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

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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 $\Delta ascC$ 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 $\Delta ascC$. The experimental infection by immersion with $\Delta aopO$ was indistinguishable from that of the parental strain, that of $\Delta aexT$ was delayed, whilst the virulence of $\Delta aopH$ was reduced significantly but not abolished. By i.p. injection, $\Delta aexT$, $\Delta aopH$ and $\Delta aopO$ 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 $\Delta ascC$ 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.

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INTRODUCTION

Aeromonas salmonicida subsp. *salmonicida* is a Gram-negative bacterium in the γ -Proteobacteria group. It is the aetiological 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 (*fstC*, *fstB*, *hupA*; Ebanks *et al.*, 2004), superoxide dismutase (*sodA*, *sodB*; Barnes *et al.*, 1996; Garduño *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,

Abbreviations: CPMG, Carr–Purcell–Meiboom–Gill (spectra); ^1H NMR, proton nuclear magnetic resonance; i.p., intraperitoneal; PCA, principal components analysis; PS, presaturation (spectra); TTSS, type III secretion system; WG, WATERGATE (spectra).

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.

DNA techniques. DNA manipulations were performed by standard genetic and molecular techniques (Ausubel *et al.*, 1998). Genomic DNA was isolated from A449 using the PureGene DNA isolation kit (Gentra Systems). Oligonucleotides were prepared by Integrated DNA Technologies. PCR was performed with either rTaq (Amersham) or Pfu (MBI Fermentas) following the manufacturers’ directions.

Construction of deletion mutant strains. All four mutant strains were created by making in-frame, unmarked deletions in the relevant gene using crossover PCR (Link *et al.*, 1997). PCR primers used are described in Table 2. Briefly, two self-complementary PCR fragments per gene were amplified from A449 chromosomal DNA. The two PCR fragments were mixed together and amplified with the two external primers to generate a large fragment encoding the relevant gene with a large internal deletion and flanking sequences. These fragments were cloned into the pir-dependent, sucrose expressing vector pWM91, which was conjugated into A449 from *E. coli* BW20767. Single crossover integrants were selected by plating on TSA supplemented with ampicillin and chloramphenicol. Double crossover mutants were isolated by selection on TSA with 15 % sucrose to select against plasmid-containing colonies.

Animal care. All animal protocols were approved by both the National Research Council Halifax Animal Care Committee and the Dalhousie University Committee on Laboratory Animals and were conducted according to Canadian Council on Animal Care guidelines. Juvenile St John River stock Atlantic salmon were obtained from a Nova Scotia hatchery certified under the Canadian Fish Health Protection guidelines. They were maintained in 100 l fibreglass tanks in single-pass dechlorinated fresh (municipal) water at 14 °C and fed

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description and origin	Reference(s) source or description
<i>Aeromonas salmonicida</i>		
A449	<i>A. salmonicida</i> subsp. <i>salmonicida</i> isolated from a brown trout in Eure, France, Cm ^R	W. Kay* (pers. comm.)
ΔascC	A449 ΔascC, Cm ^R	This work
ΔaexT	A449 ΔaopE, Cm ^R	This work
ΔaopH	A449 ΔaopH, Cm ^R	This work
ΔaopO	A449 ΔaopO, Cm ^R	This work
<i>Escherichia coli</i>		
EC100D pir-116	K-12, pir-116	Metcalf <i>et al.</i> (1994)
BW20767	K-12, pir ⁺ , conjugation ⁺	Metcalf <i>et al.</i> (1996)
Plasmids		
pWM91	oriR6Kγ, sacB, mobRP4, Ap ^R	Metcalf <i>et al.</i> (1996)
pWM-ascC	pWM91 with ascC flanking regions, Ap ^R	This work
pWM-aexT	pWM91 with aexT flanking regions, Ap ^R	This work
pWM-aopH	pWM91 with aopH flanking regions, Ap ^R	This work
pWM-aopO	pWM91 with aopO flanking regions, Ap ^R	This work

