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Aeromonas salmonicida Ati2 is an effector protein of the type three secretion system

Stéphanie Dallaire-Dufresne,[1,2,3] Xavier Barbeau,[1,2] Darren Sarty,[4]† Katherine H. Tanaka,[1,2,3] Alix M. Denoncourt,[1,2,3] Patrick Lagüe,[1,2] Michael E. Reith[4] and Steve J. Charette[1,2,3]

Correspondence
Steve J. Charette
steve.charette@bcm.ulaval.ca

[1]Institut de biologie intégrative et des systèmes, Faculté des sciences et de génie, Université Laval, Quebec City, Quebec G1V 0A6, Canada

[2]Département de biochimie, de microbiologie et de bio-informatique, Faculté des sciences et de génie, Université Laval, Quebec City, Quebec G1V 0A6, Canada

[3]Centre de recherche de l'Institut universitaire de cardiologie et de pneumologie de Québec, Quebec City, Quebec G1V 4G5, Canada

[4]Aquatic and Crop Resource Development, National Research Council Canada, Halifax, Nova Scotia B3H 3Z1, Canada

The bacterium *Aeromonas salmonicida*, a fish pathogen, uses the type three secretion system (TTSS) to inject effector proteins into host cells to promote the infection. The study of the genome of *A. salmonicida* has revealed the existence of Ati2, a potential TTSS effector protein. In the present study, a structure–function analysis of Ati2 has been done to determine its role in the virulence of *A. salmonicida*. Biochemical assays revealed that Ati2 is secreted into the medium in a TTSS-dependent manner. Protein sequence analyses, molecular modelling and biochemical assays demonstrated that Ati2 is an inositol polyphosphate 5-phosphatase, which hydrolyses PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 in a way similar to VPA0450, a protein from *Vibrio parahaemolyticus* having high sequence similarity with Ati2. Mutants of Ati2 with altered amino acids at two different locations in the catalytic site displayed no phosphatase activity. Wild-type and mutant forms of Ati2 were cloned into expression systems for *Dictyostelium discoideum*, a soil amoeba used as an alternative host to study *A. salmonicida* virulence. Expression tests allowed us to demonstrate that Ati2 is toxic for the host cell in a catalytic-dependent manner. Finally, this study demonstrated the existence of a new TTSS effector protein in *A. salmonicida*.

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INTRODUCTION

The type three secretion system (TTSS) is an organelle of Gram-negative bacteria that delivers bacterial proteins into eukaryotic cells (Büttner, 2012; Cornelis, 2006). TTSSs are employed by a wide array of bacterial species in pathogenic and symbiotic interactions with both animals and plants. The TTSS apparatus, or injectisome, is a multimeric complex that forms a needle-like structure spanning the inner and outer bacterial membranes and protruding from the surface. Upon contact with host cells, a pore is formed in the host membrane by the TTSS translocator proteins and then effector proteins are injected through the needle into the target cell.

In the host cell, the effector proteins mimic the activities of host-signalling proteins to influence the behaviour of the cell (Dean, 2011). By altering the activities of their target cells, pathogenic, TTSS-containing bacteria can evade the host's immune response. In the *Yersiniae*, the TTSS suppresses the inflammatory response and inhibits phagocytic engulfment (Navarro *et al.*, 2005) while in *Salmonella* the TTSS promotes cell invasion, intracellular survival and replication (Ibarra & Steele-Mortimer, 2009). Bacteria forming symbiotic associations with a eukaryotic host use the TTSS to promote conditions within the host conducive to bacterial colonization (Preston, 2007). Generally, the genes encoding the TTSS structural apparatus and translocators are conserved among bacteria, while the set of effector proteins varies.

Aeromonas salmonicida subsp. *salmonicida* (*A. salmonicida*) is the causative agent of an infectious bacteraemia of salmonid fish called furunculosis (Hiney & Olivier, 1999). *A. salmonicida* encodes a TTSS primarily located on a 155 kb plasmid (pAsa5), although one effector protein (AexT) and its chaperone are encoded on the chromosome

†Present addresses: Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia B3H 1X5, Canada.

Abbreviations: INPP5B, inositol-1,4,5-trisphosphate 5-phosphatase; TTSS, type three secretion system.

(Reith *et al.*, 2008). Genes for two other effector proteins, AopH and AopO, and their chaperones are present on the pAsa5 plasmid (Dacanay *et al.*, 2006; Reith *et al.*, 2008).

