AopP, a type III effector protein of *Aeromonas salmonicida*, inhibits the NF- κ B signalling pathway

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Aeromonas salmonicida subsp. *salmonicida* contains a functional type III secretion system that is responsible for the secretion of the ADP-ribosylating toxin AexT. In this study, the authors identified AopP as a second effector protein secreted by this system. The *aopP* gene was detected in both typical and atypical *A. salmonicida* isolates and was found to be encoded on a small plasmid of approximately 6.4 kb. Sequence analysis indicates that AopP is a member of the YopJ family of effector proteins, a group of proteins that interfere with mitogen-activated protein kinase (MAPK) and/or nuclear factor kappa B (NF- κ B) signalling pathways. AopP inhibits the NF- κ B pathway downstream of I κ B kinase (IKK) activation, while a catalytically inactivated mutant, AopPC177A, does not possess this inhibitory effect. Unlike other effectors of the YopJ family, such as YopJ and VopA, AopP does not inhibit the MAPK signalling pathway.

INTRODUCTION

The aeromonads are members of the family *Aeromonadaceae* (Colwell *et al.*, 1986), found within the γ subclass of the *Proteobacteria*. During the past two decades, the number of phenotypically defined species and DNA hybridization groups within the genus *Aeromonas* has grown rapidly, and, at present, the genus comprises 17 well-defined genomic species. *Aeromonas* species are widespread throughout the environment, occurring in fresh, brackish and marine waters. Many of the species are pathogenic, causing disease in both human and animal hosts. Human isolates of *Aeromonas* are primarily associated with gastrointestinal disease. However, these isolates are also associated with extraintestinal disorders, such as wound infections and septicaemia. In animals, *Aeromonas* species are associated with disease in both warm- and cold-blooded vertebrates, including fish, frogs, snakes and birds.

The species *Aeromonas salmonicida* is included among the aeromonad fish pathogens and comprises both typical and atypical isolates. Typical isolates, which belong to the

subspecies *salmonicida*, are a homologous group that are primarily associated with systemic infections in salmonid fish. Atypical isolates, however, form a biochemically and genotypically heterogeneous group that also vary in terms of their host and disease symptoms (Belland & Trust, 1988; Umelo & Trust, 1998; Gudmundsdóttir, 1998, 2003; Lund & Mikkelsen, 2004). While the significance of atypical *A. salmonicida* infections in both wild and cultivated fish stocks is now becoming clear (Gudmundsdóttir, 1998), the majority of the work carried out on *A. salmonicida* in the past has concerned typical isolates. In fact *A. salmonicida* subsp. *salmonicida* is probably the most extensively studied bacterial fish pathogen, largely due to its widespread distribution and impact on aquaculture.

Much of the work carried out on *A. salmonicida* has focused on the identification of potential virulence factors expressed by the bacterium. To date, several such factors have been reported. These include bacterial surface structures, such as the surface layer protein (Noonan & Trust, 1997) and type IV pili (Masada *et al.*, 2002), as well as extracellular proteins, including serine protease, glycerophospholipid:cholesterol acyltransferase and several haemolysins (Nomura *et al.*, 1988; Lee & Ellis, 1990; Hirono & Aoki, 1993; Vipond *et al.*, 1998).

A. salmonicida isolates have also been shown to harbour genes encoding a type III secretion system (TTSS) (Burr *et al.*, 2002, 2005). Such secretion systems are virulence

Abbreviations: EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; HA, haemagglutinin; IKK, I κ B kinase; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; NF- κ B, nuclear factor kappa B; TNF- α , tumour necrosis factor alpha; TTSS, type three secretion system; wt, wild-type.

The GenBank/EMBL/DDBJ accession number for the plasmid pAsal1 sequence reported in this paper is AJ508382.

mechanisms, common among Gram-negative bacteria, which enable the secretion and translocation of effector proteins, produced in the bacterial cytoplasm, directly into the cytosol of target eukaryotic cells (Ghosh, 2004). Once within the eukaryotic cytosol, effector proteins are able to disrupt the cytoskeleton or interfere with cell signalling cascades (Cornelis & Van Gijsegem, 2000).

Studies carried out with a typical *A. salmonicida* isolate, strain JF2267, have found that the TTSS is responsible for the translocation of the ADP-ribosylating toxin AexT into target fish cells (Burr *et al.*, 2003a). To date, AexT is the only type III effector protein produced by members of the genus *Aeromonas* to have been described. In this study, we show that *A. salmonicida* produces a second type III effector protein, which we have termed AopP.

AopP was found to belong to the YopJ family of type III effector proteins, whose members inhibit specific cell signalling cascades. YopJ, the first member of the YopJ family of effector proteins to be characterized, is expressed by pathogenic *Yersinia* species and prevents the activation of the superfamily of mitogen-activated protein kinase (MAPK) kinases, which include MAPK kinases (MKKs) and the I κ B kinase (IKK) complex (Orth, 2002). As a result of these activities, signalling via the MAPK and nuclear factor kappa B (NF- κ B) pathways is prevented. In this way, YopJ is able to block cytokine production and promote apoptosis in the target host cell. While AopP shares sequence homology with YopJ, it differs in its spectrum of inhibitory activities. We show that AopP inhibits the NF- κ B signalling pathway downstream of I κ B phosphorylation but does not affect MAPK signalling.

METHODS

Bacterial strains, plasmids and culture conditions. A summary of the bacterial strains and plasmids used in this study is provided in Table 1. *Escherichia coli* strains were routinely grown in Luria-Bertani (LB) agar or broth at 37 °C. *Pseudomonas aeruginosa* strain ATCC 27853 was grown on LB plates at 37 °C. *A. salmonicida* strains were grown in tryptic soy broth or on LB agar at 18 °C, unless otherwise indicated. When indicated, antibiotics were added to the culture media at the following final concentrations: 100 μ g ampicillin ml⁻¹, 40 μ g kanamycin ml⁻¹ and 40 μ g chloramphenicol ml⁻¹.

