Expression of and secretion through the *Aeromonas salmonicida* type III secretion system

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Aeromonas salmonicida subsp. *salmonicida* is the aetiological agent of furunculosis, a disease of farmed and wild salmonids. The type III secretion system (TTSS) is one of the primary virulence factors in *A. salmonicida*. Using a combination of differential proteomic analysis and reverse transcriptase (RT)-PCR, it is shown that *A. salmonicida* A449 induces the expression of TTSS proteins at 28 °C, but not at its more natural growth temperature of 17 °C. More modest increases in expression occur at 24 °C. This temperature-induced up-regulation of the TTSS in *A. salmonicida* A449 occurs within 30 min of a growth temperature increase from 16 to 28 °C. Growth conditions such as low-iron, low pH, low calcium, growth within the peritoneal cavity of salmon and growth to high cell densities do not induce the expression of the TTSS in *A. salmonicida* A449. The only other known growth condition that induces expression of the TTSS is growth of the bacterium at 16 °C in salt concentrations ranging from 0.19 to 0.38 M NaCl. It is also shown that growth at 28 °C followed by exposure to low calcium results in the secretion of one of the TTSS effector proteins. This study presents a simple *in vitro* model for the expression of TTSS proteins in *A. salmonicida*.

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INTRODUCTION

Aeromonas salmonicida subsp. *salmonicida* is a Gram-negative rod that is the aetiological agent of furunculosis, a disease of farmed and wild salmonids. The disease is characterized by the presence of haemorrhagic and necrotic lesions in the gills, gut and muscle. This disease results in significant costs for salmon farmers through both direct losses and indirect costs associated with treatment and vaccination. Control of *Aeromonas* infections in aquaculture includes vaccination and the use of antibiotics (Ellis, 1997). However, the vaccines currently in use display considerable variability in efficacy and episodes of epizootic infections are common on fish farms (Lund *et al.*, 2003). To better understand *A. salmonicida* pathogenicity, the regulation and expression of *A. salmonicida* virulence factors require further exploration. A number of potential virulence factors for *A. salmonicida* have previously been identified, including the VapA surface layer protein, glycerolphospholipid-cholesterol acyltransferase (GCAT) and a serine protease, AspA (Lee & Ellis, 1990; Sheeran & Smith, 1981). The specific roles played by these putative virulence factors remain to be determined.

Numerous Gram-negative animal and plant bacterial pathogens utilize type III secretion systems (TTSSs) to establish host infections and evade the host's immune response (Cornelis & Van Gijsegem, 2000; Hueck, 1998). The TTSS is a complex assembly of proteins responsible for protein secretion through the bacterial inner and outer membranes, and translocation of effector proteins through the plasma membrane of the eukaryotic target cell. These bacterial effector proteins bring about profound changes in host-cell cytoskeletal dynamics, cell-to-cell communication and signal transduction (Cornelis & Van Gijsegem, 2000; Hueck, 1998). Pathogens possessing a TTSS cause a wide range of symptoms, including mild to severe diarrhoea, chronic infection of the lung and fatal septicaemia (Cornelis & Van Gijsegem, 2000). The TTSS has been characterized in a number of pathogenic bacteria, including *Yersinia* spp., *Salmonella* spp., *Shigella* spp. and in enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli* (reviewed by Cornelis *et al.*, 1998; Frankel *et al.*, 1998; Galan & Collmer, 1999; Jarvis *et al.*, 1995; Nhieu & Sansonetti, 1999). The TTSS machinery is evolutionarily related to bacterial flagella (Macnab, 1999; Minamino & Macnab, 1999) and is composed of at least 20 proteins involved in the structural machinery, chaperone activity, regulatory and effector functions.

Bacteria use a number of strategies to regulate expression of their respective TTSS machinery (Hueck, 1998). These

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Abbreviations: ACN, acetonitrile; 2-DE, 2-dimensional gel electrophoresis; OMP, outer-membrane protein; TTSS, type III secretion system.

strategies include cell-contact-mediated as well as contact-independent mechanisms. In *Yersinia*, cell contact induces the secretion of the effector proteins. A secretion factor that negatively regulates TTSS expression is itself secreted, leading to a general derepression of the TTSS. In *Yersinia* species, this cell-contact-mediated secretion (Pettersson *et al.*, 1996; Rosqvist *et al.*, 1994) can be mimicked *in vitro* by growing the bacterium in the absence of calcium (Cornelis *et al.*, 1998). The expression of TTSS can also be induced by environmental signals that mimic conditions encountered in the host. One such set of signals is the shift in the growth temperature of *Yersinia* from 26 to 37 °C in the absence of calcium (Cornelis *et al.*, 1986; Lambert de Rouvroit *et al.*, 1992). The temperature-induced activation of *Yersinia* TTSS expression and its repression by high calcium concentrations constitutes two distinct regulatory mechanisms. The temperature shift from 26 to 37 °C directly controls Yop expression at the transcriptional level (Cornelis *et al.*, 1989; Lambert de Rouvroit *et al.*, 1992), whereas high calcium concentrations inhibit the secretion pathway (Forsberg *et al.*, 1987).

Recently, Frey and co-workers have reported that *A. salmonicida* (strain JF2267) possesses the genes for a TTSS (Burr *et al.*, 2003a, 2002, 2003b; Stuber *et al.*, 2003). Using an *in vitro* fish cell line model, Burr and co-workers demonstrated that an *A. salmonicida* *ascV* knockout mutant (a gene encoding an inner-membrane component of the TTSS) was unable to secrete the ADP-ribosylating effector toxin, AexT, and that this mutant had significantly reduced cytotoxicity (Burr *et al.*, 2002). They further showed that laboratory strains deficient in the TTSS were avirulent using an *in vivo* Rainbow Trout challenge model, again suggesting that the TTSS in *A. salmonicida* plays a role in pathogenesis (Burr *et al.*, 2003b).