A. salmonicida mutants lacking the TTSS locus on pAsa5 plasmid or deleted for key injectisome proteins are avirulent in rainbow trout gonad cells (Burr *et al.*, 2002; Stuber *et al.*, 2003) or in fish challenges (Dacanay *et al.*, 2006). The contribution of the TTSS to *A. salmonicida* virulence has also been evaluated using *Dictyostelium discoideum* amoeba, an alternative host used to study *A. salmonicida* virulence (Daher *et al.*, 2011; Froquet *et al.*, 2007). This amoeba is a bacterial predator and displays a phagocytic behaviour similar to macrophages. It is regularly used as an alternative host to study the virulence of various bacterial pathogens (Dallaire-Dufresne *et al.*, 2011). *D. discoideum* has been shown to be a suitable model to study the virulence of more than 20 bacterial species mainly by testing the capacity of wild-type and mutant strains to resist amoeba predation. Moreover, the *D. discoideum* model allows the ectopic expression of proteins much more readily than fish cell cultures. As an example of the utility of this model, the toxic effect of ectopic expression of YopE from *Yersinia pseudotuberculosis*, a TTSS effector protein, has been successfully monitored in this model (Vlahou *et al.*, 2009). Furthermore, *D. discoideum* is more appropriate to test the role of proteins originating from a psychrophilic bacterium like *A. salmonicida* due to its lower growth temperature optimum compared to mammalian cells in culture (Froquet *et al.*, 2007). Accordingly, the importance of the TTSS in *A. salmonicida* virulence has been confirmed in *D. discoideum* by comparing the capacity of parental bacteria and TTSS mutants to resist amoeba predation (Daher *et al.*, 2011; Froquet *et al.*, 2007). All observations from fish, fish cells and the amoeba model demonstrate the critical role of the TTSS in *A. salmonicida* pathogenesis.

Previous work on the expression of the TTSS in *A. salmonicida* strain A449 has shown that elevated temperature (28 °C) increases the expression of TTSS transcripts and TTSS apparatus proteins can be detected in cells grown at this temperature (Ebanks *et al.*, 2006). At the normal growth temperature for A449 (18 °C), transcript levels are considerably lower and TTSS proteins are not detectable. Under low calcium conditions (5 mM EGTA), the effector AopH and the translocator AopD were detected in the culture medium of 28 °C grown cells (Ebanks *et al.*, 2006). EGTA treatment at 17 °C does not activate TTSS expression. Thus, A449 requires a combination of increased temperature and low calcium for *in vitro* secretion of TTSS effector proteins, similar to the conditions required by *Yersinia* spp. (Forsberg *et al.*, 1987; Lambert de Rouvroit *et al.*, 1992). However, *in vivo*, TTSS-dependent virulence was seen in fish reared at temperatures of 11–14 °C (Dacanay *et al.*, 2006) or in rainbow trout gonad cells incubated at 18 °C (Burr *et al.*, 2002), indicating that increased temperature is not the *in vivo* TTSS activation signal for *A. salmonicida*.

In a previous study, the three known TTSS effector genes, *aexT*, *aopH* and *aopO* of A449, were inactivated by deletion and the virulence of the resulting mutants was assayed by live challenge in Atlantic salmon (Dacanay *et al.*, 2006). The results demonstrated that none of these effector proteins has a decisive impact on *A. salmonicida* pathogenesis. This opened the door to two possibilities: (1) the cumulative effect of all effector proteins is required for the TTSS toxic effect on the host or (2) another unknown effector protein has a predominant role in TTSS toxicity.

In 2008, the sequences of the A449 chromosome and its two large plasmids (pAsa4 and pAsa5) were released (Reith *et al.*, 2008). Sequence analyses revealed that another potential effector protein of the TTSS was present on the pAsa5 plasmid. The gene encoding this protein was named *ati2* and is found in an operon with another gene (*ati1*), proposed to be the chaperone for this potential effector protein (Reith *et al.*, 2008). Based on protein sequence homology, the Ati2 protein is suspected to have an inositol polyphosphate phosphatase activity (Reith *et al.*, 2008).

An effector protein of the TTSS of *Vibrio parahaemolyticus* named VPA0450 with high similarity to Ati2 has been described (Broberg *et al.*, 2010). Interestingly this protein displayed an inositol polyphosphate 5-phosphatase activity and provoked blebbing of the plasma membrane of host cells (Broberg *et al.*, 2010). However, even with its similarity to VPA0450, the specific function of Ati2 and its role as a TTSS effector protein have not been addressed. In the present paper, we use a combination of biochemical assays, molecular modelling and ectopic expression of Ati2 in host cells to determine its involvement in TTSS function.

METHODS

Bacteria, amoebae and antibodies. *A. salmonicida* strain A449 (Michel, 1979) and its derivative Δ*ascC* mutant (Dacanay *et al.*, 2006) were grown at 17 °C or 28 °C in tryptic soy broth (TSB) or on tryptic soy agar (TSA) containing 20 μg ml^{-1} of chloramphenicol.

D. discoideum DH1-10 cells (Cornillon *et al.*, 2000) were grown at 21 °C in HL5 medium with 15 μg ml^{-1} of tetracycline as previously described (Mercanti *et al.*, 2006). Cells were subcultured twice a week in fresh medium to prevent confluence of the cell culture. When required, cells were grown in the presence of G418 (geneticin, 10 μg ml^{-1}) for plasmid maintenance and doxycycline (10 μg ml^{-1}) to induce protein expression.

