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

Désirée Fehr,1 Carlo Casanova,2 Amy Liverman,3 Hana Blazkova,2 Kim Orth,3 Dirk Dobbelelaere,2 Joachim Frey1 and Sarah E. Burr1

12Institute of Veterinary Bacteriology1 and Division of Molecular Pathology2, Vetsuisse Faculty, Universität Bern, Länggassstrasse 122, Postfach, CH-3001 Bern, Switzerland
3Department of Molecular Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX, USA

*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-κB) signalling pathways. AopP inhibits the NF-κB pathway downstream of IκB 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 septicemia. 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, IκB kinase; MAPK, mitogen-activated protein kinase; M KK, MAPK kinase; NF-κB, 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 (MKKs) and the IkB kinase (IKK) complex (Orth, 2002). As a result of these activities, signalling via the MAPK and nuclear factor kappa B (NF-kB) pathways is prevented. In this way, YopJ is able to block cytokine production and promote apotosis 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-kB signalling pathway downstream of IkB 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 μg ampicillin ml⁻¹, 40 μg kanamycin ml⁻¹, and 40 μg 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 EcoRI, 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://www.ncbi.nlm.nih.gov/ orf.cgi). 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 μM DIG (Roche Diagnostics) in order to generate a labelled probe. PCR was carried out using the following primer pair: AsORF28, 5’-GAGATTGCTAGCGGTGAG-3’, and AsORF38, 5’-TCTTCTAGAGCCGATCC-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 Qiagrep Spin Miniprep kit (Qiagen) and was digested with the restriction enzyme BamHI. 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× saline sodium citrate (SSC), 0.1 % SDS, followed by a second wash with 0.2× 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 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 ascVSatt, 5’-GTCGACATGTCAGCAGCTGACAG-3’, and ascVHindIII, 5’-AAGCTTCCAGATGTAGGAGCAAC-3’ (underlined nucleotides indicate recognition cut sites for restriction endonucleases Sall and HindIII, 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 μg ampicillin ml⁻¹ and 40 μg 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). 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 aopPC177Adw, 5’-GAAGCAATTCAGGACAGGGATTCAGG-3’, and aopPC177A-rev, 5’-GTCGAAATATCCCCGGCTGTGTTAATTGCT-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.
Production of polyclonal anti-AopP antibodies. To generate polyclonal antibodies directed against AopP, we first amplified the aopP gene using primers HISaopPspel, 5’-GAACATGTATGAATACCCCCCATCC-3’, and HISaopPNorI, 5’-GGGGGGCCGCAAACGAGGATTTTCCGTCATCAG-3’ (underlined nucleotides indicate recognition cut sites for restriction endonucleases Spel and NorI, 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 Spel and NorI, 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 potassium 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 antiserum using HiTrap affinity columns (Amersham Pharmacia, Biotech) according to the manufacturer’s instructions.

MAP kinase assay. Approximately 7.5 × 10^6 HEK293 cells were seeded and transfected using 7 μL FuGENE 6 reagent (Roche
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 pAsa1 (6371 bp), pAsa2 (5424 bp) and pAsa3 (5259 bp). As previously reported, plasmid pAsa2 is identical to plasmid pAsa1 found in the virulent *A. salmonicida* subsp. *salmonicida* isolate, strain A449, whereas plasmid pAsa3 is virtually identical to plasmid pAsa2 of strain A449 (6 base pair differences) (Boyd et al., 2003). Plasmid pAsa1 is not found in strain A449.

Sequence analysis of plasmid pAsa1 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 Yop/J/P proteins of the pathogenic *Yersinia* sp. (46 % identity, 64 % similarity), AvrA of *Salmonella enterica* Typhimurium (40 % identity, 59 % similarity) and VopA of *Vibrio paraheamolyticus* (37 % identity, 55 % similarity). Like these Yop 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 *aexT*, which is chromosomally encoded (Stuber et al., 2003). No other potential type III effector genes were found on plasmid pAsa1, or on the other small plasmids found in *A. salmonicida* subsp. *salmonicida* strain JF2267, pAsa2 and pAsa3.