The TTSS genes in *A. salmonicida* A449 have been sequenced (M. Reith & others, unpublished); however, no reliable model is available to study the expression of the TTSS proteins. In this study, we demonstrate that *A. salmonicida* strain A449 induces the expression of its TTSS in response to growth at elevated temperatures. In addition, we show that *A. salmonicida* transcriptionally up-regulates the expression of the TTSS in response to increased NaCl in the growth medium at low temperatures. Growth at elevated temperatures in the absence of calcium results in TTSS-specific secretion of proteins into the medium.

METHODS

***A. salmonicida* strains and growth conditions.** *Aeromonas salmonicida* subsp. *salmonicida* strain A449, isolated from a diseased brown trout (*Salmo trutta*) in Normandy, France, in 1975, was provided by Dr William Kay (University of Victoria, Victoria, BC, Canada). The *A. salmonicida* subsp. *salmonicida* strain A449 genome is currently in the process of being sequenced and is therefore a natural choice for our studies. An S-layer-knockout strain (A449 Δ 03-06; Dacanay *et al.*, 2006) and an *ascC*-knockout (A449 Δ 03-23) was also used. All strains were cultured to mid-exponential growth

from glycerol stocks in tryptic soy broth (TSB; Difco) with agitation at temperatures ranging from 16 to 28 °C. For the majority of our experiments showing increased expression of type III secretion genes and proteins, the bacterium was inoculated into TSB and grown for periods of up to 18 h at 16 and 28 °C. Low-iron conditions were created by the addition of 120 μ M 2,2'-dipyridyl to TSB. For type III secretion protein expression studies, we used a defined medium (Griffin's medium, pH 7.0, containing 0.1% Casamino acids). Griffin's is a synthetic medium that is able to support *A. salmonicida* growth (O'Leary *et al.*, 1956). Bacterial numbers were estimated by measuring OD₆₀₀ or by direct colony counts on tryptic soy agar (TSA; Difco).

Construction of an *ascC* deletion mutant. An unmarked, in-frame *ascC* deletion mutant was constructed using crossover PCR (Link *et al.*, 1997) and introduced into *A. salmonicida* A449 by allelic exchange. Briefly, the upstream and downstream regions flanking *ascC* were amplified by PCR using the primers *ascC*-Ni (5'-CCCATCCACTAAACTTAAACAGCTCAAGGGTGCCATATCATC-3'), *ascC*-No (5'-TGTGCTCGAGCCTTGGGTCAGGATCACTTC-3'), *ascC*-Ci (5'-TGTTTAAGTTTAGTGGATGGGCGATGAGCTGGAAGTGCC-3') and *ascC*-Co (5'-ATATCACGATGCGGCCGCCACCTGACGGTAGTTTGGT-3'). The *ascC*-Ni and *ascC*-Ci primers contain a complementary 21 bp region that allows the joining of the PCR fragments in a second reaction using only the *ascC*-No and *ascC*-Co primers and the two PCR products as template. The assembled fragment was digested with *NotI* and *XhoI*, ligated to pWM91 (Metcalf *et al.*, 1996), a broad-host-range plasmid containing the R6K γ origin of replication that requires the Π replication protein encoded by *pir*, and the ligation mix was transformed into the *pir*-containing strain EC100D (Epicentre Biotechnologies). The resulting clone was confirmed by DNA sequencing and transformed into *E. coli* BW20767 (Metcalf *et al.*, 1996) for mobilization into *A. salmonicida*. The plasmid was transferred to *A. salmonicida* A449 by conjugation. Double crossovers were selected for resistance to 15% sucrose at 17 °C, since pWM91 contains the *B. subtilis* *sacB* gene, and sensitivity to ampicillin. Proper integration of the construct was checked by PCR using primers flanking the deletion. A full description of the *ascC*-knockout is available in Dacanay *et al.* (2006).

***In vivo* culture of *A. salmonicida*.** Atlantic salmon (*Salmo salar* Linnaeus 1758) were obtained from a hatchery certified under the Canadian Fish Health Protection Regulations. The *in vivo* culture was performed as described previously (Ebanks *et al.*, 2004). Briefly, *in vivo*-cultured bacteria were obtained by placing a bacterial suspension inside an autoclaved 12- to 14-kDa molecular-mass-cut-off dialysis tubing (Spectrapor; Spectrum Laboratories) and implanted in the abdominal cavities of anaesthetized juvenile Atlantic salmon. Fish were euthanized 24 or 48 h post-surgery with a lethal overdose of TMS. The implants were retrieved by dissection, then the contents were either placed directly in Qiagen RNeasy Protect or the bacteria were pelleted at 5000 g, washed once in cold PBS and frozen at -80 °C. Bacterial numbers within the implants were determined by direct colony counts. Typical bacterial counts were approximately 1×10^8 – 2×10^8 c.f.u.

Membrane protein extraction. Membrane proteins were obtained using a carbonate extraction method reported by Fujiki *et al.* (1982) with minor modifications. The PBS-washed bacterial pellet was resuspended in cold 50 mM Tris/HCl, 2 mM EDTA buffer, pH 8.0, containing a protease inhibitor cocktail (cocktail set 2; Calbiochem). The bacterial suspension was lysed using a French press at 16000 p.s.i. and centrifuged at 5000 g to remove unbroken cells. The supernatant was then extracted for 1 h with 10 vols ice-cold 100 mM sodium carbonate, pH 11.0. Membranes were separated from the cytosol by centrifugation at 115000 g (Beckman TLA 100.3 rotor) for 1 h. The membrane-containing pellet was resuspended in 50 mM

Tris/HCl, pH 8.0, and centrifuged again at 115 000 *g* for 30 min to remove contaminating cytosolic proteins. The final pellet was resuspended in rehydration buffer (7 M urea, 2 M thiourea, 2 % CHAPS).