Rabbit antibodies against AexT were obtained by amplifying a gene fragment by PCR using the primers aexT-exF and aexT-exR (Table 1), cloning it in pET15b and expressing the 6× His-tagged AexT protein in *E. coli* Tuner DE3. The expressed protein was purified using Ni^{2+} affinity chromatography and was sent to Pacific Immunology for antibody production. Antibodies were then purified from the rabbit serum using purified AexT immobilized on HiTrap NHS-activated HP columns (Amersham Biosciences). Rabbit antibodies against Ati2 were generated by Pacific Immunology using a peptide (CNIKQKEGRDRPDFGEL) conjugated to KLH as immunogenic. Antibodies were purified from rabbit serum using peptides immobilized with the Sulfolink kit (Pierce) according to the manufacturer's instructions. Antibody titres and specificity were tested by Western

Table 1. Primers used in this study

Primer	Sequence (5′-3′)
ati2* gene amplification primers	
Ati2-*Bgl*II-FWD1	AGATCTAGATCTATGTCTACAATTCAAATTAATAGCCAA
Ati2-*Pst*I-*Spe*I-REV2	CTGCAGCTGCAGACTAGTAAGATTGGCAACGAACTGCTGGTT
***aexT* expression amplification primers**	
aexT-exF	GTGTCATATGAAGCAGGCTGCATTTGG
aexT-exR	ATCGTCTAGACGCCAAATCGAGCCCGCGCA
Primers for N104A mutagenesis†	
Ati2-N104A-FWD	GCTCAAGCAAACCAAAAGATGCCGGAA
Ati2-N104A-REV	ATAGGTCAAAGTTAAGACCTTAAC
Primers for D261A mutagenesis†	
Ati2-D261A-FWD	GCTCTAAATGAGCGTGAAAAACGTATC
Ati2-D261A-REV	ACCAGTGATCAGAACCTCTTCTTTC
Primers for sequencing	
Ati2 seq-REV1	AAGACCTTAACGTTGCCACTGCCT
Ati2 seq-REV2	ACCAGTGATCAGAACCTCTTCTTTC
Ati2 seq-FWD1	AAGCTAACGCCAAAGAAGGCGATG
SP6	ATTTAGGTGACACTATAG
T7	TAATACGACTCACTATAGGG

*The nucleotides underlined correspond to the restriction site added to the amplicon (*Bgl*II beside the start codon and *Pst*I and *Spe*I after the last coding codon of the gene).
†The FWD primers were 5′-phosphorylated and contained the mutated codon (alanine, underlined).

blotting against gels containing 28 °C grown cell lysates and the purified protein.

In vitro* secretion by TTSS.** The A449 strain and the Δ*ascC* mutant were grown overnight at 28 °C, which induces the expression of TTSS proteins, and cultures were then pelleted, washed with TSB and diluted to an OD of 0.5. Duplicate cultures were then incubated in the presence or absence of 5 mM EGTA for 4 h. Cells were pelleted and washed with PBS followed by centrifugation at 4 °C. The pellets were resuspended in PBS, SDS-PAGE sample buffer was added and the mixture boiled for 5 min. For secretion analysis, the culture supernatant was passed through a 0.22 μm filter, precipitated with 1/10 volume of ice-cold 100 % trichloroacetic acid (TCA) and incubated on ice for 30 min. Samples were centrifuged for 10 min (15 000 ***g) at 4 °C and precipitated proteins were washed twice with acetone and air dried. Proteins were solubilized in SDS-PAGE sample buffer and boiled for 5 min. Equivalent amounts of protein-containing samples were separated on 10 % SDS-PAGE gels and proteins were electro-transferred onto Immobilon-P membrane (Millipore). Blots were probed with Ati2 and AexT antibody and developed with anti-rabbit horseradish peroxidase secondary antibody (Cell Signalling).

Sequence alignment and model building. The sequence identity between Ati2 and VPA0450, its *V. parahaemolyticus* orthologue, was calculated using a standard protein BLAST (BLASTP) query on the NCBI/BLAST web server (http://blast.ncbi.nlm.nih.gov, data not shown) (Altschul *et al.*, 1990). Ati2 (495 residues) and VPA0450 (497 residues) protein sequences were obtained from UniProt Consortium (UniProt Consortium, 2012) (UniProt accession codes A4SUE7 and Q87J05 for Ati2 and VPA0450, respectively). Protein sequences were submitted to NCBI/BLAST using the Protein Data Bank (PDB) database to find templates for homology modelling. Two structures were identified as best targets, PDB accession codes 3N9V and 3MTC. Both codes refer to crystal structures, including the catalytic site, of the human type II inositol-1,4,5-trisphosphate 5-phosphatase (Uniprot code P32019, hereafter called INPP5B), and share a high structural similarity denoted by a root-mean-square deviation of 0.2 Å between backbone atoms. However, unlike 3N9V, 3MTC includes an inositol in its active site. Consequently, the latter was chosen as the template for homology modelling. A multiple sequence alignment of the full-length sequences of Ati2, VPA0450 and INPP5B using the default parameters of T-Coffee v8.91 (Notredame *et al.*, 2000) showed that Ati2 and VPA0450 were 24.3 % and 23.8 % identical to INPP5B, respectively and 39.7 % and 39.5 % similar. This resulting alignment was used for building the homology model of Ati2 and VPA0450 using MOE (www.chemcomp.com) with default parameters as described previously (Karlsson *et al.*, 2008). Presentation of the sequence alignment was generated using TeXShade (Beitz, 2000).

Cloning of the *ati2* gene. The *ati2* gene was amplified by PCR from an A449 strain lysate (Daher *et al.*, 2011), using primers Ati2-*Bgl*II-FWD1 and Ati2-*Pst*I-*Spe*I-REV2 (Table 1). The *ati2* amplicon was inserted into plasmid pSP73 (Promega) using the restriction sites *Bgl*II and *Pst*I. The ligated plasmid was then introduced into *Escherichia coli* MC1061 (Casadaban & Cohen, 1980). The resulting pSP73-*ati2* plasmid as well as all other plasmid constructs done in this study and resulting from a PCR amplification (see below) were verified by DNA sequencing using primers indicated in Table 1 to ensure they contained no unwanted mutations.

