AEROMONAS EXOENZYME T OF AEROMONAS SALMONICIDA IS A BIFUNCTIONAL PROTEIN THAT TARGETS THE HOST CYTOSKELETON

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Type III protein secretion has recently been shown to be important in the virulence of the fish pathogen Aeromonas salmonicida. The ADP-ribosylating toxin Aeromonas exoenzyme T, or AexT, is one effector protein targeted for secretion via this system. In this study, we identified muscular and non-muscular actin as substrates of AexT’s ADP-ribosylating activity. Furthermore, we show that AexT also functions as a GTPase activating protein (GAP), displaying GAP activity against monomeric GTPases of the Rho family, specifically Rho, Rac and Cdc42. Transfection of fish cells with wild type AexT resulted in depolymerization of the actin cytoskeleton and cell rounding. Point mutations within either the GAP or the ADP-ribosylating active sites of AexT (R143 as well as E398 and E401 respectively) abolished enzymatic activity, yet did not prevent actin filament depolymerization. However, inactivation of the two catalytic sites simultaneously did. These results suggest that both the GAP and ADP-ribosylating domains of AexT contribute to its biological activity. This is the first bacterial virulence factor having a specific actin ADP-ribosylation activity and GAP activity towards Rho, Rac and Cdc42, both enzymatic activities contributing to actin filament depolymerization, to be described.

Aeromonas salmonicida subsp. salmonicida (A. salmonicida) is the causative agent of furunculosis, a systemic disease that affects salmonid fish (salmon, trout and char). A. salmonicida expresses a variety of extracellular toxins, many of which have been implicated in virulence. Several of these factors, including the pore-forming toxin aerolysin, serine protease and the phospholipase GCAT, are secreted into the environment via the well characterized type II or general secretory pathway (1,2). However, like many Gram-negative pathogens, A. salmonicida also possesses a type III protein secretion system (3-5). Such secretion systems translocate toxins, or type III effector proteins, directly into the cytosol of eukaryotic cells. Here, the effector proteins are able to modulate cell signalling pathways, or alternatively, disrupt the dynamics of the cytoskeleton (6-7).

To date, genes encoding four type III effector proteins have been identified in A. salmonicida; aexT, aopP, aopO and aopH (8-10). While functional studies of AopO and AopH have not yet been carried out, sequence homology with type III effector proteins YopO and YopH of Yersinia sp. suggest that AopO and AopH modify the actin cytoskeleton of target eukaryotic cells. In contrast, AopP has been shown to interfere with the NF-κB signalling pathway by inhibiting translocation of NF-κB into the nucleus of target cells (10).

AexT, the first type III effector of A. salmonicida to be characterized, has been shown to function as an ADP-ribosylating toxin (8). However, its substrate has never before been identified. AexT also displays significant sequence homology to the bifunctional type III effector proteins ExoS and ExoT, expressed by Pseudomonas aeruginosa. Both ExoS and ExoT are known ADP-ribosylating toxins (11) but also function as GTPase activating, or GAP proteins (12,13).

Here we report that AexT is also a bifunctional protein possessing both ADP-ribosylating and GAP activities. We identify actin as the only specific target of AexT’s ADP-ribosylating activity and show that AexT also exhibits GAP activity against the monomeric GTPases Rho, Rac and Cdc42. Both enzymatic activities are involved in
Aext-dependent actin filament depolymerization in eukaryotic cells.

Experimental Procedures

**Bacterial strains—Cloning** was routinely carried out in *Escherichia coli* strain XL1-Blue (14). *E. coli* strain BL21(DE3) (15) was used for the expression of recombinant Aext proteins and strain S17-1 (16) was used as the donor strain in bacterial conjugation.

The wild type (wt) isolate *A. salmonicida* strain JF2267 (8) used in this study was routinely cultured on Luria-Bertani agar plates at 18 °C.

**Site-directed mutagenesis**—The wt aext gene was amplified from *A. salmonicida* strain JF2267 by PCR using primers *aext*HIS-fwd and *aext*HIS-rev (Table 1) before being cloned into the vector pGEM-T Easy (Promega). Site-directed mutagenesis via the overlap extension-PCR method (17) was then carried out using mutagenesis primers as indicated in Table 1. Resulting PCR products were digested with DpnI to eliminate the methylated (parental) DNA (18) and subsequently used to transform *E. coli* XL1-Blue. Mutations were verified by DNA sequencing before cloning into the expression vectors pETHIS-1 (Novagen) and pEGFP-N2 (BD Biosciences).

**Overexpression of Aext and Aext mutants—Vector pETHIS-1**, containing either wt or mutated aext genes, was transformed into *E. coli* BL21(DE3) cells for expression. Induction and subsequent purification of recombinant proteins was performed as described previously (19).