2-Dimensional gel electrophoresis (2-DE). Protein samples (200–500 µg total carbonate-extracted protein) were diluted 1:10 with rehydration buffer containing 2 mM DTT and 0.5 % carrier ampholytes, and applied to 18 cm Immobiline drystrips, pH 4–7 (Amersham–Pharmacia). Preliminary experiments using a pH gradient of 3–10 showed that the vast majority of the proteins appeared in the pH range 4–7. Isoelectric focusing was carried out using the Multiphor II system (Amersham–Pharmacia) using linear pH gradient strips. Focusing was initiated at 30 V for 10 h and then increased to 8000 V over a 6 h period, and held at 8000 V for 3 h. The sample strips were then equilibrated in 50 mM Tris/HCl, pH 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % SDS, 65 mM DTT, then equilibrated for a second time with 50 mM Tris/HCl, pH 8.8, 6 M urea, 30 % (v/v) glycerol, 2 % SDS, 135 mM iodoacetamide. The second dimension was run on a 1 mm thick, 14 %T (total acrylamide and cross-linker) SDS-PAGE gel for 2100 V h. Proteins were visualized using silver staining (Shevchenko *et al.*, 1996) and spots of interest were excised for identification by mass spectrometry.

Secreted protein analysis. Cultures of *A. salmonicida* were grown overnight at 28 °C in TSB, washed in Griffin's medium, resuspended in Griffin's medium with or without 5 mM EGTA and incubated for 4 h at 28 °C. The cells were separated from the culture supernatant by centrifugation (15 000 *g* for 15 min), the resultant supernatant was filtered through a 0.2 µm filter and the secreted proteins were precipitated with ice-cold 10 % trichloroacetic acid in acetone. The samples were then centrifuged at 15 000 *g* for 15 min and the precipitated proteins were washed twice with ice-cold acetone, followed by air-drying. The proteins were directly solubilized in SDS-PAGE sample buffer. The proteins were separated by SDS-PAGE on a 12 %T polyacrylamide gel, visualized by silver staining (Shevchenko *et al.*, 1996) and bands of interest were excised for identification by mass spectrometry.

In-gel trypsin digestion. The excised gel spots were placed into 96-well plates, washed three times with 100 µl 50 % acetonitrile (ACN) in 25 mM NH₄HCO₃ for 10 min and then dehydrated in 200 µl 100 % ACN for 10 min. Proteins were reduced with 200 µl 10 mM DTT in 25 mM NH₄HCO₃ at 56 °C for 1 h, followed by alkylation in 200 µl 25 mM NH₄HCO₃ containing 55 mM iodoacetamide in the dark at room temperature for 45 min. The reduced and alkylated gel pieces were washed twice with 100 µl 25 mM NH₄HCO₃ and 50 % ACN in 25 mM NH₄HCO₃ for 10 min. Gel pieces were dehydrated with 200 µl 100 % ACN for 20 min and placed in a

Speedvac for 10 min to remove residual ACN. The gel pieces were rehydrated by adding 20 µl 12.5 ng trypsin ml⁻¹ (Promega) in 25 mM NH₄HCO₃ and incubated at 37 °C overnight. The tryptic peptides were eluted from the gel with two sequential extractions with 20 µl 5 % formic acid and concentrated in a Speedvac.

Mass spectrometry protein identification. Peptides were separated by reverse phase chromatography using an Eksigent nanoLC system equipped with a 15 cm × 100 mm (i.d.) Chromolith caprod C₁₈ column (Merck). The HPLC separation was carried out by injecting the sample and washing in solvent A (5 % ACN, 0.2 % formic acid) for 5 min followed by using a linear ACN gradient from 15 to 50 % B (90 % ACN, 0.2 % formic acid) over 20 min at a flow rate of 5 ml min⁻¹. The HPLC was interfaced to an AB/MDS-SCIEX QTrap mass spectrometer via a nanoflow source. Data were acquired in the information-dependent acquisition mode, i.e. the *m/z* values of the tryptic peptides were measured using a mass spectrometry scan in the linear ion trap. These data were used to generate a peak list of peptides for tandem MS analysis with parent ion selection in Q1, collision-induced dissociation occurring in Q2 and fragment ion scans performed in the linear ion trap. The tandem mass spectra were submitted to the database search program MASCOT (Matrix Science) to identify the proteins. MASCOT results were filtered using a Perl script that removed peptides with a score below the 95 % significance threshold or a peptide mass error greater than 0.5 Da.

RNA extraction and reverse transcriptase (RT)-PCR. For each culture, an aliquot (usually 0.5 ml culture with an OD₆₀₀ of 0.6–1.0) was collected in RNAprotect Bacteria Reagent (Qiagen), according to the manufacturer's protocol. All cell density readings were adjusted to an OD₆₀₀ below 0.4. At these OD₆₀₀ readings, the cell density was empirically determined to be linear with absorption and contained 2 × 10⁸ cells ml⁻¹. Following cell lysis with 1 mg lysozyme ml⁻¹ for 5 min at room temperature, total RNA was isolated using the RNeasy Mini Kit (Qiagen). RNA was treated with DNase I using Ambion's DNA-free protocol. First strand cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Each cDNA reaction contained 1 µg RNA and 5 µM random decamers (Ambion). Control reactions lacking reverse transcriptase were performed in parallel to verify that the RNA samples did not contain any contaminating genomic DNA. The cDNA was used as a template for PCR amplification (0.8 µl cDNA reaction per 20 µl PCR reaction). The primers used for PCR were based on *A. salmonicida* A449 genomic DNA sequences (unpublished) and are listed in Table 1, along with the PCR product sizes. The *tapA* gene was used as an internal control since its expression is not altered under a wide range of culture conditions (J. M. Boyd & others,