Creation of point mutations in *ati2* gene. The *ati2* gene, carried by the plasmid pSP73, was mutated at codons corresponding to two amino acids conserved among inositol polyphosphate 5-phosphatase catalytic sites (Whisstock *et al.*, 2000) by a PCR-based site-directed mutagenesis protocol (Costa *et al.*, 1996). The primer pairs used resulted in the following amino acid changes: asparagine 104 to alanine (N104A) and aspartate 261 to alanine (D261A) (Table 1). These two specific amino acids have been targeted since they correspond to two critical amino acids involved in the catalytic site

of other enzymes of the same family (Whisstock *et al.*, 2000, 2002) (see Results).

Production of GST-tagged Ati2 and VPA0450 proteins. The wild-type and mutant versions of the *ati2* gene were subcloned in phase with glutathione *S*-transferase (GST) in plasmid pGEX-4T3 (GE Healthcare) after digestion of inserts with *Bgl*II and *Xho*I and pGEX-4T3 with *Bam*HI and *Xho*I (New England Biolabs). Subsequently, pGEX-4T3 constructs containing *ati2* gene inserts as well as the empty pGEX-4T3 were transformed into *E. coli* BL21 to produce recombinant proteins. The plasmid expressing the GST-tagged VPA0450 protein, which was previously described (Broberg *et al.*, 2010), has also been used.

GST-tagged protein expression was done by the addition of 0.2 mM isopropylthiogalactoside (IPTG) to bacterial cultures for 24 h at 18 °C with stirring at 200 r.p.m. Then the cells were pelleted by centrifugation at 4 °C before resuspension in 50 ml of lysis buffer (50 mM NaCl, 50 mM Tris/HCl pH 8, 5 mM EDTA, 0.15 mM PMSF, 1 mM DTT). The cells were lysed using an EmulsiFlex C3 (Avestin) at 20 000 p.s.i. Triton X-100 was added to the lysates to a final concentration of 1 % to reduce protein aggregation. The bacterial lysates were left on ice for 30 min before being centrifuged at 15 500 r.p.m. for 20 min at 4 °C in a Sorvall RC5B + with an SA-600 rotor. The supernatant was collected and mixed with 1 ml of glutathione Sepharose 4B beads (GE Healthcare) at a concentration of 50 % in wash buffer (50 mM Tris/HCl pH 8, 5 mM EDTA, 150 mM NaCl, 0.15 mM PMSF, 1 mM DTT). The mixture was incubated at room temperature for 30 min with gentle agitation and then centrifuged for 5 min at 500 ***g***. The beads were recovered and washed two times with 10 ml of wash buffer. The beads were resuspended in 2 ml of wash buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Quantification of purified recombinant protein was performed by SDS-PAGE electrophoresis using a dilution-series of BSA as quantitative reference.

Inositol polyphosphate phosphatase assay. Equivalent quantities, with correction for their molecular mass in kDa, of GST and GST-tagged Ati2 and VPA0450 proteins (i.e. 0.6 to 1.6 μg of purified proteins) attached to the beads were incubated in the presence of diluent (20 mM Tris, 3 mM $MgCl_2$, 0.5 mM EGTA, 150 mM NaCl, pH 7.2) for 1 h with gentle stirring. Up to four different types of phosphatidylinositols [PtdIns(3,4)P_2, PtdIns(3,5)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3] from Echelon Biosciences were tested in the presence of purified proteins to determine the capacity of the latter to hydrolyse phosphate from these lipids. The phosphoinositides were added in excess (4000 pmol) to each reaction in a final volume of 25 μl. The reaction was incubated at room temperature (~21 °C) for 17 h with gentle agitation. This incubation temperature was chosen to reflect more accurately the conditions in which Ati2 would operate during infection and were thus lower than the temperature previously used (30 °C) for the study of VPA0450 (Broberg *et al.*, 2010). The incubation time was determined in preliminary assays and was set to obtain the highest signal inside the range proposed by the manufacturer, e.g. 200 to 2 000 pmol of free phosphate. The enzymic reaction was stopped by adding 25 μl of 100 mM *N*-ethylmaleimide. The free phosphate produced in each sample was determined with the Malachite Green Assay kit (Echelon Biosciences) according to the manufacturer's instructions.

Effect of ectopic expression of Ati2 in amoeba. The wild-type and mutant versions of the *ati2* gene were subcloned in phase with the Green Fluorescent Protein (GFP) in pDM317 (Veltman *et al.*, 2009b) and pDM334 plasmids (Veltman *et al.*, 2009a) after digestion of both inserts and plasmids with *Bgl*II and *Spe*I (New England Biolabs). These plasmids allow constitutive or inducible expression in *D. discoideum*, respectively.

D. discoideum DH1-10 cells (1.5×10^7 cells) were electroporated with 5 to 7.5 μg of pDM plasmids containing or not the various versions of the *ati2* gene. Electroporations were performed using a pre-chilled 2 mm electrode spacing cuvette in a Gene Pulser (Bio-Rad) with the following parameters: 1.2 kV, 3 μF, 200 Ω with an expected time constant of about 0.3 ms. The cells were incubated for 15 min in a solution of 100 mM $MgCl_2$ and 100 mM $CaCl_2$, and for 24 h in HL5 medium with tetracycline at 21 °C. Cells containing plasmids were selected using G418 (10 μg ml^{-1}) added to the medium for at least a week until the appearance of colonies in Petri dishes.