**ADP-ribosylation assay**—In vitro ADP-ribosylation assays were carried out in 50 mM triethanolamine buffer (pH 7.5) containing 5 mM MgCl₂, 10 mM DTT, 10 mM thymidine and 5×10⁵ cpm [³²P]NAD (spect. act. 800 Ci mmol⁻¹; Perkin Elmer, Boston, MA). Lysates of EPC or Vero cells (20 μg), muscular or non-muscular actin (1 or 4.5 μg), Rho-GST or Rac-GST fusion proteins (1 μg) and recombinant wt Aext (10 μM) were added to a total volume of 20 μl. After incubation at 37 °C for 30 min, samples were subjected to SDS-PAGE and processed by autoradiography.

Kinetic analysis was carried out as previously described (20) using 0.5 μM Aext. NAD concentrations were 5, 3.3, 1, 0.33, 0.1 and 0.03 μM. ADP-ribosylating activity constants (Vₘₐₓ and Kₘ) were determined using Lineweaver-Burk plots. Determination of ADP-ribosylating activity of iota toxin from *Clostridium perfringens* was included for comparison.

**Peptide analysis by HPLC, sequencing and mass spectrometry**—Non-muscular actin (6 μg) was incubated with Aext (10μM) and [³²P]NAD (1 μM) or cold NAD (10 mM) for 1 hour at 37 °C. The actin was then subjected to SDS-PAGE and stained with amido-black. Actin bands were cut from the gel, washed with dH₂O, dried and suspended in 150 μl 50 mM Tris HCl, pH 8.6, containing 0.01 % Tween 20 and 0.2 μg trypsin (Promega). Following 18 hours incubation at 30 °C, the preparations were centrifuged and supernatants were recovered. Samples were injected onto a DEAE HPLC column (Interchim Hema Bio 1000 DEAE, 33 x 1 mm) linked to a C18 reverse phase HPLC column (Interchim Uptisphere ODBD, 150 x 1 mm) and eluted with a 2-70 % acetonitrile gradient in 0.1 % trifluoroacetic acid. Radioactive material in fractions from actin ADP-ribosylated with [³²P]NAD was screened by blotting 3 μl of each fraction onto a PVDF membrane and analyzing with a phosphoimager.

Protein sequencing was performed on an Applied Biosystem 494 sequencer.

Separated peptides (1-2 μl on a gold ProteinChip or H4 ProteinChip array) were identified by their mass using SELDI-TOF-MS (PCS 4000) from Bio-Rad (Focus mass = 4000 daltons; Laser intensity = 1100 nJ). The matrix (0.8 μl) was alpha-cyano-4-hydroxy cinnamic acid saturated in 50 % acetonitrile, 0.5 % trifluoroacetic acid.

**GAP activity assay**—GAP activity was determined as previously described (21). The assays were performed 3 times, each time in duplicate.

**Immunofluorescent staining of the actin cytoskeleton**—Epithelioma papulosum cyprinid (EPC) cells were transiently transfected with 2 μg of plasmid DNA using the PolyFect Reagent (Qiagen) according to the manufacturer’s instructions. Cells were incubated overnight at 18 °C. Eighteen hours post-transfection, cells were fixed with 4 % formaldehyde and then incubated in 50 mM NH₄Cl for 15 min in order to block free aldehydes remaining after fixation. Cells were
permeabilized with 0.1 % Triton X-100 for 10 min, followed by incubation for 1 hour at room temperature with TRITC-Phalloidin (Sigma). Stained cells were observed using a Nikon Eclipse 80i microscope and images were produced by digital confocal imaging using Openlab software (Improvision). All tranfections were carried out twice and a minimum of 20 transfected cells were examined for each mutant.

Marker-replacement mutagenesis—The aexT gene and flanking regions were amplified from A. salmonicida strain JF2267 using primers aexTREcoRI and aexTLSacI (Table 1) and cloned into vector pUC19 (Invitrogen). The kanamycin cassette from plasmid pSSV1186 (22) was excised and blunt ends were created using S1 nuclease (Promega) before ligation into the Apal and Stul cut sites (blunt-ended with S1 nuclease) within aexT. The inactivated aexT gene was then cloned into the mobilizable suicide vector pSUP202sac (23) and the resulting plasmid, pSUP202sac-aexT::Km was transformed into E. coli S17-1. Conjugation into A. salmonicida strain JF2267 was carried out by filter mating (16). Double-crossover mutants were selected for directly by growth on tryptic soy agar containing 15 % (wt/vol) sucrose, 40 μg of kanamycin ml⁻¹ and 20 μg of chloramphenicol ml⁻¹ at 15 °C for 7 days. Chloramphenicol was used to select against the A. salmonicida strain JF2267 is resistant to this antibiotic. PCR was then used to ensure the correct mutation was present in A. salmonicida.

Fish cell infections with A. salmonicida—Infection of EPC cells was performed as described previously (24) using a multiplicity of infection of 20:1 (bacteria to fish cells). Two hours post infection, the cells were photographed under phase-contrast microscopy (Axiovert 100; Zeiss).