DNA sequencing. Plasmid DNA was isolated from *A. salmonicida* subsp. *salmonicida* strain JF2267 using the QiaPrep Spin Miniprep kit (Qiagen). The DNA was then digested with *Eco*RI, cloned into plasmid pBSK⁺ and transformed into *E. coli* XL-1 Blue (Bullock *et al.*, 1987). An ordered set of nested deletions was then obtained using the Erase-a-Base system (Promega) according to the manufacturer's instructions.

Sequencing was performed using the dRhodamine Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's protocol, with either T3 or T7 primers flanking the cloned insert in pBSK⁺ or synthesized internal primers (Microsynth). Reaction products were analysed on an ABI Prism 3100 genetic analyser (Applied Biosystems). Sequence alignment and editing was performed using the software Sequencher (Gene Codes Corp.). Identification of potential ORFs was carried out using the ORF Finder ([http://](http://www.ncbi.nlm.nih.gov/gorf/gorf.html)

www.ncbi.nlm.nih.gov/gorf/gorf.html). Comparison of DNA sequences and their corresponding amino acid sequences was performed using BLAST (Altschul *et al.*, 1990).

Southern blot analysis. The *aopP* gene of *A. salmonicida* subsp. *salmonicida* strain JF2267 was amplified by PCR in the presence of 20 μ M DIG (Roche Diagnostics) in order to generate a labelled probe. PCR was carried out using the following primer pair: AsORF28, 5'-GAGAGTTGGCTAGCGGTGAG-3', and AsORF38, 5'-TCCTCATGGAGCGCATCCAG-3', and PCR conditions were 3 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 58 °C and 30 s at 72 °C.

Plasmid DNA was prepared using the Qiaprep Spin Miniprep kit (Qiagen) and was digested with the restriction enzyme *Bam*HI. The DNA was separated by gel electrophoresis, and Southern blotting was then performed by alkaline transfer onto positively charged nylon membranes (Roche Diagnostics). Hybridization was carried out overnight at 60 °C and the membrane was washed with 2 $\times$ saline sodium citrate (SSC), 0.1 % SDS, followed by a second wash with 0.2 $\times$ SSC, 0.1 % SDS. Hybridization reactions were detected using anti-DIG antibodies and the chemiluminescent reagent CDP-Star (Roche Diagnostics) according to the manufacturer's protocol. DNA from *A. salmonicida* subsp. *salmonicida* strain JF2267 and *P. aeruginosa* strain ATCC 27853 served as positive and negative controls, respectively.

Complementation. In order to complement the Δ *ascV* mutation in *A. salmonicida* subsp. *salmonicida* strain JF2747, the *ascV* gene and its ribosome-binding site were amplified from the wild-type (wt) strain JF2267 by PCR using primers *ascV*SallI, 5'-GTCGACATGTCGATCAGCTGATCAG-3', and *ascV*HindIII, 5'-AAGCTTCCAGATAGTGAGAGGCCAAC-3' (underlined nucleotides indicate recognition cut sites for restriction endonucleases *Sall*I and *Hind*III, respectively). PCR was carried out using a primer-annealing temperature of 58 °C and an extension time of 3.5 min. The resulting PCR product was then cloned into the broad-host-range plasmid pMMB66EH (Fürste *et al.*, 1986) and transformed into *E. coli* strain S17.1 (Simon *et al.*, 1983). The plasmid was introduced into *A. salmonicida* subsp. *salmonicida* strain JF2747 by filter mating (Simon *et al.*, 1983) for 2 days at 15 °C. Clones containing the recombinant plasmid were selected for on media containing 100 μ g ampicillin ml⁻¹ and 40 μ g kanamycin ml⁻¹, and the presence of the wt gene was verified by PCR. Expression of the cloned gene was induced by the addition of IPTG to a final concentration of 1 mM.

AopP secretion. *A. salmonicida* strains were grown in tryptic soy broth containing protease inhibitor (Complete, Roche Diagnostics), for 18 h at 18 °C. Equivalent amounts of cells and supernatants were harvested and separated on 12 % SDS-PAGE gels according to the method of Laemmli (1970). Following separation, Western blot analysis was performed using anti-AopP antibodies. Antibody binding was visualized using ECL plus Western Blotting Detection reagents (Amersham Biosciences).

Site-directed mutagenesis. A catalytically inactivated AopP mutant, AopPC177A, was constructed by recombinant PCR using the method of Vallette *et al.* (1989) with mutagenesis primers *aopPC177A*-fwd, 5'-GAAGCAATTGAGAGCAGGGATATTCAGCG-3', and *aopPC177A*-rev, 5'-GCTGAATATCCCTGCTTCTGAATTGCTTC-3' (mutations underlined). The PCR conditions were as follows: 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 58 °C, and up to 90 s at 68 °C. An extension step of 2 min at 68 °C was carried out following the last cycle in order to ensure full-length synthesis of the fragments. The final product was sequenced to ensure the correct mutation and was cloned into plasmids pSFFV or pcDNA3.1/V5-HIS.

Table 1. Bacterial strains and plasmids used in this study

A superscript T denotes a type strain.