Cells electroporated with pDM317 plasmids were fixed and stained in order to highlight the number of colonies in each culture. The medium was gently removed from Petri dishes before adding staining solution (0.25 % solution of trypan blue in 5 % acetic acid) for about 15 min. Then the Petri dishes were washed by immersing them gently in a cold water bath and allowed to dry upside down.

Cells electroporated with pDM334 plasmids were inoculated into two Petri dishes (600 000 cells/dish) with HL5 medium containing G418 and tetracycline. For each type of cell, one Petri dish was induced with doxycycline at a final concentration of 10 μg ml^{-1}, the other serving as control. Since doxycycline is diluted in ethanol, amoeba controls received the same volume of ethanol. Cells were incubated for up to 72 h at 21 °C, resuspended and counted with a haemocytometer every 24 h or after 72 h. Cells containing pDM317 served as controls in this experiment because they can grow in presence of G418.

RESULTS

Ati2 is secreted in a TTSS-dependent manner by *A. salmonicida*

The capacity of *A. salmonicida* to secrete Ati2 through its TTSS was assessed by Western blotting of cell lysates and culture supernatants (Ebanks *et al.*, 2006). In the absence of calcium (presence of EGTA), the Ati2 protein was secreted into the culture media by *A. salmonicida* A449 (Fig. 1). In the absence of EGTA, secretion was diminished as expected for TTSS-dependent secretion. In the Δ*ascC* strain, in which TTSS secretion was blocked due to the absence of the TTSS outer membrane pore protein (Ebanks *et al.*, 2006), no Ati2 was detected in the culture media, even though the protein was present in cells. The results for Ati2 were identical to those for the known TTSS effector AexT (Burr *et al.*, 2003). Ati2 secretion is thus dependent on the TTSS.

Ati2 is an inositol polyphosphate 5-phosphatase

Ati2 has a very strong sequence identity (87 %) with VPA0450, a TTSS effector from *V. parahaemolyticus*. Both Ati2 and VPA0450 display a moderate degree of sequence identity with the human INPP5B inositol polyphosphate 5-phosphatase (24.3 % and 23.8 % for Ati2 and VPA0450, respectively), for which molecular structures are available (PDB ID: 3MTC). From nine inositol polyphosphate 5-phosphatases, a previous study identified six highly conserved domains containing eight residues essential to catalytic activity (Whisstock *et al.*, 2000). Despite only moderate identity between Ati2/VPA0450 and INPP5B, three of these domains are clearly conserved (data not

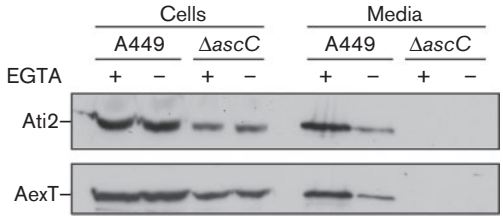

Fig. 1. Ati2 secretion by the TTSS. Ati2 and AexT were detected in the wild-type A449 strain (A449 lanes) or in the Δ*ascC* mutant (Δ*ascC* lanes) by Western blotting. Strains were grown in the presence (+) or absence (−) of EGTA and both the cell pellet (Cells) and TCA precipitated proteins from the media (Media) were analysed by Western blot using anti-Ati2 (upper panel) and anti-AexT antibodies (lower panel).

shown) and all eight essential catalytic residues are conserved as seen in T-Coffee alignments (Fig. 2).

In order to investigate experimentally if Ati2 is an inositol polyphosphate 5-phosphatase, the wild-type GST-tagged Ati2 purified protein was tested in an inositol polyphosphate phosphatase assay (Fig. 3a). This experiment demonstrated that Ati2 hydrolyses phosphatidylinositols, but with a substrate affinity slightly different from that previously shown for VPA0450 (Broberg *et al.*, 2010). Ati2 dephosphorylates PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 at approximately the same level while VPA0450 was shown to dephosphorylate PtdIns(4,5)P_2 three times more than PtdIns(3,4,5)P_3 (Broberg *et al.*, 2010). In order to understand the substrate specificity difference between Ati2 and VPA0450, the latter was also tested in exactly the same conditions as those used for Ati2. As shown in Fig. 3(b), VPA0450 displayed the same capacity to hydrolyse both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 in the experimental procedure used in this study, suggesting that Ati2 and VPA0450 have, in fact, similar catalytic activity.