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Table 2. Primers

Name	Sequence*	Description
ascC-No	tgtgctcgagCCTTGGGTCAGGATCACTTC	To amplify 5' flanking region for knockout
ascC-Ni	cccatccactaaacttaacaGCTCAAGGGTGCCATATCATC	To amplify 5' flanking region for knockout, with crossover sequence
ascC-Ci	tgtttaagtttagtgatgggCGATGAGCTGGAAGTGCC	To amplify 3' flanking region for knockout, with crossover sequence
ascC-Co	atatcacgatcgggccgcCCACCTGACGGTAGTTTGGT	To amplify 3' flanking region for knockout
aexT-No	tgtgctcgagCTCCAGCTGGTGATGGATCT	To amplify 5' flanking region for knockout
aexT-Ni	cccatccactaaacttaacaCATGATGATTGTCTTCTGTGATG	To amplify 5' flanking region for knockout, with crossover sequence
aexT-Ci	tgtttaagtttagtgatgggTGACCAGATAGCACGAAAGC	To amplify 3' flanking region for knockout, with crossover sequence
aexT-Co	atatcacgatcgggccgcCGAGCAGATCATGTTTCGAGTT	To amplify 3' flanking region for knockout
aopH-No	tgtgctcgagATGGTTATGCAGCAGGGTCA	To amplify 5' flanking region for knockout
aopH-Ni	cccatccactaaacttaacaCATGAGTGAGTCGCGAGTATAGAA	To amplify 5' flanking region for knockout, with crossover sequence
aopH-Ci	tgtttaagtttagtgatgggCTGCGGGCATAACCGTCGAT	To amplify 3' flanking region for knockout, with crossover sequence
aopH-Co	atatcacgatcgggccgcGGGAAATCGAGTTGGGCTAT	To amplify 3' flanking region for knockout
aopO-No	tgtgctcgagAACCGACCAACAGATTGAAG	To amplify 5' flanking region for knockout
aopO-Ni	cccatccactaaacttaacaCATGACTGATTACACCCAGCTT	To amplify 5' flanking region for knockout, with crossover sequence
aopO-Ci	tgtttaagtttagtgatgggCGCTAAATAACTGATCCTAATTCC	To amplify 3' flanking region for knockout, with crossover sequence
aopO-Co	atatcacgatcgggccgcGCGCAAAGCAGAAGAAAGTG	To amplify 3' flanking region for knockout

*Restriction sites are underlined; non-gene-specific regions are in lower case.

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 $\sim 10^6$ c.f.u. ml⁻¹ A449, $\Delta ascC$, $\Delta aexT$, $\Delta aopH$ or $\Delta aopO$ had been added as well as anaesthetic (15 mg tricaine methanesulphonate l⁻¹, Syndel Laboratories) to sedate the fish during the immersion. Bacterial doses were retrospectively confirmed by direct colony counts on TSA. After 30 min, the fish were removed and replaced in the resident tanks. Control fish experienced identical handling but were exposed to PBS only. For the intraperitoneal (i.p.) injection challenge, there were two tanks per group and ~30 fish per tank. Fish were anaesthetized with 50 mg TMS l⁻¹ until laterally recumbant and injected with $\sim 10^5$ c.f.u. per animal in 100 μ l PBS of either A449, $\Delta ascC$, $\Delta aexT$, $\Delta aopH$ or $\Delta aopO$. The doses were confirmed by direct colony counts on TSA. Control animals received an equal volume of PBS only.

All fish were monitored closely post-exposure. Moribund and dead fish were removed immediately; moribund animals were killed with an overdose of TMS. The posterior kidney, in accordance with standard procedures for determining *A. salmonicida* infections (Schotts, 1994), was sampled onto TSA supplemented with 20 μ g chloramphenicol ml⁻¹.

Rechallenge. Animals that had survived challenge with the parental strain or $\Delta ascC$ were rechallenged with the parental strain. For the immersion challenge, the survivors were rechallenged 85 days

after the initial immersion with $\sim 10^6$ 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 $\sim 10^5$ c.f.u. A449 per animal in 100 μ l 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, the plasma was aliquoted and stored at -20 °C.