Strain or plasmid	Properties and/or origin	Source or reference
Strains		
<i>A. salmonicida</i> subsp. <i>salmonicida</i>		
JF2267	Wild-type isolate, arctic char, Switzerland	Braun <i>et al.</i> (2002)
JF2747	$\Delta ascV::Km$, derived from JF2267	Burr <i>et al.</i> (2005)
JF3239	$\Delta ascV::Km/pMMB66EHascV^+$, derived from JF2747	This study
ATCC 33658 ^T	Salmon, UK	ATCC
CC-27	Salmon, Norway	Burr <i>et al.</i> (2005)
CC-29	Salmon, Scotland	Burr <i>et al.</i> (2005)
CC-72	Salmon, Canada	Burr <i>et al.</i> (2005)
JF3224	Brown trout, Switzerland	Burr <i>et al.</i> (2005)
Austin 98	Sediment, Scotland	Austin <i>et al.</i> (1998)
Atypical <i>A. salmonicida</i>		
NCIMB 1110 ^T	subsp. <i>achromogenes</i> , trout	B. Gudmundsdóttir
ATCC 27013 ^T	subsp. <i>masoucida</i> , salmon	B. Gudmundsdóttir
F-265/87	subsp. <i>achromogenes</i> , atlantic salmon, Iceland	B. Gudmundsdóttir
As 209	Wolf fish, UK	B. Gudmundsdóttir
As 51	Rainbow trout, Norway	B. Gudmundsdóttir
<i>P. aeruginosa</i>		
ATCC 27853	Blood culture	ATCC
<i>E. coli</i>		
XL-1 Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i>	Bullock <i>et al.</i> (1987)
BL21(DE3)	<i>F'dcm ompT hsdS(r_B⁻ m_B⁻) gal λ (DE3)</i>	Studier <i>et al.</i> (1990)
S17-1	<i>thi pro hsdR hsdM⁺ recA</i> [RP4 2-Tc::Mu-Km::Tn7(Tp ^r Sm ^r)Tra ⁺]	Simon <i>et al.</i> (1983)
Plasmids		
pGEM-T Easy	Cloning vector, Amp ^R	Promega
pBSK ⁻	Bluescript II SK ⁻ , cloning vector, Amp ^R	Stratagene
pSFFV	Expression vector, Amp ^R	Palmer <i>et al.</i> (1999)
pETHIS-1	Expression vector, Amp ^R	Novagen
pMMB66EH	Expression vector, Amp ^R ; IncQ; inducible <i>tac</i> promoter	Fürste <i>et al.</i> (1986)
pcDNA3.1/V5-HIS	Expression vector; Amp ^R	Invitrogen
MEKK1-pcDNA3.1	Expression vector; Amp ^R	D. Dobbelaere
pIkB α -FLAG	Expression vector; Amp ^R	L. Schmitz
pHA-ERK	Expression vector; Amp ^R	K. Orth

Production of polyclonal anti-AopP antibodies. To generate polyclonal antibodies directed against AopP, we first amplified the *aopP* gene using primers HISAopPSpeI, 5'-GAAGTAGTTATGAATATACCCCCATCC-3', and HISAopPNotI, 5'-GGGCGGCCGCAAACGGATTTTCCGTCATCAG-3' (underlined nucleotides indicate recognition cut sites for restriction endonucleases SpeI and NotI, respectively). The PCR conditions were as follows: 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 2 min at 68 °C. An extension step of 5 min at 68 °C was carried out following the last cycle. The resulting PCR product was cloned into the pGEM-T Easy vector (Promega) and transformed into *E. coli* strain XL-1 Blue (Bullock *et al.*, 1987). Recombinant plasmids were then digested with the restriction enzymes SpeI and NotI, and the resulting DNA fragment was ligated into the expression vector pETHIS-1 in order to generate polyhistidine tags at both the N- and C-terminal ends of the recombinant protein. The cloned *aopP* insert was sequenced to ensure correct fusion with the vector poly-HIS codons before being transformed into *E. coli* BL21(DE3) cells (Studier *et al.*, 1990) for expression of the fusion protein. LB broth (50 ml) containing ampicillin was inoculated with *E. coli* BL21(DE3)

cells harbouring plasmid pETHIS-*aopP* and incubated at 37 °C to an OD₆₀₀ of 0.3. Cells were then induced by the addition of 1 mM IPTG and grown for an additional 3 h. Following induction, the fusion proteins were purified from cell extracts under denaturing conditions, using Ni²⁺ chelate affinity chromatography columns (Qiagen) according to the manufacturer's instructions. The bound protein was eluted by decreasing the pH from 8.0 to 5.0 with 50 mM potassium phosphate buffer, 300 mM NaCl, 6 M guanidine hydrochloride. The eluted protein was then dialysed against 50 mM phosphate buffer, 300 mM NaCl, pH 8.0, before being electro-eluted from 12 % SDS-PAGE gels using an Elutrap (Schleicher & Schuell). Polyclonal antibodies against AopP were obtained by immunization of a rabbit with purified recombinant AopP-HIS protein mixed 1:1 with GERBU Adjuvant 10 (GERBU Biotechnik). IgG was isolated from the anti-AopP antisera using HiTrap affinity columns (Amersham Pharmacia, Biotech) according to the manufacturer's instructions.

MAP kinase assay. Approximately 7.5×10^5 HEK293 cells were seeded and transfected using 7 μ l FuGENE 6 reagent (Roche

Diagnostics) with 100 ng pHA-ERK haemagglutinin (HA)–extracellular signal-regulated kinase (ERK) plasmid and 100 ng pSFFV vector, YopJ-pSFFV, YopJC172A-pSFFV, AopP-pSFFV or AopPC177A-pSFFV, and were allowed to rest for 24 h. The cells were starved overnight to reduce background or stimulus-independent phosphorylation. The whole-cell lysates harvested after epidermal growth factor (EGF) treatment (100 ng ml⁻¹) for 5 min at room temperature were then separated by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting with anti-phospho-ERK and anti-HA antibodies.

I κ B α phosphorylation assay. Approximately 1×10^5 HeLa cells were seeded and transiently transfected using the PolyFect reagent (Qiagen) according to the manufacturer's instructions, and incubated at 37 °C in 5% CO₂ for 18 h to allow for expression. DNA quantities used were as follows: 1 μ g each of pI κ B α -FLAG and MEKK1-pcDNA3.1; 0.5 μ g each of YopJ-pSFFV, YopJC172A-pSFFV, AopP-pcDNA3.1/V5-HIS and AopPC177A-pcDNA3.1/V5-HIS. The proteasome inhibitor MG-262 (Affiniti) was then added to a final concentration of 250 nM, and cells were incubated for a further 4 h. For tumour necrosis factor alpha (TNF- α) stimulation, HeLa cells were transiently transfected with 1 μ g each of YopJ-pSFFV, YopJC172A-pSFFV, AopP-pcDNA3.1/V5-HIS or AopPC177A-pcDNA3.1/V5-HIS only, and stimulated with TNF- α (100 ng ml⁻¹) for 3 min. Cell lysates were prepared and separated on 12% SDS-PAGE gels according to the method of Laemmli (1970). Once separated, the proteins were electroblotted onto nitrocellulose membranes (Bio-Rad Laboratories). Immunoreactive proteins were detected with antibodies to I κ B α (C-21, sc-371; Santa Cruz Biotechnology), phospho-I κ B α (Cell Signalling Technology), MEK kinase-1 (C-22, sc-252; Santa Cruz Biotechnology), V5 (Invitrogen) or tubulin (Sigma) using horseradish peroxidase (HRP)-conjugated anti-rabbit (Cell Signalling Technology) or anti-mouse (Dako) secondary antibodies. Antibody binding was visualized using ECL plus Western Blotting Detection reagents (Amersham Biosciences).