RESULTS

AexT ADP-ribosylates cellular actin—In order to identify potential targets of AexT’s ADP-ribosylating activity, an in vitro ADP-ribosylating assay was performed using lysates of both EPC cells, a fish cell line derived from carp epithelium, and Vero cells. In each cell line, wt AexT ADP-ribosylated only one protein of approximately 45 kDa (Fig. 1A, lanes 2 and 3), corresponding to the size of cellular actin (Fig. 1A, lane 1). When the assay was performed using AexT alone, no ADP-ribosylation was detected indicating AexT does not undergo auto-ribosylation (Fig. 1A, lane 5). As a further control, we also carried out this assay using purified actin and iota toxin, a well-characterized ADP-ribosylating toxin of C. perfringens that targets all actin isoforms (25,26,28). The result with this toxin was the expected one (Fig. 1A, lane 4) thereby adding strength to our finding that actin is the substrate of AexT’s ADP-ribosylating activity.

In order to further verify our results, a second assay was performed using both muscular (bovine) and non-muscular (human platelet) actin. The GTPases Rho and Rac were also included as potential targets as these proteins are ADP-ribosylated by the AexT homologue ExoS of P. aeruginosa and are also the substrate of various other toxins such as exoenzyme C3 of Clostridium botulinum and large clostridial toxins (33). The results indicate that AexT exhibits specific ADP-ribosylating activity against actin, with the reaction against non-muscular actin being significantly stronger than that against muscular actin (Fig. 1B, lanes 1 and 2). No AexT-dependent ADP-ribosyltransferase activity could be detected against Rho and Rac (Fig. 1B, lanes 3 and 4) although ADP-ribosylation of Rho by C3, which served as a positive control, was detected (Fig. 1B, lane 5).

In order to quantitate the difference seen between ADP-ribosylation of muscular and non-muscular actin, a kinetic assay was carried out using both targets. The results, shown in Table 2, confirmed our finding that ADP-ribosylating activity of AexT is stronger against non-muscular actin. A further comparison between AexT and iota toxin indicated that ADP-ribosylating activity of AexT is lower than that of iota toxin for both isoforms of actin tested (Table 2).

In an effort to identify the actin residue or residues that are ADP-ribosylated by AexT, non-muscular actin was incubated with AexT in the presence of [³²P]NAD. Trypsin digestion was then carried out and the resulting peptides were separated by HPLC. Radioactivity was primarily recovered in fraction 51 with lesser amounts recovered in three fractions immediately adjacent (Fig. 2A). This indicated that a specific peptide contained the ADP-ribosylated residue.
To obtain a higher yield of actin ADP-ribosylated with AexT, the reaction was then carried out with 10 mM non radioactive NAD. The peptide profile of actin modified by AexT and non radioactive NAD was similar to that of unmodified actin however, peak 51 was slightly enlarged in the actin ADP-ribosylated by AexT (data not shown). Peaks 51 from both ADP-ribosylated and unmodified actin were analyzed by N-terminal sequencing and mass spectrometry. Several peptides were identified in both fractions beginning with the sequences FRCPEA, LCYVAL and CPEALF. An additional peptide beginning with the sequence TTGIVM was identified only in AexT-treated actin. Mass spectrometry by two different arrays showed that a 4391 Da peptide was present in peak 51 of AexT-treated actin but not in the corresponding peak of the control actin (Fig. 2B and C). The size of this peptide corresponds to the sequence TTGIVMDSDGVTHTVPIYEGYALPHAILR177LDLAGR with an oxidized M and an ADP-ribose and Na+ molecule (4389.33 Da). A small amount of the same peptide with the non-oxidized M and an ADP-ribose and Na+ molecule (4373.34 Da) was also recovered. Modification of R-177 by ADP-ribosylation thereby preventing trypsin cleavage at this residue explains the presence of this peptide in peak 51 from ADP-ribosylated actin but not in the equivalent peak from control actin. Taken together, these results indicate that R-177 is mono-ADP-ribosylated by AexT.

Glutamic acid residues E398 and E401 and arginine R303 are involved in ADP-ribosylating activity of AexT. ADP-ribosylating toxins retain a conserved structure that forms the enzymatic site. This region accommodates a molecule of NAD and possesses a catalytic glutamic acid residue essential for the enzymatic activity (27). The active site of certain toxins, including actin-ADP-ribosylating toxins such as iota toxin, contain not one but two glutamic acid residues whereby both residues are required for the ADP-ribosylation activity (27,28). The amino acid sequence of AexT possesses such a biglutamic acid motif within its C-terminal domain (Fig. 3A) (8). According to sequence alignment, the two glutamic acid residues within the motif of AexT are found at positions 401 and 403. To determine the influence of these residues on the ADP-ribosylating activity of AexT, mutants containing glutamic acid to alanine substitutions were generated. The ADP-ribosylating activity of the recombinant mutants was then assayed in vitro using non-muscular actin as a substrate. A single mutant, whereby the second of the two glutamic acid residues in the ADP-ribosylating domain of AexT was mutated to alanine, AexTE403A, displayed only a slight decrease in ADP-ribosyltransferase activity when compared to the wt protein (Fig. 3B, lane 2). A similar result was obtained with a double mutant whereby both glutamic acid residues were substituted, AexTE401A/E403A (Fig. 3B, lane 3). Examination of the amino acid sequence of AexT surrounding the biglutamic acid site then identified a third glutamic acid upstream of E401 at position 398 (Fig. 3A). Two more AexT mutants, AexTE398A/E403A and AexTE398A/E401A, were therefore constructed. While AexTE398A/E403A still displayed significant ADP-ribosylating activity (Fig. 3B, lane 4), the activity of mutant AexTE398A/E401A was virtually abolished (Fig. 3B, lane 5). A triple mutant, AexTE398A/E401A/E403A, containing glutamic acid substitutions in all three positions, was also found to possess virtually no ADP-ribosylating activity (Fig. 3B, lane 6). The results indicate that the two catalytic glutamic acids of AexT are E398 and E401. It is interesting to note that these residues are separated by two amino acids, instead of only one as in other ADP-ribosylating toxins possessing a biglutamic motif (Fig. 3A) (27).