Table 1. Primer sets used for RT-PCR analysis of *A. salmonicida* genes

Gene	Sequence (5'→3')		PCR product (bp)*
	Forward primer	Reverse primer	
<i>acrV</i>	GTAAGGGTTGCGGGATGAG	GCCGCTCTTCTTCAGGTCAC	416
<i>tapA</i>	ACATGAAGAAGCAATCAGGC	AGAGGTCATGCGTTAGCAG	443
<i>aexT</i>	CAGATTCAAGCAAACACCGTCGGC	CAGCCCGGTAGCCAACTCCCG	501
<i>aopH</i>	GATCAGCTGACCCTCAAGGA	GAAGCCAGGACTGCCAGAAC	524
<i>aopN</i>	GCATCAGCGAGATCGAGG	CGGCTGTGAATATCGC	430
<i>aopO</i>	AGAGCGATCTCTTCTCGTG	CTGATCGGCAAACCTCTGAC	506
<i>ascC</i>	GCATTGGAGCAACAGTCCCA	CCTTCAATCCCCTTGCGAT	476

*The size denotes the number of base pairs in the amplicon.

unpublished). An annealing temperature of 60 °C was used for all amplification reactions. All RT-PCR experiments were repeated at least twice from two independent biological replicates.

PCR analysis of plasmid thermostability in *A. salmonicida*.

Cultures of *A. salmonicida* were grown at 16 °C to an OD₆₀₀ of 0.3 in TSB. The cultures were then transferred to a 28 °C incubator and aliquots of bacteria were sampled at 0, 7, 18 and 30 h. Genomic DNA was isolated from the cultures using a Puregene genomic DNA purification kit (Gentra). The presence of the plasmid was analysed by PCR amplification using 10 ng genomic DNA as template and primers for *ascC* (located on the plasmid, see Table 1). To ensure that our PCR reaction was in the linear range, two PCR reactions of 25 and 28 cycles were run simultaneously. Primers for the *tapA* gene (located on the chromosome, see Table 1) were used as an internal control. The band intensities were obtained using Quantity One software version 4.5.2 (Bio-Rad).

Database searching. Two *A. salmonicida* databases were used for MASCOT searches: (1) a nucleotide database consisting of all contigs in the latest *A. salmonicida* genome sequence assembly (unpublished data); and (2) a protein database of all *A. salmonicida* ORFs with

hits in GenBank. The latter was constructed using a Perl script to automate the identification and searching (BLASTP) of ORFs and the compilation of the ORF sequence with a description indicating its best GenBank hit.

RESULTS

Comparative proteomic analysis of *A. salmonicida* outer-membrane proteins (OMPs) grown at 16 vs 28 °C

Growth of *A. salmonicida* close to its upper lethal temperature of 30 °C results in a loss of virulence that correlates with the loss of the S-layer VapA protein (Ishiguro *et al.*, 1981). To characterize the OMPs of *A. salmonicida* in relation to growth temperature, we grew *A. salmonicida* at both 16 and 28 °C and performed 2-DE analysis of the membrane protein extracts (Fig. 1A, B). The most obvious difference between the two growth temperatures is the loss of the

Fig. 1. 2-DE (pH 4–7) of carbonate-insoluble membrane proteins from *A. salmonicida* A449 (A, B) or *A. salmonicida* A449 Δ vapA mutant (C, D), grown in TSB at 16 °C (A, C) or 28 °C (B, D). The S-layer protein is highlighted in the box in (A). Proteins induced in response to growth at 28 °C are circled.

S-layer protein at 28 °C. In addition, several proteins from 28 °C-grown cultures have higher intensities than the corresponding proteins in 16 °C-grown cultures (Fig. 1A, B). Four of these proteins were identified as belonging to the *A. salmonicida* TTSS: AscC, AcrV, AopD and Acr2. Based on homologies with other TTSS systems, the *A. salmonicida* AscC protein is predicted to form the channel through which the TTSS proteins are secreted across the outer membrane (Allaoui *et al.*, 1993; Ginocchio *et al.*, 1994; Haddix & Straley, 1992) and is the only component of TTSSs that is clearly located in the outer membrane (Plano & Straley, 1995). The remaining TTSS proteins identified are associated with the TTSS apparatus membrane components (Table 2).

The loss of the S-layer protein at high temperature affects the relative amount of proteins that are loaded on the gel. Therefore, an apparent increase in Acr2 (for example) may be due to the absence of VapA or a true up-regulation due to temperature. To confirm the increased expression of the TTSS proteins at 28 °C, an *A. salmonicida* VapA-knockout was grown at both 16 and 28 °C and the proteomic analysis was repeated (Fig. 1C, D). Fewer spots appeared to be up-regulated than were seen in the wild-type bacteria. Of these, eight membrane-associated proteins were identified, including the four TTSS proteins identified above as well as two additional TTSS proteins (Table 2). Two isomers of AopN were identified which differed in their isoelectric points by 0.3 pH units (Fig. 1C, D). The significance of the differing pIs was not determined. The other two proteins were identified as having increased expression at 28 °C: a 16 kDa heat-shock protein A homologue (HspA) and a 17 kDa unidentified protein. The HspA is not associated with the TTSS. These results indicate that *A. salmonicida* A449 up-regulates the expression of its primary virulence mechanism when grown at elevated temperatures.