Immunofluorescence microscopy. Approximately 2×10^4 HeLa cells, grown on 12 mm glass coverslips, were transiently transfected using PolyFect reagent (Qiagen). DNA quantities used were as follows: 1 μ g of AopP-pcDNA3.1/V5-HIS, AopPC177A-pcDNA3.1/V5-HIS or pcDNA3.1/V5-HIS only. Cells were allowed 22 h for expression and were stimulated with TNF- α (100 ng ml⁻¹) for 30 min. Cells were washed twice with PBS and then fixed for 20 min in 4% formaldehyde, 10% fetal calf serum (FCS) in PBS. Cells were then permeabilized with 0.1% Triton X-100 for 10 min and washed twice in PBS. Fixed samples were incubated in blocking solution (10% FCS in PBS) for 10 min. Coverslips were incubated for 1 h with either rabbit anti-p65 antibody (C-20, sc-372; Santa Cruz Biotechnology) diluted 1/50, mouse anti-V5 antibody (Invitrogen) diluted 1/500 or anti-YopP/J antibodies (kindly provided by Guy Cornelis, Biozentrum, Basel) diluted 1/1000 in blocking solution. Coverslips were then washed three times and subsequently incubated with anti-rabbit Alexa Fluor 488 antibody (Molecular Probes) diluted 1/1500 or anti-mouse Texas red antibody (Vector Laboratories) diluted 1/800. The cell nuclei were stained with Hoechst. The coverslips were mounted on glass slides and stained cells were observed using a Nikon Eclipse 800 microscope. Images were produced by digital confocal imaging using Openlab software (Improvision).

Nucleotide sequence accession numbers. The nucleotide sequence of plasmid pAsal1 has been submitted to the EMBL Nucleotide Sequence Database under accession number AJ508382. The accession numbers of the *aopP* gene from all *A. salmonicida* strains studied are AM181130–AM181138.

RESULTS AND DISCUSSION

Nucleotide sequence analysis

In the virulent *A. salmonicida* subsp. *salmonicida* isolate, strain JF2267, genes encoding all structural components of the TTSS are located on a large plasmid of approximately 140 kb. In order to further characterize plasmid carriage in this isolate, we isolated plasmid DNA from the bacterium by alkaline lysis. The DNA obtained was then digested with restriction endonucleases, cloned and sequenced. Like most *A. salmonicida* isolates, which typically carry three to four small plasmids that range in size from approximately 4 to 8 kb (Belland & Trust, 1989; Hanninen *et al.*, 1995; Giles *et al.*, 1995), *A. salmonicida* subsp. *salmonicida* strain JF2267 was found to harbour three small plasmids, which we have termed pAsal1 (6371 bp), pAsal2 (5424 bp) and pAsal3 (5259 bp). As previously reported, plasmid pAsal2 is identical to plasmid pAsal found in the virulent *A. salmonicida* subsp. *salmonicida* isolate, strain A449, whereas plasmid pAsal3 is virtually identical to plasmid pAsa2 of strain A449 (6 base pair differences) (Boyd *et al.*, 2003). Plasmid pAsal1 is not found in strain A449.

Sequence analysis of plasmid pAsal1 revealed the presence of five significant ORFs (Fig. 1A, Table 2). Of particular interest was the identification of a gene encoding a potential type III effector protein, which we have termed *aopP*. The *aopP* gene is 900 bp in length and encodes a predicted protein, AopP, of 299 aa. The amino acid sequence of AopP reveals sequence similarity to YopJ/P proteins of the pathogenic *Yersinia* sp. (46% identity, 64% similarity), AvrA of *Salmonella enterica* Typhimurium (40% identity, 59% similarity) and VopA of *Vibrio parahaemolyticus* (37% identity, 55% similarity). Like these YopJ family members, amino acid sequence alignment revealed that AopP contains a catalytic triad, formed by a histidine, a glutamic acid and a cysteine residue, which is necessary for the activity of these proteins (Fig. 1B) (Denecker *et al.*, 2001; Orth, 2002).

It is interesting to note that the location of the *aopP* gene on a small plasmid is different from that of the *A. salmonicida* subsp. *salmonicida* type III effector gene *axtT*, which is chromosomally encoded (Stuber *et al.*, 2003). No other potential type III effector genes were found on plasmid pAsal1, or on the other small plasmids found in *A. salmonicida* subsp. *salmonicida* strain JF2267, pAsal2 and pAsal3.

Detection of *aopP* in *A. salmonicida* field isolates

A number of *A. salmonicida* field isolates, including both typical and atypical strains, were investigated for the presence of *aopP*. Plasmid DNA was isolated from each strain and examined for the presence of *aopP* by Southern blot hybridization (Fig. 2A). The results indicated that all the typical *A. salmonicida* strains investigated harboured the *aopP* gene, while three out of five atypical isolates possessed

Fig. 1. Genetic analysis of pAsal1 and AopP. (A) Map of plasmid pAsal1. Gene position and the direction of transcription are indicated by arrows. (B) Amino acid sequence alignment of the catalytic core of AopP of *A. salmonicida* subsp. *salmonicida* (accession no. NP_710166), YopJ of *Yersinia pseudotuberculosis* (AAT28340), YopJ of *Yersinia pestis* (AAC69766), YopP of *Yersinia enterocolitica* (NP_863566), AvrA of *S. enterica* Typhimurium (AAB83970) and VopA of *V. parahaemolyticus* (AAT08443). Asterisks designate catalytic residues.

the gene (Fig. 2B). Strains ATCC 27013^T and As51, the two atypical strains that did not give a positive signal for *aopP*, were also negative when the Southern blot was repeated using genomic DNA (data not shown).