ADP-ribosylating toxins can be divided into two groups according to conserved residues in the catalytic site. The cholera toxin-group of ADP-ribosylating toxins, which includes iota toxin and other ADP-ribosylating toxins that target actin, contain a conserved arginine residue that is required to maintain the stability of the active site pocket. In the diphtheria toxin-group, a histidine residue rather than an arginine, carries out this function (27). Sequence alignment of AexT with cholera toxin suggests that R303 or R306 in AexT could be the conserved, functional arginine residue of the cholera toxin-group (Fig. 4A). Two AexT mutants, whereby these arginine residues were mutated to alanine, were constructed and their ADP-ribosylating activity was compared to that of wt AexT. While the mutant R306A retained full enzymatic activity, AexT carrying the mutation R303A displayed an almost complete loss of ADP-ribosylating activity.
(Fig. 4B). This result indicates that AexT retains the main features of the cholera-like toxins.

**AexT alters the actin cytoskeleton and mutation of the catalytic ADP-ribosylating residues does not prevent AexT-dependent actin depolymerization—Eukaryotic cell transfections, carried out using aexT constructs cloned in-frame with enhanced green fluorescent protein (EGFP), and immunofluorescence microscopy were performed in order to assay the effect of AexT expression on EPC cells. Fluorescence microscopy showed that cells transfected with the pEGFP-N2 vector only displayed a typical morphology and a well-defined actin cytoskeleton (Fig. 5A). In contrast, all cells transfected with wt pAexT-EGFP had a rounded appearance and no longer displayed actin stress fibers (Fig. 5B). The AexT-dependent changes in the actin cytoskeleton are reminiscent of the effects induced by actin-ADP-ribosylating toxins. When the mutants AexTE398A/E401A and AexTE398A/E401A/E403A, both of which have significantly reduced ADP-ribosylating activity, were expressed in EPC cells, significant actin depolymerization was still observed in at least 18 of 20 transfected cells examined (90 %), resulting in the presence of actin clouds and a rounded cell morphology (Fig. 5C and D). This indicates that wt AexT induces disruption of actin filaments and cell rounding and that mutations within the ADP-ribosylating site do not prevent this effect. Therefore, an additional activity is likely involved in the actin filament disruption caused by AexT.

**AexT exhibits GAP activity towards GTPases Rho, Rac, and Cdc42—Sequence alignment of AexT with type III effector proteins that are known to act as GAP proteins identified a potential RhoGAP consensus motif (29) in the N-terminal domain of the protein (Fig. 6A). The RhoGAP motif contains an arginine residue that is known to be essential for efficient catalysis (30) and in AexT, this residue is found at position 143 (Fig. 6A).

The results of an in vitro assay of GTP hydrolysis performed with recombinant wt AexT indicated that almost 100 % of the $[^{32}P]$-GTP bound to Rac, Cdc42 and Rho was hydrolyzed following 5 min incubation at room temperature (Fig. 6B, C, D). In each case, the GAP activity of wt AexT was similar to that of RhoGAP, which served as a positive control. In contrast, an AexT mutant whereby the arginine residue at position 143 was substituted with a lysine residue, AexTR143K, showed a marked decrease in GTP hydrolysis. Five min following the addition of this mutant, approximately 60 % of bound $[^{32}P]$-GTP was hydrolyzed by Rac and Cdc42 (Fig. 6B, C), and approximately 30 % was hydrolyzed by Rho (Fig. 6D). Similar results were obtained with the negative control where no GAP protein was present. These results demonstrate that AexT possesses GAP activity towards all three GTPases tested and indicates that the arginine residue found within the RhoGAP consensus motif is required for this activity.