Stress test. Sixty-six days after exposure by immersion and 14 days after the cessation of mortality, half of the surviving animals exposed to the parental strain, $\Delta ascC$ and PBS-negative control groups 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 χ^2 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

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 *Yersinia yopH* and *yopO/yopK*, 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 morbid 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

Protein	Predicted function	Mol. mass (Da)*	Closest orthologue†	Identity (%)‡	Location	Accession no.§
AscC	Outer-membrane secretin	67 401	YE YscC	70	Plasmid	DQ386863
AexT	Secreted toxin, ADP-ribosyltransferase	50 102	PA ExoT	58	Chromosome	DQ386860
AopH	Secreted toxin, tyrosine-phosphatase	50 411	YP YopH	56	Plasmid	DQ386861
AopO	Secreted toxin, serine/threonine protein kinase	79 565	YE YopO	65	Plasmid	DQ386862

*Predicted, before processing.
†YE, *Yersinia enterocolitica*; YP, *Yersinia pseudotuberculosis*; PA, *Pseudomonas aeruginosa*.
‡Amino acid identity. Calculated with BioEdit (Hall, 1999; <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) using the Blosum62 matrix.
§For strain A449.

Table 4. Cumulative morbidity, hazard ratio and median survival data for immersion and i.p. challenges of Atlantic salmon with a virulent strain of *A. salmonicida* (A449) or one of four isogenic mutants

Strain	Immersion			Intraperitoneal		
	Cumulative morbidity†	Hazard ratio‡	Median survival§	Cumulative morbidity	Hazard ratio	Median survival
A449	60.0 % (48/80) ^a	–	27 days	76.3 % (45/59) ^c	–	5 days
PBS	0.0 % (0/80) ^{b*}	–	Not defined	0.0 % (0/62) ^{f*}	–	Not defined
$\Delta ascC$	0.0 % (0/80) ^{b*}	–	Not defined	0.0 % (0/60) ^{f*}	–	Not defined
$\Delta aexT$	47.5 % (38/80) ^a	1.37	39 days	71.0 % (44/62) ^c	0.96	5 days
$\Delta aopH$	35.0 % (28/80) ^{c*}	1.68	49 days	67.2 % (41/61) ^c	0.98	5 days
$\Delta aopO$	52.5 % (42/80) ^a	0.99	29 days	84.9 % (56/66) ^c	0.86	5 days

*Indicates survival curve significantly different from that of A449 (Mantel–Haenszel test; $P < 0.05$).

†Data are percentage cumulative morbidity (number of morbid animals/total number of animals). Different superscript letters denote significant differences in cumulative morbidity between groups (G -test, $P < 0.05$).

‡Hazard ratio, ratio of mortalities in parental strain group to mortalities in mutant strain group.

§Median survival, time in days to 50 % mortality within a group. Not defined, could not be calculated.

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 $\Delta aopH$.

Intraperitoneal challenge

Intraperitoneal (i.p.) injection with $\sim 10^5$ c.f.u. per animal of either A449 or the isogenic mutant strains $\Delta aexT$, $\Delta aopH$ or $\Delta aopO$ 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 $\Delta ascC$ 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: $\Delta aexT$ (71.0 % cumulative morbidity), $\Delta aopH$ (67.2 %) and $\Delta aopO$ (84.9 %). The survival curves were not significantly different (Mantel–Haenszel test; $P > 0.05$). The hazard ratio was ~ 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 $\Delta ascC$ 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 $\Delta ascC$ -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 $\Delta ascC$ 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, $\Delta ascC$ or PBS, the latter two groups experienced high morbidity; PBS (57.5 % cumulative mortality) and $\Delta ascC$ (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 $\Delta ascC$ group (G -test; $P < 0.0001$).

Again, animals initially exposed to PBS or $\Delta ascC$ 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 ^1H NMR spectra of plasma

Metabolite profiles from plasma samples drawn from survivors of the immersion and rechallenge were compared by ^1H 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

Table 5. Cumulative morbidity and median survival data for Atlantic salmon re-exposed to a virulent strain of *A. salmonicida* (A449) after a prior exposure to the virulent strain, the avirulent isogenic $\Delta ascC$ mutant strain or PBS

Initial and second exposures were either both by immersion or both by intraperitoneal injection.

First strain	Second strain	Immersion		Intraperitoneal	
		Cumulative morbidity†	Median survival‡	Cumulative morbidity	Median survival
PBS	A449	57.5 % (23/40) ^{a*}	15 days	78.6 % (22/28) ^c	5 days
A449	A449	4.2 % (1/24) ^b	Not defined	28.6 % (2/7) ^d	Not defined
$\Delta ascC$	A449	57.9 % (22/38) ^{a*}	15 days	63.3 % (19/30) ^c	5 days
PBS	PBS	7.5 % (3/40) ^b	Not defined	0.0 % (0/32) ^e	Not defined

*Indicates survival curve significantly different from that of A449/A449 (Mantel–Haenszel test; $P < 0.05$).