Sequence comparison of *aopP* from *A. salmonicida* field isolates

We sequenced the *aopP* gene of all *A. salmonicida* strains that gave a positive signal in the Southern blot analysis. All typical strains and one atypical strain, As209, showed 100 % sequence identity to the *aopP* gene found in *A. salmonicida* subsp. *salmonicida* strain JF2267. The *aopP* gene of the atypical strains F-265/87 and NCIMB 1110^T were identical to one another and differed in 4 base pairs, when compared to the *aopP* gene of strain JF2267. The nucleotide substitutions in strains NCIMB 1110^T and F-265/87 and the resulting amino acid residue changes did not affect the three enzymic residues within the catalytic triad.

Secretion of AopP

As AopP showed sequence similarity to type III secreted proteins, we examined secretion of AopP into the external environment by all 12 *A. salmonicida* isolates. The results indicated that four typical *A. salmonicida* isolates, strains JF2267, CC-27, CC-72 and JF3224, were able to secrete AopP. Previous studies have also shown that all four of these strains possess the gene cluster that encodes the type III secretion apparatus in *A. salmonicida* (Burr *et al.*, 2003b, 2005). Only one typical isolate that is known to possess type III secretion genes and that gave a positive signal for *aopP* by Southern blot hybridization was unable to secrete AopP, strain CC-29 (Fig. 3A). The remaining typical *A. salmonicida* isolates, strains ATCC 33658^T and Austin 98, do not harbour a functional TTSS (Stuber *et al.*, 2003; Burr *et al.*, 2005) and were not able to secrete AopP (Fig. 3A), even though both strains expressed the AopP protein (results not shown).

Our results also indicate that the atypical *A. salmonicida* strains NCIMB1110^T and F-265/87 are able to secrete AopP, whereas strain As209 does not secrete the protein, although it does possess the *aopP* gene (Figs 2B and 3A). In agreement

Table 2. Characteristics of significant ORFs encoded on pAsal1

Designation	Description	Length (bp)	Homologous gene	Percentage amino acid identity/similarity	Homologue accession no.
<i>repA</i>	Replication protein (ColE2 family)	975	<i>repA</i> of <i>Vibrio cholerae</i> O1	46/61	BAD88720
<i>orfC1</i>	Unknown function	324	<i>orfC1</i> of <i>Ralstonia solanacearum</i>	38/55	NP_052314
<i>mobA</i>	Mobilization protein	1263	<i>mbkA</i> of <i>E. coli</i>	28/43	AY929248
<i>tnp</i>	Potential transposase, ISAs3	1215	<i>tnp</i> of <i>Y. pestis</i> KIM	67/81	NP_671111
<i>aopP</i>	Potential type III effector	900	<i>yopP</i> of <i>Y. enterocolitica</i>	46/64	AAK69256

Fig. 2. Detection of *aopP* in *A. salmonicida* strains. (A) Plasmid DNA was digested with the restriction enzyme *Bam*HI and separated by agarose gel electrophoresis. (B) Southern blot hybridization of (A) using a DIG-labelled probe against *aopP*. Typical and atypical strains are indicated. Genomic DNA from *P. aeruginosa* was used as a negative control.

with these findings, strains NCIMB 1110^T and F-265/87 are known to possess a TTSS, while strain As209 does not (Burr *et al.*, 2005).

To confirm whether the AopP protein is secreted via the TTSS, we examined secretion of AopP in two derivatives of the wt *A. salmonicida* subsp. *salmonicida* strain JF2267, an isogenic type III secretion mutant, strain JF2747, and a complemented strain, JF3239. Strain JF2747 possesses a mutation in the gene *ascV*, which encodes a structural inner-membrane component of the TTSS. As a result, this strain is unable to secrete proteins via the TTSS (Burr *et al.*, 2003a, 2005). In strain JF3239, the $\Delta ascV$ mutation has been complemented *in trans* with plasmid pMMB66EH $\Delta ascV^+$. All three strains were grown overnight in tryptic soy broth, and the cell pellets and culture supernatants were then subjected to Western blot analysis using polyclonal anti-AopP antibodies. The results indicated that AopP is found in the culture supernatant of the wt strain JF2267 but not in the supernatant of the $\Delta ascV$ mutant, strain JF2747. This finding indicates that AopP is secreted into the external

Fig. 3. Type III-dependent secretion of AopP. (A) Typical and atypical *A. salmonicida* strains were grown overnight in tryptic soy broth and the presence of AopP in the culture supernatants was assayed by SDS-PAGE and Western blotting using anti-AopP antibodies. The presence of the *aopP* gene and a type III secretion gene cluster in each strain is indicated. Purified recombinant AopP-HIS protein was used as a positive control. (B) Bacterial cultures were grown overnight in tryptic soy broth and the presence of AopP in the cell pellets and culture supernatants (SN) was analysed by SDS-PAGE and Western blotting using polyclonal anti-AopP antibodies. wt, *A. salmonicida* subsp. *salmonicida* strain JF2267; $\Delta ascV$, isogenic $\Delta ascV$ mutant, strain JF2747; $\Delta ascV/ascV^+$, $\Delta ascV$ mutation complemented *in trans*, strain JF3239. Recombinant AopP-HIS protein was used as a positive control.

environment via the TTSS pathway. *In trans* complementation of the $\Delta ascV$ mutation restored the ability to secrete AopP (Fig. 3B), confirming that secretion of AopP is type III-secretion dependent.