Mutant versions of Ati2 were also constructed and tested in the inositol polyphosphate phosphatase assay (Fig. 3a). The two mutants were Asn 104 changed for an alanine (N104A) and Asp 261 replaced by an alanine (D261A). Ati2′s amino acids N104 and D261, which are present in the catalytic site (Fig. 2b) and conserved among inositol polyphosphate 5-phosphatases, are essential for the activity of inositol polyphosphate 5-phosphatases (Whisstock *et al.*, 2002). More specifically, N104 and D261 correspond to N18 and D232 of human 43 kDa 5-phosphatase, respectively (Whisstock *et al.*, 2000). In the catalytic site of 43 kDa 5-phosphatase, nucleophilic attack of the phosphoinositide is initiated by D232. N18 is required for the proper orientation of D232 through a hydrogen bond (Whisstock *et al.*, 2002). Mutations of N18 or D232 in the human 43 kDa 5-phosphatase completely abolished the catalytic activity of this enzyme (Whisstock *et al.*, 2000). As expected, the N104A and D261A mutations in Ati2 abolished its catalytic activity, showing experimentally that

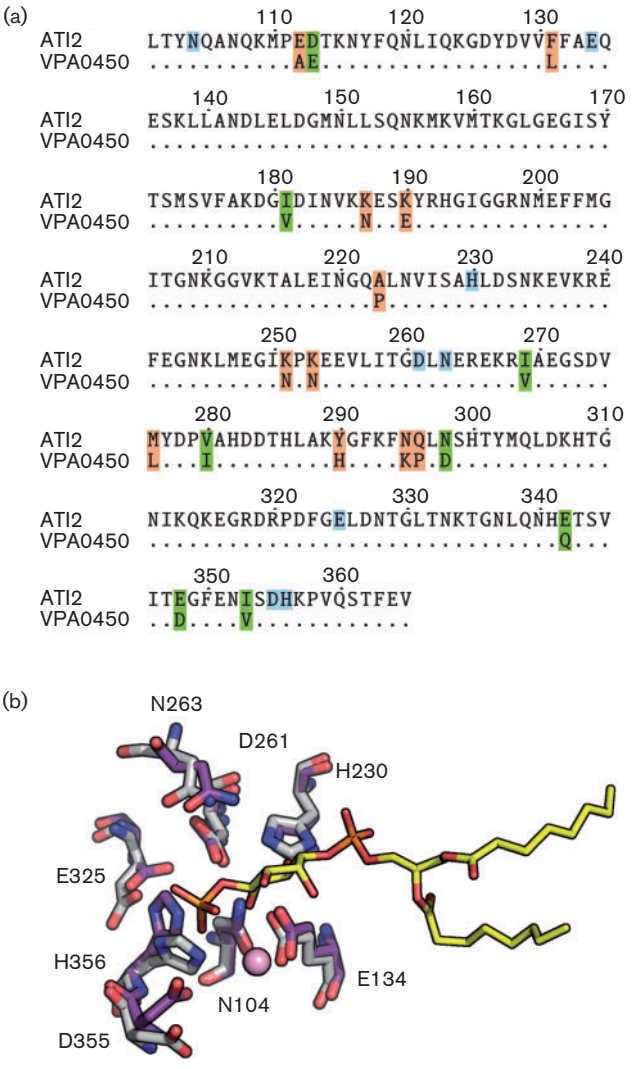

Fig. 2. Ati2 shares sequence and structural homologies with inositol polyphosphate 5-phosphatases. (a) Sequence alignment of Ati2 and VPA0450. Only the region of the sequence used for homology modelling is shown. Identical residues are represented as dots, functionally equivalent residues are coloured in green and non-conserved residues in orange. The eight essential catalytic residues described by Whisstock *et al.* (2000) are coloured in blue. (b) Structural representation of the eight essential catalytic residues. Homology model of Ati2 is in grey and INPP5B (PDB ID 3MTC) in purple. Phosphatidylinositol 4-phosphate (PtdIns4P) is shown as yellow sticks and the magnesium ion coordinated in the catalytic site as a pink sphere. Residue numbers refer to Ati2.

these amino acids are essential for Ati2 activity as they are in other enzymes of the same family.

Ati2 has a toxic effect on host cells

In order to evaluate the toxicity of the Ati2 protein in the host cell, a GFP fusion of Ati2 and its mutants (N104A and D261A) was ectopically expressed in *D. discoideum* cells. In a

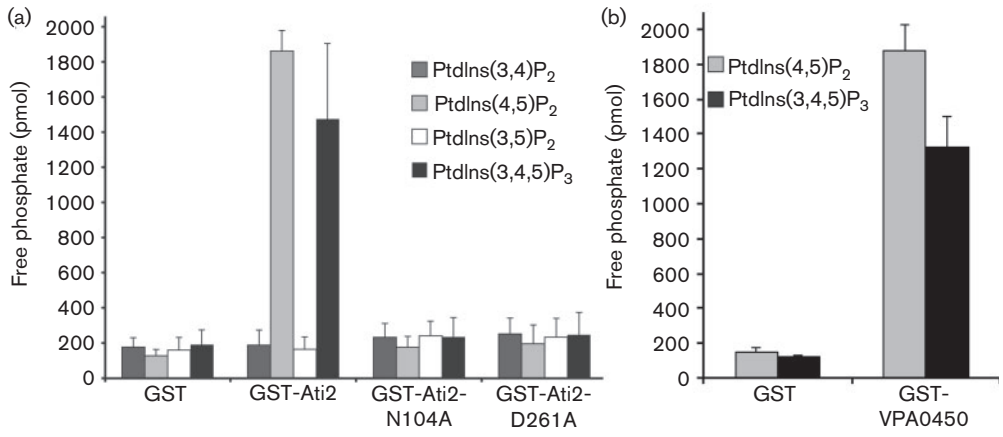

Fig. 3. Malachite green assay demonstrating the inositol polyphosphate 5-phosphatase activity of Ati2 and VPA0450 proteins. The means and SD (error bars) of four independent experiments are shown in (a) and three in (b).

first step, the effect of the constitutive expression of Ati2 and its mutants (N104A and D261A) on *D. discoideum* growth was examined by electroporation of the pDM317 plasmids, which allowed constitutive ectopic expression of the Ati2 proteins (Fig. 4). The wild-type form of Ati2 is toxic since virtually no colonies were detected 10 days after electroporation while the two mutants that do not possess the phosphatase activity were permissive to host cell growth in a way similar to the control and gave >200 colonies in every electroporation (Fig. 4). These results suggested that the active form of Ati2 is toxic to the host cells.