AexT-dependent actin cytoskeleton alteration is mediated by both actin-ADP-ribosylation and GAP activity towards Rho-GTPases—The effect of the GAP domain of AexT on actin depolymerization in EPC cells was also assayed by cell transfection, using plasmid pEGFP-N2 expressing AexTR143K. After 18 hours incubation, at least 60 % of fish cells transfected with this construct displayed an altered actin cytoskeleton and a rounded morphology (Fig. 7A) similar to that seen in cells transfected with wt AexT (Fig. 5B). Following 22 hours incubation, at least 80 % of transfected cells displayed these morphological changes. This indicates that, like the loss of ADP-ribosylating activity, loss of GAP activity alone does not prevent actin depolymerization by AexT.

Finally, transfection with a quadruple mutant, AexTR143K/E398A/E401A/E403A, whereby the essential residues within the GAP and the ADP-ribosylating domains were mutated, was performed. At least 90 % of examined cells transfected with this mutant displayed a well-defined cytoskeleton and no cell rounding was observed (Fig. 7B). This suggests that both the GAP and ADP-ribosylating domains of AexT have the ability to affect the actin cytoskeleton independently of one another. Similar results were obtained when the mammalian cell line, COS-7, was used in the transfection assay (data not shown).

**AexT contributes to the cytopathology of A. salmonicida—**The cytopathic effect of AexT was assessed by infecting cultured EPC cells with wt A. salmonicida strain JF2267 and an isogenic ΔaexT mutant containing a deletion of both the GAP and ADP-ribosylating domains (Fig. 8A). While...
infection with wt *A. salmonicida* strain JF2267 induced cell rounding and detachment within 2 hours post infection (Fig. 8B), EPC cells infected with the isogenic Δ*aexT* mutant displayed no marked morphological changes (Fig. 8C). These cells were similar in appearance to cells incubated with PBS only (Fig. 8D). After 3 hours incubation, EPC cells infected with the Δ*aexT* mutant began to display cell rounding and after 4 hours, these cells were similar in appearance to those infected with the wt strain (data not shown). The results indicate that while AexT induces a cytopathic effect on target fish cells, other proteins expressed by *A. salmonicida* also contribute to the cytopathic effect of the bacterium.

**DISCUSSION**

AexT was found to be a bifunctional protein with two distinct enzymatic activities, ADP-ribosylating activity and GAP activity. We have shown that, *in vitro*, AexT ADP-ribosylates both muscular and non-muscular actin with the reaction against non-muscular actin being the stronger. AexT is therefore, a member of the large group of ADP-ribosylating toxins that target actin. Other members of this groups include the clostridial binary toxins (31-33), vegetative insecticidal proteins (VIP) from *Bacillus cereus* and *Bacillus thuringiensis* (32,34), the *Streptococcus pyogenes* toxin SpyA (35) as well as the potential type III effector molecule SpvB of *Salmonella enterica* (36,37). Like the ADP-ribosylating effector SpvB (37) and toxins such as C2 toxin of *C. botulinum* (32), AexT preferentially modifies non-muscular actin versus muscular actin.

Another marked similarity found between AexT, SpvB and C2 toxin is the site of modification on the actin molecule. AexT was found to mono-ADP-ribosylate actin at residue R-177. This residue is located at the contact site between actin monomers. ADP-ribosylation of monomeric actin prevents contact with other actin monomers thereby preventing polymerization (27). R-177 has also been shown to the modification site of SpvB and C2 toxin as well as of iota toxin (26,38,39).

Despite a low level of sequence homology, ADP-ribosylating toxins possess a conserved structure that forms the site of ADP-ribosylation. This active site is formed by a β-strand followed by an α-helix bend together creating a cavity in which the nicotinamide ring of NAD is anchored during catalysis (27). In all ADP-ribosylating toxins, the catalytic residue is a glutamic acid. This residue forms a hydrogen bond with the 2'-hydroxyl group on the nicotinamide ribose of NAD, which mediates the transferase reaction. Toxins of the cholera toxin-group have another glutamic acid or glutamine residue located two amino acids upstream of the catalytic glutamic acid. C3 has the Q-X-E sequence whereas toxins from the iota and C2 families, cholera toxin, as well as other ADP-ribosyl transferases possess an E-X-E motif (27). Type III-secreted ADP-ribosylating toxins such as ExoS, ExoT, and SpvB also retain an E-X-E motif (37,40). Detailed functional studies of iota toxin have shown that both of the glutamic acid residues in the E-X-E motif are required for ADP-ribosyltransferase activity (28,41). An E-X-E motif has previously been identified in AexT and, by sequence alignment, was found to encompass residues E401 and E403 (Fig 3A) (8). Surprisingly however, our current study has found that recombinant AexT mutants containing glutamic acid to alanine substitutions of these residues, AexTE403A and AexTE401A/E403A, still retained significant ADP-ribosylating activity (Fig. 3B). This is in marked contrast to clostridial binary toxins and the type III secreted ADP-ribosyltransferases ExoS, ExoT, and SpvB whereby mutation of the glutamic acid residues within the E-X-E motif abolishes ADP-ribosylation activity and subsequent cell effects (32,42,43). For example, mutation of the second glutamic acid within the E-X-E motif of ExoS and within the *S. pyogenes* toxin SpyA is sufficient to eliminate ADP-ribosylating activity (35,44). In the case of the *C. perfringens* iota toxin, mutation of either of the two glutamic acid residues within the E-X-E site is sufficient to abolish the protein’s activity (28). Still another study has found that a double mutant of SpvB, whereby both glutamic acid residues had been replaced by aspartates, was no longer active (37).