Lack of inhibition of MAPK signalling pathway upstream of ERK

The AopP homologues YopJ/P and VopA are able to inhibit the MAPK signalling pathway of target eukaryotic cells (Palmer *et al.*, 1999; Orth *et al.*, 1999; Trosky *et al.*, 2004; Zhou *et al.*, 2005). We therefore assayed whether AopP also possesses this ability. As substitution of the cysteine residue within the catalytic triad is enough to abolish the enzymic activity of YopJ and its homologues (Orth *et al.*, 2000; Trosky *et al.*, 2004), we also constructed a catalytically

inactivated mutant of AopP, AopPC177A, whereby the cysteine at position 177 was changed to an alanine. HEK293 cells were transfected with plasmids expressing AopP, YopJ or the catalytically inactivated forms of these effectors (AopPC177A and YopJC172A), together with pHA-ERK. Cells were then stimulated with EGF to induce ERK activation. wt YopJ, but not wt AopP or the catalytically inactive effectors, was able to inhibit EGF-induced ERK activation (Fig. 4A). We conclude that, in contrast to YopJ and VopA, AopP is unable to inhibit the MAPK signalling pathway. As in the case of AopP, the *S. enterica* Typhimurium homologue AvrA has also been found to be unable to inhibit MAPK signalling in mammalian cells (Trosky *et al.*, 2004).

AopP inhibits the NF- κ B signalling pathway downstream of IKK

We investigated whether AopP interferes with the NF- κ B signalling pathway by assaying the effect of AopP on I κ B α phosphorylation. HeLa cells were transiently transfected with plasmids expressing I κ B α -FLAG, MEKK1 (as an internal inducer of endogenous IKK), and the wt or catalytically inactivated forms of AopP or YopJ. Western blot analysis of cell lysates using antibodies against the phosphorylated form of I κ B α showed that expression of wt AopP and the catalytically inactive forms of both effectors had no effect on the phosphorylation of I κ B α (Fig. 4B). In contrast, expression of wt YopJ resulted in significantly reduced phosphorylation of I κ B α (Fig. 4B). These data suggest that AopP does not interfere with IKK activation per se. To further rule out an inhibitory activity of AopP on the NF- κ B pathway upstream of IKK, we next tested whether AopP affected IKK activity triggered by stimulation of the TNF receptor. HeLa cells were transiently transfected with plasmids encoding wt or catalytically inactivated forms of AopP or YopJ, and stimulated for 3 min with TNF- α . Western blot analysis using anti-phospho-I κ B α antibodies showed that neither AopP nor its catalytically inactive mutant inhibited TNF- α -induced IKK activation; on the contrary, in AopP-expressing cells, a slight increase in levels of phospho-I κ B α could be observed (Fig. 4C, top panels, lane 4). Expression of YopJ, on the other hand, led to a reduction in I κ B α phosphorylation by ~50 % compared to the catalytically inactive YopJC172A mutant (Fig. 4C, top panels, lanes 2 and 3). Interestingly, YopJ expression also further reduced the low levels of constitutive IKK activity that can be detected in unstimulated HeLa cells (Fig. 4C, lower panels, lane 1).

The lack of inhibition by AopP cannot be attributed to low levels of AopP expression. Immunofluorescence analysis of HeLa cells transfected with either plasmid AopP-pcDNA3.1/HIS-V5 or YopJ-pSFFV revealed comparable transfection efficiencies and confirmed that both proteins, AopP and YopJ, were sufficiently expressed (Fig. 4D). Our results are consistent with previous findings that indicate that YopJ blocks IKK activation (Orth *et al.*, 1999;

Collier-Hyams *et al.*, 2002), and indicate that AopP does not interfere with IKK-mediated I κ B α phosphorylation.

As the *Salmonella* homologue of AopP, AvrA, is also unable to prevent phosphorylation of I κ B α , yet is capable of inhibiting the NF- κ B pathway downstream of IKK β activation (Collier-Hyams *et al.*, 2002), we used immunolocalization of the p65 subunit of NF- κ B to further study the potential interference of AopP with the NF- κ B pathway. HeLa cells were transiently transfected with plasmids expressing wt AopP, the catalytically inactive mutant AopPC177A or the vector only. After 22 h incubation, the transfected cells were stimulated with TNF- α for 30 min. As can be seen in Fig. 5, TNF- α induced nuclear translocation of p65 in cells transfected with the vector only. However, expression of wt AopP in HeLa cells greatly reduced the nuclear translocation of p65 following stimulation with TNF- α . In contrast, the catalytically inactive mutant AopPC177A did not prevent translocation of p65 from the cytoplasm into the nucleus.

Taken together, our results indicate that, in contrast to YopJ, AopP affects the NF- κ B pathway at a point downstream of I κ B α phosphorylation (Fig. 6). Furthermore, AopP was found to have no inhibitory effect on the MAPK signalling pathway. It has recently been shown that YopJ functions as a deubiquitinating enzyme that negatively regulates the MAPK and NF- κ B signalling pathways by removing lysine63- and lysine48-linked ubiquitin moieties from regulatory proteins (Zhou *et al.*, 2005). In the NF- κ B pathway, YopJ impedes the lysine63-linked ubiquitination processes required for IKK activation, and proteasomal degradation of I κ B α is prevented by cleaving lysine48-linked ubiquitin chains. Our findings indicate that AopP does not show the same promiscuity as YopJ. AopP blocked neither the MAPK pathway nor I κ B α phosphorylation. The fact that p65 translocation is blocked, however, might suggest that the activity of AopP is restricted to preventing I κ B α degradation. AopP is therefore another example of how members of the YopJ family, despite relatively high sequence similarity, display marked diversity in their signalling pathway inhibitory profiles (Fig. 6).