Host cells are not likely constitutively exposed to TTSS effector proteins during the infection process. The induction of ectopic expression of an effector protein in cells probably more adequately mimics the injection of effector proteins by the TTSS during the infection. Consequently, cells containing pDM334 plasmid for Ati2 and its mutants (N104A and D261A) were exposed to doxycycline to induce ectopic protein expression. Without doxycycline, the inducible vectors are permissive to cell growth (data not shown). Cells having pDM334 containing the wild-type or mutated Ati2 were divided into two groups: one exposed to doxycycline and the other serving as non-induced control. The initial concentration of the two populations was identical. It was possible to observe a growth difference between control and induced cells. Cells expressing wild-type Ati2 had a clear decrease in population size already perceptible 24 h after induction of protein expression, but more obvious after 72 h (Fig. 5a). Expression of non-functional Ati2 (N104A and D261A) resulted in a population size similar to the control (Fig. 5b). These results confirmed that a functional form of Ati2 affects the growth or survival of the host cells.

DISCUSSION

In the present study, we demonstrated that Ati2 was secreted by *A. salmonicida* TTSS and acted as an effector protein by altering the growth/survival of host cells through its inositol polyphosphate 5-phosphatase activity.

While it has previously been shown that VPA0450 has a marked preference for PtdIns(4,5)P_2 (Broberg *et al.*, 2010), our results indicated that both Ati2 and VPA0450 can hydrolyse PtdIns(3,4,5)P_3 at levels only slight less than PtdIns(4,5)P_2. However, the difference in substrate specificity seen in our study compared to the previous one is likely related to the difference in how the inositol polyphosphate phosphatase assay has been performed. Nevertheless, in both studies, VPA0450 displayed clear

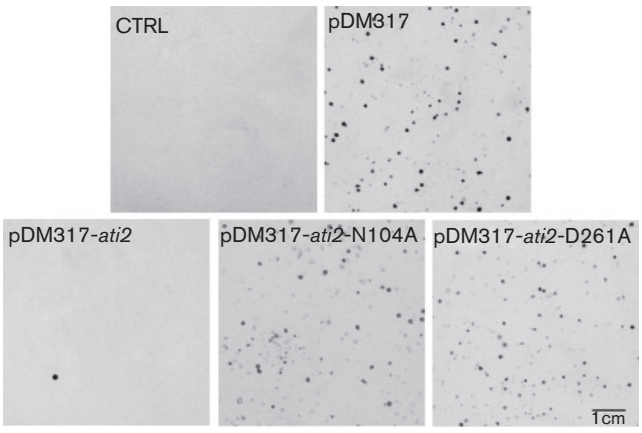

Fig. 4. Toxic effect of constitutive expression of Ati2 in host cells. The images show colonies of *D. discoideum* cells in Petri dishes following electroporation of pDM317 plasmids. Control amoebae (CTRL) were transfected without plasmid and thus have no resistance to the antibiotic selection. Cells electroporated with plasmid pDM317 expressing only GFP were used as positive control to illustrate the growth of amoebae. These tests were conducted at least four times and representative results are shown.

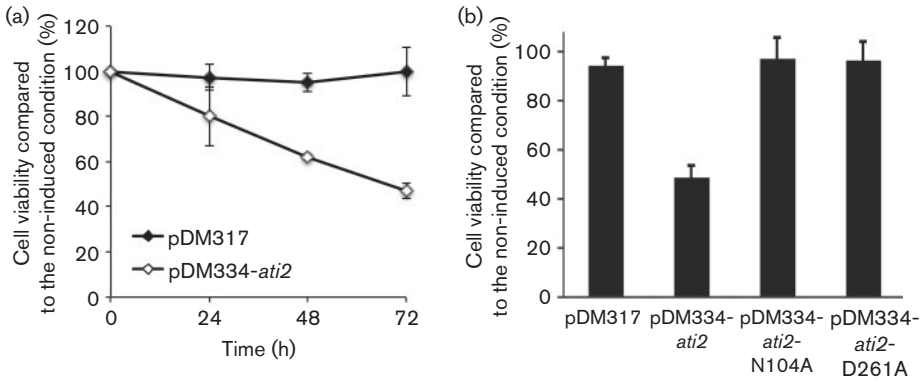

Fig. 5. Toxic effect of induced expression of Ati2 in host cells. The percentage of cell viability was obtained by comparing the number of cells in the induced condition and the non-induced control from 0 to 72 h in three experiments (a) and after 72 h in four experiments (b). Data are means and SD (error bars).

hydrolytic activity of PtdIns(3,4,5)P_3 in addition to PtdIns(4,5)P_2.