In AexT, we have found an additional glutamic acid residue located directly upstream of the E-X-E motif, at position 398 (Fig 3A). In contrast to the AexT mutants AexTE401A/E403A and AexTE398A/E403A, which retain significant ADP-ribosyltransferase activity, the mutant
AexTE398A/E401A displayed almost no ADP-ribosylating activity (Fig. 3B). This finding suggests that the key glutamic acid residues in AexT are E398 and E401 although this is not consistent with the position of the E-X-E motif. Interestingly, ExoT also contains a glutamic acid two amino acids upstream of the E-X-E motif at a position equivalent to E398 in AexT (Fig. 3A). Yet, substitution of the E-X-E motif of ExoT by A-A-A results in loss of cell rounding (42). Another study has found that mutation of the E-X-E motif to D-X-D results in a specific loss of ADP-ribosylating activity (45). Therefore, despite the sequence homology of the catalytic site, AexT and ExoT do not require equivalent glutamic acid residues for their enzymatic activity. Structural analysis of these proteins, in particular of the catalytic site, would help to solve why AexT does not appear to conform to the functional E-X-E consensus sequence.

Unlike secreted toxins including actin-ADP-ribosylating toxins, many type III effector proteins have a bifunctional activity (33). Among them, the AexT homologues ExoS and ExoT display ADP-ribosylation and GAP activities. Like ExoS and ExoT, AexT was shown, in vitro, to possess GAP activity for monomeric GTPases of the Rho family, RhoA, Rac1 and Cdc42 (Fig. 6). During the preparation of this manuscript, this result was confirmed by an independent study, which expressed only the N-terminal region of AexT (amino acids 93-255) (46). In contrast to ExoS which ADP-ribosylates numerous cell proteins (47-49) and to ExoT, which targets Crk proteins (45), AexT specifically ADP-ribosylates cellular actin. Indeed, the bifunctional activity of AexT is specifically directed against the actin cytoskeleton including modification of actin monomers and down regulation of Rho proteins leading to actin filament depolymerization.

Fish cells transfected with a plasmid expressing wt AexT displayed a distinct, rounded morphology and the actin filaments were clearly disrupted. This observation is consistent with both the ADP-ribosylating and GAP activities of AexT. It is not surprising therefore, that despite the strong GAP activity of AexT, transfection of EPC cells with a plasmid expressing AexTR143K, the mutant that no longer possesses GAP activity (Fig. 6), did not prevent the cytopathic effect of AexT (Fig. 7A). This finding is in contrast to studies of the type III effector YopE where expression of a corresponding mutant (R144A) completely inhibited the disruption of the microfilament structure of HeLa cells (50). However, YopE, which is shorter in length than AexT, does not possess an ADP-ribosylating domain. In contrast, a single mutation of R149K in the bifunctional protein ExoT did not abolish the cytopathic effect of this toxin. Disruption of the actin cytoskeleton was still visible when HeLa cells were transfected with ExoTR149K indicating that the ADP-ribosylating domain of ExoT also contributes to the biological functions of this protein (42). Our observation that expression of AexTR143K within transfected EPC cells causes an altered cytoskeleton, indicates that the ADP-ribosylating domain of AexT also contributes to this protein’s activity.

Transfection of EPC cells with plasmids expressing mutants that no longer possess ADP-ribosylating activity, the double mutant AexTE398A/E401A and triple mutant AexTE398A/E401A/E403A, also resulted in significant alteration of the actin cytoskeleton and a rounded appearance similar to that seen when cells were transfected with the wt protein (Fig. 5B, C, and D). Together with the transfection results of the GAP mutant, these results indicate that both the GAP and ADP-ribosylating activities of AexT are able to affect the cytoskeleton independently of one another. Accordingly, no disruption of the actin cytoskeleton was seen when EPC cells were transfected with a quadruple mutant, AexTR143K/E398A/E401A/E403A, which is devoid of both GAP and ADP-ribosylating activities (Fig. 7B).

When epithelial fish cells were infected with the wt A. salmonicida isolate, strain JF2267, the cells displayed extensive cell rounding and retraction two hours post infection (Fig. 8B). In contrast, cells inoculated with an isogenic aexT mutant did not display any morphological changes (Fig. 8C). This is consistent with our findings that AexT affects the eukaryotic cytoskeleton. However, after a longer incubation time, cells infected with the ΔaexT mutant also began to display a rounded appearance. We should note that these findings are inconsistent with a previous study by Braun et al., which indicated a ΔaexT mutant had no toxic effect on RTG-2 cells even after prolonged incubation (8). It has since become clear that the type III secretion genes of A. salmonicida strain JF2267, the strain used in both the
previous study and our current study, are easily lost during laboratory cultivation (51). It is possible therefore, that the long-term effect reported by Braun et al. was due to a loss of the type III secretion system itself and not by the mutation in aexT. The ΔaexT mutant created in this study was analyzed by PCR to ensure the strain still maintained the type III secretion genes that are responsible for the translocation of AexT into the eukaryotic cytosol.