Comparative proteomic analysis of *A. salmonicida* cytosol grown at 16 vs 28 °C

We studied the effect of growth at elevated temperature on the cytosolic proteins. Fig. 2 shows the comparative 2-DE of the cytosolic extracts of *A. salmonicida* A449 grown at 16 and 28 °C. Four spots were consistently differentially expressed in the 28 °C cytosolic gel. Mass spectrometric analysis of these spots resulted in the identification of all four spots. Three of the identified proteins (AcrH, AcrV and AopN) belonged to the *A. salmonicida* TTSS. AcrH is the homologue of the *Yersinia* LcrH protein, which has been reported to function as a chaperone for the *Yersinia* YopD protein (Francis *et al.*, 2000; Wattiau *et al.*, 1994). The other up-regulated protein identified was a homologue of *Salmonella enterica* OMP S1.

Transcriptional profile of *A. salmonicida* temperature-induced expression of TTSS by RT-PCR

Having observed that growth at 28 °C induces expression of some of the *A. salmonicida* TTSS-associated proteins, we then examined the induction of the TTSS in bacteria grown at 17, 20, 24 and 28 °C. To more quickly assay the expression of the TTSS induction, we used RT-PCR. Primers specific for the most abundantly expressed TTSS gene, *ascC*, and one *Yersinia* effector protein homologue, *aexT*, were designed and their relative expression was assessed by RT-PCR (Fig. 3). The qualitative data show that both the mRNAs for the *ascC* structural gene and for the *aexT* effector gene were up-regulated by growing *A. salmonicida* A449 at 28 °C. There was a slight increase in both the *ascC* and *aexT* mRNAs as the temperature was increased from 17 to 24 °C; however, the largest change in expression occurred at 28 °C for both genes.

Table 2. Summary of proteins identified from 2-DE gels

Protein	Putative function*	Cellular fraction†	TTSS protein	Sequence coverage (%)‡	Score§	No. of peptides	Mass (kDa)
AscC	Secretin	M	Yes	46	1621	27	67
AcrV	Protective antigen, anti-host factor	M/C	Yes	51	1873	29	37
AopD	Translocation apparatus	M	Yes	54	191	4	35
AopN	Regulation of translocation	M/C	Yes	51	159	3	32
AscG	Chaperone	M	Yes	13	64	1	12
Acr2	Chaperone	M	Yes	33	192	4	13
AcrH	Chaperone	C	Yes	21	72	2	21
OMPS1	Unknown	C	No	9	63	2	38
HspA	Heat-shock protein	M	No	39	362	6	15
Ati-1	Unknown	M	Unknown	NI	—	—	18

*Putative functions are based on homologies in other bacteria.

†Cellular fraction in which the protein was identified: M, membrane fraction; C, cytosolic fraction.

‡Sequence coverage refers to the percentage of the total protein identified by peptide sequencing. NI, Not identified.

§Score from MASCOT search; scores above ~25 are highly significant.

||Molecular mass of the predicted protein.

Fig. 2. 2-DE (pH 4–7) of *A. salmonicida* cytosolic proteins in response to growth in TSB at 16 (A) or 28 °C (B). Proteins up-regulated in response to growth at 28 °C are indicated on the gel.

The mRNA level of the constitutively expressed *tapA* did not vary with temperature. Taken together, these results suggest that growth at elevated temperatures above 24 °C up-regulates the transcription of both structural and effector TTSS genes in *A. salmonicida* with maximum induction occurring with growth at 28 °C.

Fig. 3. Reverse transcriptase PCR analysis of expression of *A. salmonicida* A449 *aexT*, *ascC* and *tapA* genes as a function of growth temperature. PCR templates were first strand cDNA (+RT), RNA (–RT) or genomic DNA (gDNA). The number of PCR cycles for the corresponding product is indicated in parentheses. Representative experiments from at least two independent biological replicates are shown.

Expression of TTSS genes under *in vivo* or low-iron growth conditions as assessed by RT-PCR

In other TTSS-containing bacteria, a number of *in vitro* and *in vivo* conditions have been found that result in the induction of the type III virulence genes (Hueck, 1998). RT-PCR was used to determine whether growth of *A. salmonicida* under low iron conditions and *in vivo*, which is also a low-iron environment (Ebanks *et al.*, 2004), could induce expression of the TTSS genes. These experiments were carried out by either growing *A. salmonicida* A449 within the peritoneal cavity of salmon or inducing low-iron conditions by growing the bacterium in TSB with 120 µM 2,2'-dipyridyl at 17 °C followed by total RNA isolation. The gene expression levels of three *A. salmonicida* effector genes (*aexT*, *aopH*, *aopO*), the gene for the putative stop valve protein (*aopN*), the *ascC* structural gene, the *acrV* gene, which might be a functional element of the protein translocation apparatus, and the *tapA* control were investigated. Qualitatively, the expression of five TTSS genes (*acrV* was not tested under these conditions) was unaffected by growth *in vivo* (Fig. 4). Therefore, serum factors within the salmon do not lead to the up-regulation of the *A. salmonicida* TTSS. Similarly, the expression levels of all six TTSS genes were the same when grown under either iron-rich or iron-limiting conditions.