By their substrate selectivity and hydrolysis capacity of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, VPA0450 and Ati2 are more likely related to INPP5B, Synaptojanin1 and OCRL in the type II phosphoinositide 5-phosphatase family (Schmid *et al.*, 2004). However, more complex analyses with additional phosphoinositides would be required to more clearly link the bacterial phosphoinositide 5-phosphatases to their eukaryotic counterparts.

In HeLa cells, VPA0450 promotes the creation of host cell membrane blebs apparently by disrupting the interaction between the actin cytoskeleton and the membrane. This blebbing is proposed to lead to the efficient and accelerated lysis of infected cells by additional TTSS effectors in the late stages of infection (Broberg *et al.*, 2010). With a similar enzyme activity, Ati2 may also cause plasma membrane instability and promote host cell lysis. However, based on experiments done with rainbow trout gonad cells, *A. salmonicida* TTSS induces rounding of the cells and their detachment from the plastic support without indication of cell lysis (Burr *et al.*, 2002; Stuber *et al.*, 2003).

Another mechanism by which Ati2 may promote virulence other than cell lysis would be by interfering with the phagocytic/digestion activities of fish macrophages and other immune system cells. An investigation of invasion of adherent head kidney leukocytes by wild-type *A. salmonicida* A449, a mutant deleted for three TTSS effectors (AexT, AopH, AopO; referred to as Δ*aop3*) and the Δ*ascC* mutant showed no difference in invasion success between A449 and Δ*aop3*, while invasion by the Δ*ascC* mutant was reduced five- to sixfold (Fast *et al.*, 2009). These results suggest a role in leukocyte invasion for the TTSS effector(s) not deleted in the Δ*aop3* strain. At present, Ati2 is the only additional known TTSS effector in *A. salmonicida* A449, indicating a role for it in host cell invasion, perhaps through disruption of phagocytosis or digestion by macrophage-like cells.

In *D. discoideum*, blocking the formation of PtdIns(4,5)P_2 leads to the inhibition of endocytosis and a decrease in phagocytosis (Zouwail *et al.*, 2005), while PtdIns(3,4,5)P_3 is involved in the signalling pathways of macropinocytosis, phagocytosis and chemotaxis (Buczynski *et al.*, 1997; Cardelli, 2001; Dormann *et al.*, 2004; Kortholt *et al.*, 2007). In both cases, the dephosphorylation of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 would prevent the recruitment of various host proteins like PH domain-containing proteins involved in signalling pathways required among others for actin regulation (Dormann *et al.*, 2004). *D. discoideum* cells used in this study are grown in liquid medium where they internalize nutrients by macropinocytosis. In *D. discoideum*, expression of Ati2, by dephosphorylating PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, would decrease both phagocytosis and macropinocytosis and thus inhibit growth by both starving the cells and blocking nutrient uptake.

A previous study has visualized the localization of a TTSS effector protein in *D. discoideum* cells (Vlahou *et al.*, 2009). YopE, a TTSS effector protein of *Yersina pseudotuberculosis*, was found to be associated with intracellular membrane compartments and to affect the actin cytoskeleton. A similar analysis was attempted for Ati2 using cells transformed with the GFP fusion plasmids pDM317 and pDM334 containing both wild-type and mutant forms of Ati2. In fact, based on previous work on the 43 kDa human 5-phosphatase (Whisstock *et al.*, 2002), asparagine 104 and aspartate 261 are involved in the nucleophilic attack of the phosphoinositides' phosphate group but not in the binding of the substrate to the enzyme. These two mutants were expected to bind to the substrate without hydrolysing it. Consequently, the N104A and D261A mutants should accumulate in the cell compartment where substrate is present. However, no fluorescence was observed for GFP-Ati2 proteins, while fluorescence was detected in control

cells expressing only GFP (data not shown). We also tried to detect the GFP-Ati2 proteins by Western blot with an anti-GFP antibody. The expression of GFP-Ati2(N104A) and GFP-Ati2(N261A) was detectable but the signal was very low compared to cells expressing only GFP as positive control (data not shown). These results suggest that only low levels of Ati2 are required to impact cell growth and survival. They also imply that the GFP-Ati2 proteins are likely unstable in *D. discoideum* cells and rapidly degraded. Amoebae, which are continually confronted by various hostile bacteria in the wild, have probably developed efficient countermeasures to resist the action of intracellular bacterial toxins.

In the course of these studies, the A449 strain was found to be avirulent in the *D. discoideum* predation assay, apparently due to reduced secretion of proteases (Daher *et al.*, 2011). A449 has been in culture for more than 30 years (Michel, 1979) and this domestication has likely led to subtle changes in its characteristics. More recently isolated strains such as 01-B526 (Daher *et al.*, 2011) were highly virulent in the *D. discoideum* assay, but are not amenable to the construction of knockout mutants using the same methods as A449 (data not shown). These issues preclude the testing of an *ati2* mutant in the *D. discoideum* assay to determine its importance in *A. salmonicida* virulence.

In conclusion, the present study demonstrated that Ati2 is a newly identified TTSS effector protein of *A. salmonicida*. Ati2 acts as an inositol polyphosphate 5-phosphatase, effectively hydrolysing both PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 and presumably impacting host cell membrane stability and/or phagocytic activity.

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