In an effort to determine whether AopP contributes to the virulence of *A. salmonicida* subsp. *salmonicida* *in vivo*, we attempted to generate a defined *aopP* knock-out mutant by gene inactivation and plasmid replacement. However, in all our attempts, we were unable to knock out the wt copy of the *aopP* gene, possibly due to the high copy number of plasmid pAsal1, which is estimated to be approximately 50 copies per cell. By serial passaging of *A. salmonicida* subsp. *salmonicida* strain JF2267, we were able to cure the strain of plasmid pAsal1. However, this resulted in the concomitant loss of the plasmid that encodes the structural genes of the TTSS, the system that governs the secretion of AopP. We were therefore unable to properly assay the effect of the loss of pAsal1 on the virulence of this strain. Because AopP is able to inhibit the NF- κ B signalling cascade by repressing translocation of the eukaryotic transcription

Fig. 4. AopP does not block MAP-kinase signalling upstream of ERK and does not inhibit IκBα phosphorylation. (A) HEK293 cells were transfected with plasmid pSFFV-HA-ERK expressing the indicated type III effectors or their catalytically inactivated forms or with the vector only. Forty hours post-transfection, the cells were treated for 5 min with EGF, and cell lysates were subjected to SDS-PAGE and Western blotting using anti-phospho-ERK and anti-HA antibodies as indicated. (B) HeLa cells were transfected with plasmids expressing IκBα-FLAG, MEKK1 (as an internal inducer of endogenous IKK), and the indicated effector proteins AopP and YopJ or their catalytically inactivated forms. Cells were allowed 18 h for expression and then incubated with 250 nM proteasome inhibitor MG-262 for 4 h. Cell lysates were prepared and analysed by SDS-PAGE and Western blotting using the antibodies indicated. (C) HeLa cells were transiently transfected with plasmids AopP-pcDNA3.1/HIS-V5, AopPC177A-pcDNA3.1/HIS-V5, YopJ-pSFFV or YopJC172-pSFFV only and stimulated with TNF-α for 3 min as indicated. Cell lysates were subjected to SDS-PAGE and analysed by Western blotting using anti-phospho-IκBα and anti-tubulin antibodies as indicated. (D) AopP and YopJ expression in HeLa cells. HeLa cells were transfected with plasmids AopP-pcDNA3.1/HIS-V5 or YopJ-pSFFV and visualized using an anti-V5 or an anti-YopJ antibody, respectively. Nuclei were stained with Hoechst.

Fig. 5. AopP inhibits nuclear translocation of NF- κ B. Immunofluorescent labelling of p65 (green) and AopP-V5 (red) in epithelial cells. Adherent HeLa cells were transfected with plasmids pcDNA3.1/V5-HIS, AopP-pcDNA3.1/V5-HIS or AopPC177A-pcDNA3.1/V5-HIS and stimulated with TNF- α (100 ng ml⁻¹) for 30 min. AopP and p65 were labelled using anti-V5 and anti-p65 antibodies, respectively. White arrowheads mark cells transfected with AopP or AopPC177A. In the bottom row (p65+AopP), the pictures were merged.

factor NF- κ B into the nucleus, we anticipate that this protein has the potential to regulate the host inflammatory response. However, the exact role of AopP in the natural disease process may be more complex and will require further investigation, particularly as some virulent *A. salmonicida* subsp. *salmonicida* strains do not possess the *aopP* gene (Boyd *et al.*, 2003).

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REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Austin, D. A., Robertson, P. A., Wallace, D. K., Daskalov, H. & Austin, B. (1998). Isolation of *Aeromonas salmonicida* in association with purple-pigmented bacteria in sediment from a Scottish loch. *Lett Appl Microbiol* **27**, 349–351.
- Belland, R. J. & Trust, T. J. (1988). DNA:DNA reassociation analysis of *Aeromonas salmonicida*. *J Gen Microbiol* **134**, 307–315.
- Belland, R. J. & Trust, T. J. (1989). *Aeromonas salmonicida* plasmids: plasmid-directed synthesis of proteins *in vitro* and in *Escherichia coli* minicells. *J Gen Microbiol* **135**, 513–524.
- Boyd, J., Williams, J., Curtis, B., Kozera, C., Singh, R. & Reith, M. (2003). Three small, cryptic plasmids from *Aeromonas salmonicida* subsp. *salmonicida* A449. *Plasmid* **50**, 131–144.
- Braun, M., Stuber, K., Schlatter, Y., Wahli, T., Kuhnert, P. & Frey, J. (2002). Characterization of an ADP-ribosyltransferase toxin (AexT) from *Aeromonas salmonicida* subsp. *salmonicida*. *J Bacteriol* **184**, 1851–1858.
- Bullock, W. O., Fernandez, J. M. & Short, J. M. (1987). XL1-Blue: a high efficiency plasmid transforming *recA* *Escherichia coli* strain with β -galactosidase selection. *Biotechniques* **5**, 376–378.

Fig. 6. Model depicting the inhibitory activity of AopP on mammalian signalling pathways compared to that of other YopJ homologues.