The observation that the ΔaexT mutant has a cytopathic effect after prolonged incubation indicates that other virulence factors expressed by A. salmonicida also contribute to the cytopathogenicity of the bacterium. This is supported by a recent report whereby an aexT mutant displayed only a subtle reduction in virulence, in vivo, when compared to the parent strain (9). As previous studies have shown that the cytopathic effect of A. salmonicida strain JF2267 is dependent upon a functional type III secretion system (3,24) these other factors must be type III effector proteins. This conclusion is also supported by two independent in vivo studies carried out in rainbow trout (5) and Atlantic salmon (9), both of which have shown that inactivation of the type III secretion system attenuates A. salmonicida virulence.

AexT homologues ExoS, ExoT, and YopE are primarily involved in antiphagocytosis permitting Pseudomonas and Yersinia, respectively, to escape host defense (7,40). The functional role of the double activity on the actin cytoskeleton of AexT in the pathogenesis of Aeromonas is not yet known. However, recent results have shown that a functional type III secretion system, responsible for the translocation of AexT into target fish cells, prevents uptake of A. salmonicida by peripheral blood leukocytes (4). We speculate that AexT-dependent actin depolymerization plays a role in this process.

In conclusion—AexT produced by A. salmonicida displays bifunctional enzymatic activity, ADP-ribosylation of cellular actin and GAP activity towards Rho-GTPases. This is the first bacterial toxin known to disrupt actin filaments in target cells via these two specific targets.

REFERENCES


**FOOTNOTES**

*We thank Barbara Mueller for excellent technical assistance with tissue culture. This work was supported by the Swiss National Science Foundation, grant number SNF Nr. 3100A0-10159 and by funding of the Institute Pasteur.*
The abbreviations used are: *A. salmonicida*, *A. salmonicida* subsp. *salmonicida*; GAP, GTPase activating protein; wt, wild type; EPC cells, epithelioma papulosum cyprinid cells; EGFP, enhanced green fluorescent protein.

FIGURE LEGENDS

FIG. 1. AexT ADP-ribosylates muscular and non-muscular actin. *A*, 1 μg non-muscular actin (lane 1), and 20 μg lysates of Vero cells (lane 2) or EPC cells (lane 3) were incubated with recombinant wt AexT (10 μM) and 5×10^5 cpm [32P]NAD in ADP-ribosylation buffer as described under “Experimental Procedures”. 0.1 μM iota toxin incubated with 1 μg non-muscular actin (lane 4), 10 μM AexT incubated alone (lane 5) and 20 μg lysate of EPC cells alone (lane 6). Following incubation for 30 min at 37 °C, proteins were separated by SDS-PAGE and processed by autoradiography. *B*, 1 μg of muscular actin (lane 1), non-muscular actin (lane 2), Rho-GST (lane 3) and Rac-GST (lane 4) were incubated together with recombinant AexT and 5×10^5 cpm [32P]NAD for 30 min at 37 °C, C3 incubated with Rho-GST (lane 5). Samples were subjected to SDS-PAGE and processed with autoradiography. Standards (kDa) are indicated in the left margin.


FIG. 3. Residues E398 and E401 are involved in the ADP-ribosylating activity of AexT. *A*, amino acid alignment of the biglutamic acid site of AexT with other ADP-ribosylating toxins. *A. salmonicida* AexT (accession no. CAE17664), *P. aeruginosa* ExoT (L46800), *P. aeruginosa* ExoS (L27629), iota toxin of *C. perfringens* (CAA51959), C2 toxin of *C. botulinum* (BAA32536), labile toxin (LT) from *E. coli* (P06717), choler toxin (CT) from *V. cholerae* (P01555), SpvB from *S. enterica* (NP_073228), SpyA of *S. pyogenes* (ABF35422), vegetative insecticidal protein 2 (VIP2) of *B. cereus* (IQS1D) and NarE of *Neisseria meningitidis* (NP_274362). Arrows indicate conserved glutamic acid residues. *B*, non muscular actin (4.5 μg) was incubated with recombinant AexT mutants (10 μM) and 5×10^5 cpm [32P]NAD in ADP-ribosylation buffer as described under “Experimental Procedures”. Following incubation for 30 min at 37 °C, proteins were separated by SDS-PAGE and processed with autoradiography. Numbers below the lanes refer to glutamic acid residues that were mutated to alanine residues. Standards (kDa) are indicated in the left margin.