Investigation of TTSS expression under other conditions

In some organisms, expression of the TTSS is controlled by quorum sensing (Sircili *et al.*, 2004). Therefore, we wanted

Fig. 4. RT-PCR analysis of *A. salmonicida* A449 TTSS genes *aexT*, *aopH*, *aopO*, *aopN*, *ascC*, *acrV* and *tapA* in response to growth inside *Salmo salar* (*in vivo*), growth *in vitro* in TSB at 17 °C, TSB+120 µM dipyrindyl at 17 °C, or TSB at 28 °C (*in vitro*). PCR templates were first strand cDNA (+RT), RNA (-RT), genomic DNA (gDNA) or water. The number of PCR cycles for the corresponding product is indicated in parentheses. Representative experiments from at least two independent biological replicates are shown.

to determine if *A. salmonicida* grown at 17 °C regulates its TTSS as a function of cell density. The experiment was carried out by harvesting total RNA from *A. salmonicida* A449 grown at 17 °C to cell densities ranging from an OD₆₀₀ of 0.6 to 8.2, followed by RT-PCR. As shown in Fig. 5(a), qualitative observations show that mRNA expression of the *aexT* and the *ascC* genes did not increase when cells were grown to densities ranging from 0.6 to 8.5. However, equivalent cell densities at 28 °C resulted in increased expression of both *aexT* and *ascC* genes (Fig. 5b). This experiment suggests qualitatively that cell densities ranging from 0.6 to 8.2 at 17 °C do not affect the expression of the *A. salmonicida* A449 TTSS.

In the experiments described so far, growth times were between 12 and 24 h at a constant temperature of 28 °C. We wanted to determine if a short exposure of *A. salmonicida* A449 to 28 °C could induce the expression of the TTSS genes. This experiment was carried out by growing *A. salmonicida* A449 at 16 °C, followed by exposure to 28 °C for 30 or 120 min, after which total RNA was prepared and RT-PCR performed. As shown in Fig. 5(c), exposure of *A. salmonicida* A449 to 28 °C for as little as 30 min induced the expression of both *aexT* and *ascC*, but not *tapA*. Exposure of the bacteria to 28 °C for 120 min showed the same qualitative level of expression as that at 30 min exposure. To address if temperature difference is the true signal for TTSS expression, we grew the bacterium at 16 °C overnight and then exposed the cultures to 4 °C for 2 h, followed by 30 min exposure to 16 °C. Total RNA was isolated from the 16 °C overnight culture, the culture exposed to 4 °C and the 4–16 °C culture, and the relative mRNA expression levels of the *ascC* gene and the *tapA* control gene were assessed qualitatively by RT-PCR. We found no differences in the

Fig. 5. RT-PCR analysis of *A. salmonicida* A449 *aexT*, *ascC* and *tapA* genes in response to growth density at 17 °C (a), growth at 28 °C (b), heat shock at 28 °C for 30 and 120 min (c), growth in TSB with added NaCl at 17 °C (d) and growth at 17 °C in the presence of EGTA (e). PCR templates were first strand cDNA (+RT), RNA (-RT) or genomic DNA (gDNA). The number of PCR cycles for the corresponding product is indicated in parentheses. Representative experiments from at least two independent biological replicates are shown.

expression levels of the *ascC* structural gene when exposed to 4 °C for 30 min, nor when the temperature was raised by placing the 4 °C culture at 16 °C for 2 h (data not shown).

One environmental factor known to induce the expression of TTSS genes in other organisms is increased NaCl levels (Walker & Miller, 2004; Young & Young, 2002). Preliminary experiments established that *A. salmonicida* A449 could grow in salt concentrations up to 380 mM NaCl in TSB at 16 °C. However, the growth rate was much slower than when grown in TSB at 28 °C (data not shown). To evaluate if salt concentration plays a role in the expression of *A. salmonicida* A449 TTSS genes, bacteria were grown in TSB with 0.19 and 0.38 M NaCl (normal salt content in TSB is 0.085 M), and the qualitative mRNA levels were determined by RT-PCR. Growth in 0.19 M salt at 17 °C induces the expression of the *ascC* and *aexT* mRNAs above the level seen for the OD₆₀₀ matched control (OD₆₀₀ = 0.6) (Fig. 5d). Increasing the salt concentration to 0.38 M NaCl further increases the expression of both mRNAs when compared to the OD₆₀₀ matched control (OD₆₀₀ = 1.05).

In some organisms, the pH of the growth environment has been shown to induce the expression of TTSS genes (Nakayama & Watanabe, 1995). *A. salmonicida* is sensitive to the pH of the culture medium since it cannot grow at pH values below 6.2 (data not shown). Using proteomic analysis of the *A. salmonicida* A449 OMPs, we saw no evidence of expression of the AscC protein when the bacteria were grown at pH 6.2, nor when the bacteria were grown at pH 7.2 followed by 4 h at pH 5.2 (data not shown). These results suggest that *A. salmonicida* A449 does not induce the expression of its TTSS genes in response to low pH stress.

Comparative proteomic analysis of *A. salmonicida* proteins secreted at 28 °C

One of the first and most characterized environmental factors influencing the secretion of TTSS gene products is calcium ion concentration (Hueck, 1998). In *Yersinia*, *in vitro* secretion of the Yop effector proteins occurs in calcium-depleted medium (Forsberg *et al.*, 1987; Straley & Bowmer, 1986). This low-calcium response probably mimics cell-contact-mediated secretion of TTSS proteins (Pettersson *et al.*, 1996). Chelation of calcium using EGTA and other metal chelators at 37 °C, has been shown to lead to leakage of TTSS effector proteins and the subsequent transcriptional up-regulation of TTSS genes. We wanted to determine whether *A. salmonicida* secretes its effector proteins in response to calcium chelation and growth at 28 °C. Unfortunately, growth at 28 °C also results in increased cell death and lysis and the resulting contamination of the cell culture supernatant by cytosolic proteins complicates the analysis. To overcome this problem, an *ascC* deletion mutant that is blocked in TTSS secretion was used as a control for proteins secreted through the TTSS. *A. salmonicida* A449 and A449 Δ *ascC* were grown overnight in TSB at 28 °C, equivalent OD₆₀₀ cells harvested, washed and resuspended in defined media with or without EGTA at 28 °C for 4 h. The