†Data are percentage cumulative morbidity (number of morbid animals/total number of animals). Different superscript letters denote significant differences in cumulative mortality between groups (G-test, $P < 0.05$).

‡Median survival, time in days to 50 % mortality within a group. Not defined, could not be calculated.

immersion with A449 ($n = 9$); (IV) exposure by immersion to $\Delta ascC$ /rechallenge by immersion with A449 ($n = 8$) and (V) exposure by immersion to $\Delta ascC$ only ($n = 21$). PS, WG and CPMG spectra were recorded, giving three independent datasets, which were analysed separately by PCA (Aries *et al.*, 1991; Eriksson *et al.*, 1999). The spectral data of the naïve control group (PBS/PBS; group I) were compared with the spectra from each of the four bacteria-exposed groups (II–V) by analyses of scores plots (Fig. 1a–f). Fig. 1(a–d) shows the scores plots for PS spectra from groups II, III, IV and V compared to group I. The scores plots show clearly that the metabolite profiles of plasma from the A449/A449 group (group II) clustered together, occupying a distinct region separate from that of the PBS/PBS group (Fig. 1a), showing that the metabolic response to infection of the A449/A449 group was significantly different from that of naïve controls. The spectral profiles of groups III, IV and V clustered with those of the naïve group (Fig. 1b–d), indicating there was no difference in the metabolite profiles for these groups. Similar data were obtained after analysis of the WG and CPMG spectra; scores plots showing the comparison of the A449/A449 and PBS/PBS groups are shown in Fig. 1(e, f).

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 $\Delta ascC$ 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 $\Delta ascC$ 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 $\Delta ascC$ 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 $\Delta ascC$ 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 $\Delta ascC$ 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).

Fig. 1. (a–d) PCA scores plots (PC1 vs PC2) based on ^1H 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 ΔascC . The scores present the relationship between ^1H NMR spectral profiles of group I (PBS/PBS control) ($\blacklozenge$) with group II (A449/A449, a) ($\square$); with group III (PBS/A449, b) ($\triangle$); with group IV (ΔascC /A449, c) ($\circ$); and with group V (ΔascC only, d) ($\diamond$). 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.

Metabonomic analysis was also performed on these plasma samples. Metabonomics uses PCA of ^1H 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_2O 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 ΔascC .

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 (Trülsch *et al.*, 2004). Both ΔyopO and ΔyopE mutant strains of *Y. enterocolitica* O:8 were capable of colonizing mice and causing an overt disease following oral challenge; ΔyopO behaved as wild-type and was lethal whereas ΔyopE was able to colonize mice but did not cause an overt disease

and was eventually cleared. Virulence was abolished in the $\Delta yopH$ mutant strain. Similar findings are reported for *Y. pseudotuberculosis* TTSS effectors (Logsdon & Mecsas, 2003). In this study both $\Delta aexT$ and $\Delta aopO$ behaved as the parental strain; there was only a subtle reduction in the virulence of $\Delta aexT$. Whereas virulence was significantly reduced in $\Delta aopH$, 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 $\Delta aexT$ and $\Delta aopO$ in contrast to a more obvious effect on virulence in $\Delta aopH$ suggest that AopO and AexT either share intracellular targets or target the same process(es); thus in $\Delta aexT$ and $\Delta aopO$ 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, 2005; 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. The level of overt disease caused by $\Delta aopH$ was significantly reduced and $\Delta aexT$ was also attenuated. Full virulence was restored to these effector mutants when they were administered by injection, a route that does not require invasion. The apparent inability of the $\Delta ascC$ mutant to invade would explain the absence of involvement of the host's acquired immune system in the response to this strain.

Unlike the effector mutant strains, virulence was not restored to $\Delta ascC$ 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-unidentified exterior structure of the fish (Hiney *et al.*, 1997). Furthermore, the pathognomic clinical sign for furunculosis is a focal dermomyonecrotic lesion (furuncle) that arises from dermally, 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 it 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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