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 the *aopP* gene.
the gene (Fig. 2B). Strains ATCC 27013T 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 1110T 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 1110T 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 33658T 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 NCIMB1110T 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

<table>
<thead>
<tr>
<th>Designation</th>
<th>Description</th>
<th>Length (bp)</th>
<th>Homologous gene</th>
<th>Percentage amino acid identity/similarity</th>
<th>Homologue accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>repA</td>
<td>Replication protein (ColE2 family)</td>
<td>975</td>
<td>repA of Vibrio cholerae O1</td>
<td>46/61</td>
<td>BAD88720</td>
</tr>
<tr>
<td>orfC1</td>
<td>Unknown function</td>
<td>324</td>
<td>orfC1 of Ralstonia solanacearum</td>
<td>38/55</td>
<td>NP 052314</td>
</tr>
<tr>
<td>mobA</td>
<td>Mobilization protein</td>
<td>1263</td>
<td>mbbA of E. coli</td>
<td>28/43</td>
<td>AY929248</td>
</tr>
<tr>
<td>tsp</td>
<td>Potential transposase, ISA3</td>
<td>1215</td>
<td>tsp of Y. pestis KIM</td>
<td>67/81</td>
<td>NP 671111</td>
</tr>
<tr>
<td>aopP</td>
<td>Potential type III effector</td>
<td>900</td>
<td>yopP of Y. enterocolitica</td>
<td>46/64</td>
<td>AAK69256</td>
</tr>
</tbody>
</table>
with these findings, strains NCIMB 1110\textsuperscript{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 \textit{A. salmonicida} subsp. \textit{salmonicida} strain JF2267, an isogenic type III secretion mutant, strain JF2747, and a complemented strain, JF3239. Strain JF2747 possesses a mutation in the gene \textit{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 \textit{ascV} mutation has been complemented \textit{in trans} with plasmid pMMB66EH\textit{ascV}\textsuperscript{+}. 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 \textit{ascV} mutant, strain JF2747. This finding indicates that AopP is secreted into the external environment via the TTSS pathway. \textit{In trans} complementation of the \textit{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-κB signalling pathway downstream of IKK**

We investigated whether AopP interferes with the NF-κB signalling pathway by assaying the effect of AopP on IκBζ phosphorylation. HeLa cells were transiently transfected with plasmids expressing IκBζ-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 IκBζ showed that expression of wt AopP and the catalytically inactive forms of both effectors had no effect on the phosphorylation of IκBζ (Fig. 4B). In contrast, expression of wt YopJ resulted in significantly reduced phosphorylation of IκBζ (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-κB 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-IκBζ 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-IκBζ could be observed (Fig. 4C, top panels, lane 4). Expression of YopJ, on the other hand, led to a reduction in IκBζ 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 IκBζ phosphorylation.

As the Salmonella homologue of AopP, AvrA, is also unable to prevent phosphorylation of IκBζ, yet is capable of inhibiting the NF-κB pathway downstream of IKKα activation (Collier-Hyams et al., 2002), we used immunolocalization of the p65 subunit of NF-κB to further study the potential interference of AopP with the NF-κB 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-κB pathway at a point downstream of IκBζ 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-κB signalling pathways by removing lysine63- and lysine48-linked ubiquitin moieties from regulatory proteins (Zhou et al., 2005). In the NF-κB pathway, YopJ impedes the lysine63-linked ubiquitination processes required for IKK activation, and proteasomal degradation of IκBζ 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 IκBζ phosphorylation. The fact that p65 translocation is blocked, however, might suggest that the activity of AopP is restricted to preventing IκBζ 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-κB 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 IkBα 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 IkBα-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, AopPC172A-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-IkBα 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.
factor NF-κB 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**


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Fig. 5. AopP inhibits nuclear translocation of NF-κB. 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.

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


