discounted.

Unlike the effector mutant strains, virulence was not restored to ΔascC by i.p. injection, suggesting that there is a second step in the pathogenesis of furunculosis that requires an active TTSS after host invasion. This is likely to be either TTSS-mediated cytotoxicity (Burr et al., 2003b), which in A. salmonicida has been shown to be contact mediated (Olivier et al., 1992), or a secondary invasive step such as macrophage residence as suggested by Garduño et al. (2000).

Very little is known on the portal of entry for A. salmonicida in natural infections. Work on the covert infection state has shown that prior to invasion and progression to an overt disease state A. salmonicida resides on an as-yet-unknown structure of the fish (Hiney et al., 1997). Furthermore, the pathognomonic clinical sign for furunculosis is a focal dermomyonecrotic lesion (furuncle) that arises from dermal, rather than more deeply located A. salmonicida microcolonies (Bernoth, 1997; Roberts & Rodger, 2001). The requirement of the TTSS of A. salmonicida for invasion and possibly colonization of exterior surfaces prior to invasion is consistent with this.

This study has also revealed that A. salmonicida pathogenesis is a more complex process than initially appears. Further investigation of both the bacterial virulence factors and host immune responses is required to better understand this disease.

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