FIG. 4. Arginine R303 is required for the ADP-ribosylating activity of AexT. *A*, amino acid alignment of the region surrounding the conserved arginine residue of cholera toxin (CT; accession no. P01555) with AexT (accession no. CAE17664). Dots indicate similar amino acids, asterisks indicate identical amino acids. The conserved arginine of cholera toxin is underlined. Arrows indicate potential corresponding residues in AexT. *B*, non muscular actin (4.5 μg) was incubated with recombinant AexT mutants (10 μM) and 5×10^5 cpm [32P]NAD in ADP-ribosylation buffer as described under “Experimental Procedures”. Following incubation for 30 min at 37 °C, proteins were separated by SDS-PAGE and processed with autoradiography. Numbers below the lanes refer to arginine residues that were mutated to alanine residues. Standards (kDa) are indicated in the left margin.

FIG. 5. AexT alters the eukaryotic actin cytoskeleton. EPC cells were transiently transfected with 2 μg of pEGFP (*A*), pEGFP-fused to wt *aexT* (*B*) or pEGFP-fused to *aexT* mutants as indicated (*C* and *D*). Following incubation over night at 18 °C, the cells were fixed and stained with TRITC–phalloidin for 1 hour at room temperature. Stained cells were observed and photographed using immunofluorescence microscopy.

FIG. 6. AexT displays GAPase activating activity. *A*, amino acid alignment of the potential RhoGAP sequence in AexT with that of known GAP proteins. *A. salmonicida* AexT (accession no. CAE17664), *P. aeruginosa* ExoT (L46800), *P. aeruginosa* ExoS (L27629), *Y. pseudotuberculosis* YopE (P08008), *Salmonella* SptP (NP 461799), RhoGAP consensus sequence. Arrow indicates the conserved arginine residue. *B*, *C* and *D*, GTPases Rac1 (*B*) Cdc42 (*C*) and RhoA (*D*) were loaded
with \([\gamma^{32}\text{P}]\text{GTP}\) as described under “Experimental Procedures”. RhoGAP and AexT (100 nM) were added to the preloaded GTPases (200 nM) and incubated at room temperature for the indicated time intervals. GTPase activity was then analysed using a filter-binding assay. Error bars indicate standard deviation between replicates. RhoGAP ■; wt AexT ∆; AexTR143K ○; Blank ●.

FIG. 7. GAPase activating and ADP-ribosylating activities contribute to AexT-dependent alteration of the actin cytoskeleton. EPC cells were transiently transfected with 2 \(\mu\)g of pEGFP-fused to aexT mutants as indicated (A and B). Following incubation over night at 18 °C, the cells were fixed and stained with TRITC–phalloidin for 1 hour at room temperature. Stained cells were observed and photographed using immunofluorescence microscopy.

FIG. 8. AexT contributes to the cytopathic effect of \(A. \text{salmonicida}\). A, a schematic representation of the construction of the isogenic \(\Delta\text{aexT}\) mutant. Grey box represents the kanamycin cassette. Essential amino acids residues are indicated. B, C and D, EPC cells were infected with either wt \(A. \text{salmonicida}\) strain JF2267 (B), the isogenic \(\Delta\text{aexT}\) mutant (C) or PBS only (D). Multiplicity of infection used was 20:1 (bacteria to fish cells) and cells were photographed under phase-contrast microscopy 2 hours post infection.
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$a$ lowercase letters indicate nucleotides added to create restriction enzyme recognition sites

$b$ underlined letters indicate mutation generated by site-directed mutagenesis
TABLE 2. Maximum velocity ($V_{\text{max}}$) and Michaelis-Menten constant ($K_m$) values for AexT and iota toxin using non-muscular and muscular actin as substrates.

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<td>AexT</td>
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<td>Iota toxin</td>
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<td>0.90</td>
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a) enzymatic activity detected but too low to be quantitated
Figure 1
Figure 2

A

51

B

AεxT + Actin

Actin only

C

AεxT + Actin

Actin only
Figure 3

A

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B

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wt 403 401 398 398 398 403 403 401 401 401 403 403
Figure 4

A

AexT 297-EYQLNRSRQLQKEQLDAGQLIDQGMSTAFKSTP
CT 20-DDKLYRADSRPPDEIKQSGGLMPRQGSEYFDRG-

AexT 332-TEQLIKTRGLTGGDAFNEVAEGQVHDVAYLSTS
CT 53-TQMNINLYDHRGQV-----TQTGFRHDGYVSTS

B

kDa

66 - 45 -

1 2 3

wt 303 306
Figure 5
Figure 6

A

AexT 139- NGPLRSCT
ExoT 145- DAGLRSLAT
ExoS 142- DAGLRSLST
YopE 140- SGPLRGSTT
SptP 205- NGPRLSMT
RhoGAP consensus GXXRXSG

B

C

D

32P-GTP bound (% of time zero)

Time (min)
Figure 7

A
R143K

B
R143K/E398A/E401A/E403A
Figure 8
Aeromonas exoenzyme of aeromonas salmonicida is a bifunctional protein that targets the host cytoskeleton

Désirée Fehr, Sarah Ellen Burr, Maryse Gibert, Jacques d’Alayer, Joachim Frey and Michel Robert Popoff

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