extracellular medium was then harvested, and the secreted proteins were precipitated and analysed by one-dimensional SDS-PAGE. As shown in Fig. 6, growth of *A. salmonicida* A449 at 28 °C followed by exposure to EGTA resulted in the heightened secretion of two bands in the extracellular medium at approximately 48 and 30 kDa. Analysis of both bands resulted in the identification of the same protein (AopH, predicted molecular mass of 50 kDa), an *A. salmonicida* homologue of the *Y. pestis* Yop protein-tyrosine phosphatase TTSS effector protein. Presumably, the lower molecular mass band is a degradation product of the upper band. These bands were not apparent in the *ascC* non-polar mutant exposed to EGTA, consistent with the inability of this mutant to express the AscC channel protein. Initial experiments at 17 °C, with and without EGTA, showed no difference in the secreted proteins at that temperature. This indicates that temperature is indeed critical and that EGTA itself does not cause the specific secretion. In high-calcium medium (no EGTA), wild-type *A. salmonicida* A449 secreted a 34 kDa protein identified as AopD that was not detectable in the low-calcium (EGTA) lane. The AopD band was also absent from the A449 Δ *ascC* mutant culture supernatant, suggesting that its secretion is TTSS-dependent. AopD is the homologue of *Yersinia* YopD that forms the translocon pore in the target cell.

Fig. 6. Silver-stained 1D SDS-PAGE of the extracellular medium of wild-type *A. salmonicida* A449 and the *A. salmonicida* A449 Δ *ascC* mutant in the presence or absence of the calcium chelator EGTA. Gel sections that appeared to have differentially secreted proteins and that were subsequently excised and analysed by mass spectrometry are highlighted in boxes.

PCR analysis of plasmid thermostability in *A. salmonicida*

It has been reported previously that growth of some strains of *A. salmonicida* at elevated temperatures (25 °C and above) can result in the loss of the major plasmid carrying the type III secretion genes (Stuber *et al.*, 2003). We wanted to determine whether *A. salmonicida* A449 cultured at 28 °C results in the loss of the major plasmid carrying the type III secretion genes. The result of this experiment is shown in Fig. 7. Growth of *A. salmonicida* A449 at 17 °C followed by growth for up to 30 h at 28 °C did not result in a reduction of the plasmid-specific amplification of the *ascC* gene product. PCR amplification of the chromosomal *tapA* gene served as an internal control. Densitometric analysis of the *ascC/tapA* intensity ratios showed a mean ratio of 0.32 ± 0.056 . These results clearly show that at least with *A. salmonicida* A449, and under the culture conditions of these experiments, growth of this bacterium at temperatures up to 28 °C does not result in the loss of the plasmid carrying the type III secretion genes.

DISCUSSION

Many animal pathogens sense that they have infected their host via environmental indicators, such as the host's body temperature (37–41 °C), osmolarity, pH, low iron levels and various other host-derived signalling molecules (Hueck, 1998; Konkel & Tilly, 2000). The intimate coupling of TTSS protein production with infection ensures efficient use of energetically expensive machinery. We have shown that the TTSS of *A. salmonicida* A449 is induced upon exposure of the bacteria to temperatures in the range of 24–28 °C. This expression of the TTSS can be induced with exposure to elevated temperatures for as little as 30 min. Growth of *A. salmonicida* under stress conditions such as low iron, and exposure to host serum, low pH and high cell densities did not result in increased expression of the TTSS. The results suggest that the temperature-induced up-regulation of the

TTSS in *A. salmonicida* is a specific response and not a general stress response.

In general, most organisms that induce the TTSS in response to temperature do so as an indication that the bacterium is inside a homeothermic host (Hueck, 1998). *A. salmonicida* temperature-induced up-regulation of the TTSS is unexpected since the typical hosts for the bacterium are poikilotherms. In the case of salmonids, the fish are rarely, if ever, exposed to temperatures above 20 °C. Thus, temperature-induced expression of the TTSS may not be associated with pathogenesis in salmon. Alternatively, it may indicate a recent gain through horizontal gene transfer (Gophna *et al.*, 2003). A number of *Aeromonas* spp. cause a wide variety of infections in homeotherms, including septicæmia, wound infections, meningitis, pneumonia and gastroenteritis (Figueras *et al.*, 2000; Janda & Abbott, 1998). Using a hybridization assay, Chacón and co-workers showed that approximately 50 % of human clinical isolates of *Aeromonas* possess the TTSS with a higher prevalence among *Aeromonas hydrophila* and *Aeromonas veronii* (Chacon *et al.*, 2004). It would be interesting to see if temperature induces the up-regulation of the TTSS proteins in these *Aeromonas* species that can grow at 37 °C.

We wanted to test if the temperature-induced expression of the TTSS proteins in *A. salmonicida* also results in the assembly of a functionally competent secretion complex. Chelation of calcium by EGTA at 28 °C resulted in the TTSS-specific secretion of at least one effector protein (AopH). This suggests that the translocation apparatus is assembled and can transport proteins from the bacterial cytoplasm to the extracellular space. Calcium chelation also resulted in lower levels of AopD being secreted into the extracellular medium. This suggests that low calcium concentrations may play a role in inhibiting the secretion of AopD at high temperatures. No evidence of this phenomenon was evident in the strain A449 $\Delta ascC$ mutant, suggesting that this is a TTSS-specific event. Work by Williams & Straley (1998) has demonstrated that *Y. pestis* YopD is involved in the negative regulation of the low-calcium response in addition to its role in translocation of Yops. While we do not understand the significance of AopD in the high-calcium secreted fraction, the results are consistent with the recent suggestion that YopD might also play a role in feedback inhibition of the TTSS (Lee *et al.*, 2001; Wulff-Strobel *et al.*, 2002).