- Burr, S. E., Stuber, K., Wahli, T. & Frey, J. (2002). Evidence for a type III secretion system in *Aeromonas salmonicida* subsp. *salmonicida*. *J Bacteriol* **184**, 5966–5970.
- Burr, S. E., Stuber, K. & Frey, J. (2003a). The ADP-ribosylating toxin, AexT, from *Aeromonas salmonicida* subsp. *salmonicida* is translocated via a type III secretion pathway. *J Bacteriol* **185**, 6583–6591.
- Burr, S. E., Wahli, T., Segner, H., Pugovkin, D. & Frey, J. (2003b). Association of Type III secretion genes with virulence of *Aeromonas salmonicida* subsp. *salmonicida*. *Dis Aquat Organ* **57**, 167–171.
- Burr, S. E., Pugovkin, D., Wahli, T., Segner, H. & Frey, J. (2005). Attenuated virulence of an *Aeromonas salmonicida* subsp. *salmonicida* type III secretion mutant in a rainbow trout model. *Microbiology* **151**, 2111–2118.
- Collier-Hyams, L. S., Zeng, H., Sun, J., Tomlinson, A. D., Bao, Z. Q., Chen, H., Madara, J. L., Orth, K. & Neish, A. S. (2002). Cutting edge: *Salmonella* AvrA effector inhibits the key proinflammatory, anti-apoptotic NF- κ B pathway. *J Immunol* **169**, 2846–2850.
- Colwell, R. R., Macdonell, M. T. & De Ley, J. (1986). Proposal to recognize the family *Aeromonadaceae* fam. nov. *Int J Syst Bacteriol* **36**, 473–477.
- Cornelis, G. R. & Van Gijsegem, F. (2000). Assembly and function of type III secretory systems. *Annu Rev Microbiol* **54**, 735–774.
- Denecker, G., Declercq, W., Geuijen, C. A., Boland, A., Benabdillah, R., van Gurp, M., Sory, M. P., Vandenabeele, P. & Cornelis, G. R. (2001). *Yersinia enterocolitica* YopP-induced apoptosis of macrophages involves the apoptotic signaling cascade upstream of Bid. *J Biol Chem* **276**, 19706–19714.
- Fürste, J. P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M. & Lanka, E. (1986). Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* **48**, 119–131.
- Ghosh, P. (2004). Process of protein transport by the type III secretion system. *Microbiol Mol Biol Rev* **68**, 771–795.
- Giles, J. S., Hariharan, H. & Heaney, S. B. (1995). The plasmid profiles of fish pathogenic isolates of *Aeromonas salmonicida*, *Vibrio anguillarum*, and *Vibrio ordalii* from the Atlantic and Pacific coasts of Canada. *Can J Microbiol* **41**, 209–216.
- Gudmundsdóttir, B. K. (1998). Infections by atypical strains of the bacterium *Aeromonas salmonicida*. *Buvisindi* **12**, 61–72.
- Gudmundsdóttir, B. K., Hvanndal, I., Bjornsdottir, B. & Wagner, U. (2003). Analysis of exotoxins produced by atypical isolates of *Aeromonas salmonicida*, by enzymatic and serological methods. *J Fish Dis* **26**, 15–29.
- Hanninen, M. L., Ridell, J. & Hirvela-Koski, V. (1995). Phenotypic and molecular characteristics of *Aeromonas salmonicida* subsp. *salmonicida* isolated in southern and northern Finland. *J Appl Bacteriol* **79**, 12–21.
- Hirono, I. & Aoki, T. (1993). Cloning and characterization of three hemolysin genes from *Aeromonas salmonicida*. *Microb Pathog* **15**, 269–282.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lee, K. K. & Ellis, A. E. (1990). Glycerophospholipid:cholesterol acyltransferase complexed with lipopolysaccharide (LPS) is a major lethal exotoxin and cytotoxin of *Aeromonas salmonicida*: LPS stabilizes and enhances toxicity of the enzyme. *J Bacteriol* **172**, 5382–5393.
- Lund, V. & Mikkelsen, H. (2004). Genetic diversity among A-proteins of atypical strains of *Aeromonas salmonicida*. *Dis Aquat Organ* **61**, 257–262.
- Masada, C. L., LaPatra, S. E., Morton, A. W. & Strom, M. S. (2002). An *Aeromonas salmonicida* type IV pilin is required for virulence in rainbow trout *Oncorhynchus mykiss*. *Dis Aquat Organ* **51**, 13–25.
- Nomura, S., Fujino, M., Yamakawa, M. & Kawahara, E. (1988). Purification and characterization of salmolyisin, an extracellular hemolytic toxin from *Aeromonas salmonicida*. *J Bacteriol* **170**, 3694–3702.
- Noonan, B. & Trust, T. J. (1997). The synthesis, secretion and role in virulence of the paracrystalline surface protein layers of *Aeromonas salmonicida* and *A. hydrophila*. *FEMS Microbiol Lett* **154**, 1–7.
- Orth, K. (2002). Function of the *Yersinia* effector YopJ. *Curr Opin Microbiol* **5**, 38–43.
- Orth, K., Palmer, L. E., Bao, Z. Q., Stewart, S., Rudolph, A. E., Bliska, J. B. & Dixon, J. E. (1999). Inhibition of the mitogen-activated protein kinase kinase superfamily by a *Yersinia* effector. *Science* **285**, 1920–1923.
- Orth, K., Xu, Z., Mudgett, M. B., Bao, Z. Q., Palmer, L. E., Bliska, J. B., Mangel, W. F., Staskawicz, B. & Dixon, J. E. (2000). Disruption of signaling by *Yersinia* effector YopJ, a ubiquitin-like protein protease. *Science* **290**, 1594–1597.
- Palmer, L. E., Pancetti, A. R., Greenberg, S. & Bliska, J. B. (1999). YopJ of *Yersinia* spp. is sufficient to cause downregulation of multiple mitogen-activated protein kinases in eukaryotic cells. *Infect Immun* **67**, 708–716.
- Simon, R., Priefer, U. & Puhler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Biotechnology* **1**, 784–791.
- Stuber, K., Burr, S. E., Braun, M., Wahli, T. & Frey, J. (2003). Type III secretion genes in *Aeromonas salmonicida* subsp. *salmonicida* are located on a large thermolabile virulence plasmid. *J Clin Microbiol* **41**, 3854–3856.
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990). Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185**, 60–89.
- Trosky, J. E., Mukherjee, S., Burdette, D. L., Roberts, M., McCarter, L., Siegel, R. M. & Orth, K. (2004). Inhibition of MAPK signaling pathways by VopA from *Vibrio parahaemolyticus*. *J Biol Chem* **279**, 51953–51957.
- Umelo, E. & Trust, T. J. (1998). Physical map of the chromosome of *Aeromonas salmonicida* and genomic comparisons between *Aeromonas* strains. *Microbiology* **144**, 2141–2149.
- Vallette, F., Mege, E., Reiss, A. & Adesnik, M. (1989). Construction of mutant and chimeric genes using the polymerase chain reaction. *Nucleic Acids Res* **17**, 723–733.
- Vipond, R., Bricknell, I. R., Durant, E., Bowden, T. J., Ellis, A. E., Smith, M. & MacIntyre, S. (1998). Defined deletion mutants demonstrate that the major secreted toxins are not essential for the virulence of *Aeromonas salmonicida*. *Infect Immun* **66**, 1990–1998.
- Zhou, H., Monack, D. M., Kayagaki, N., Wertz, I., Yin, J., Wolf, B. & Dixit, V. M. (2005). *Yersinia* virulence factor YopJ acts as a deubiquitinase to inhibit NF- κ B activation. *J Exp Med* **202**, 1327–1332.