Two other proteins, an HspA homologue and an unidentified protein (herein referred to as Ati-1, for *Aeromonas* temperature-induced protein), were up-regulated at 28 °C and have no association with other TTSSs in the literature. Because growth at 28 °C is very close to the lethal temperature limit for *A. salmonicida*, it not unexpected to see increased expression of heat-shock proteins. *A. salmonicida* HspA is a small, highly conserved, heat-induced heat-shock protein. These small heat-shock proteins have been reported to have a large number of functions, ranging from thermo-tolerance to acting as molecular chaperones (Hendrick & Hartl, 1993). A TBLASTN search against the partially

Fig. 7. PCR analysis of *A. salmonicida* A449 plasmid stability as a function of growth temperature. *A. salmonicida* A449 was cultured at 17 °C (0 h), after which the culture was transferred to a 28 °C incubator and samples were taken after 7, 18 and 30 h. The presence of the plasmid was assayed by PCR using plasmid-specific primers (*ascC*). Primers for the *tapA* gene (located on the chromosome) were used as an internal control. The control PCR reactions had no DNA template added. The number of PCR cycles is indicated in parentheses.

sequenced *A. salmonicida* genome suggests that the bacterium has a single copy of the HspA gene which is not localized on a plasmid, nor does it reside within the vicinity of any other TTSS genes, suggesting it is not a TTSS protein. The other protein (Ati-1) could not be identified; however, it was consistently up-regulated at 28 °C.

We have also found that growth of *A. salmonicida* in salt concentrations ranging from 0.19 to 0.38 M NaCl (Fig. 5) qualitatively induces the expression of TTSS genes in *A. salmonicida* A449. The most interesting aspect of the low-temperature salt induction of the *A. salmonicida* A449 TTSS is the relationship between salt and high temperature with respect to the TTSS. In *Yersinia enterocolitica*, pathogenic strains carry the pYV plasmid encoding the Ysc-Yop TTSS, which operates at 37 °C (Foultier *et al.*, 2003). However, the biovar 1B *Y. enterocolitica* strains possess a second TTSS, designated the Ysa, which is induced under conditions of low temperature and high salt (0.20–0.49 M NaCl) (Haller *et al.*, 2000; Young & Young, 2002). However, we found no increased expression of TTSS genes at 0.15 M salt (physiological salt concentration) in the *in vivo* implant experiment (Fig. 4). This suggests that 0.15 M NaCl is below the concentration for TTSS induction, or factors in addition to salt must play a role in the *in vivo* expression of the TTSS in *A. salmonicida*. We have shown that as little as 0.19 M NaCl in TSB can activate TTSS gene expression in *A. salmonicida*. Thus exposure of *A. salmonicida* to sea water (0.48 M NaCl) may induce expression of the TTSS.

Work by Stuber *et al.* (2003) has shown that the type III secretion genes in *A. salmonicida* subsp. *salmonicida* JF2267 are located on a 140 kDa plasmid. They demonstrated plasmid loss for this strain within the first 6 h of growth at 25 °C by using Southern hybridization with probes for *ascV*. In contrast, *A. salmonicida* JF2267 cultured at 18 °C retained *ascV* even after 24 h incubation. Stuber *et al.* (2003) further showed that secretion of the extracellular ADP-ribosyltransferase toxin (AexT) was lost when the bacterium was cultured at 25 °C. In the *A. salmonicida* A449 strain used in the current study, the plasmid containing the majority of the TTSS genes is 155 kb in size (M. Reith & others, unpublished). In contrast to the JF2267 strain used by Stuber *et al.* (2003), we observed that growth of *A. salmonicida* subsp. *salmonicida* A449 at 28 °C resulted in the induction of the TTSS and that the plasmid is stably maintained at 28 °C for up to 30 h (Fig. 7). Previous research by Ishiguro *et al.*, (1981), showing loss of virulence of *A. salmonicida* when cultured at high temperatures, demonstrated that this loss of virulence was correlated with the loss of the S-layer protein and was not the result of plasmid loss. Our results are consistent with those of Ishiguro and co-workers rather than the plasmid loss mechanism presented by Stuber and co-workers. We have observed, however, that growth of *A. salmonicida* at elevated temperatures activates transposons, with the resultant loss of other genes, primarily the VapA protein gene (Belland & Trust, 1985). Strain JF2267 used by Stuber *et al.* (2003) is a virulent strain isolated from an

Arctic char (*Savelinus alpinus*) (Braun *et al.*, 2002). The discrepancy in the reported stability of the plasmids encoding the TTSS suggests strain-specific variability in the genetic as well as the regulatory components of the TTSS.

In further examining the results from a limited number of published papers, we can see evidence for genetic and regulatory differences in the TTSS of different *A. salmonicida* strains (Burr *et al.*, 2003a, b, 2002; Stuber *et al.*, 2003). Work by Burr and co-workers, using the *A. salmonicida* subsp. *salmonicida* strain JF2646, showed secretion of the AexT toxin under low-calcium conditions at 18 °C. However, in strain A449, we saw no evidence by using RT-PCR of TTSS low-calcium-induced expression of *aexT* or *ascC* at 17 °C (Fig. 5e). In our hands, evidence for the low calcium secretion of the TTSS in *A. salmonicida* A449 was only seen when the culture was exposed to a temperature of 28 °C.

In conclusion, we have demonstrated that *A. salmonicida* subsp. A449 induces the expression of its TTSS in response to growth at high temperatures. We have also shown that at the permissive temperature and in low-calcium medium, *A. salmonicida* A449 can secrete effector proteins into the extracellular medium, in a TTSS-dependent manner. We have also demonstrated that salt concentrations ranging from 0.19 to 0.39 M NaCl could also induce the expression of the TTSS in *A. salmonicida*. This study provides simple *in vitro* models to study the expression of TTSS proteins in *A. salmonicida*.

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