Published in final edited form as:

Microb Pathog. 2010 September ; 49(3): 122–134. doi:10.1016/j.micpath.2010.05.011.

Unraveling the mechanism of action of a new type III secretion system effector AexU from *Aeromonas hydrophila*

Johanna C. Sierra¹, Giovanni Suarez¹, Jian Sha¹, Wallace B. Baze², Sheri M. Foltz¹, and Ashok K. Chopra^{1, *}

¹Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555

²University of Texas M.D. Anderson Cancer Center, Bastrop, TX 78602

Abstract

We recently characterized a T3SS effector, AexU, from a diarrheal isolate SSU of *Aeromonas hydrophila*, which exhibited ADP-ribosyltransferase (ADPRT) activity. Here we provided evidence that AexU also possessed GTPase-activating protein (GAP) activity, which was mainly responsible for host cell apoptosis and disruption of actin filaments. Earlier, we showed that the $\Delta aexU$ null mutant was attenuated in a mouse model, and we now demonstrated that while the parental *A. hydrophila* strain could be detected in the lung, liver, and spleen of infected mice, the $\Delta aexU$ mutant was rapidly cleared from these organs resulting in increased survivability of animals. Further, AexU prevented phosphorylation of c-Jun, JNK and I κ B α and inhibited IL-6 and IL-8 secretion from HeLa cells. Our data indicated that AexU operated by inhibiting NF- κ B and inactivating Rho GTPases. Importantly, however, when the $\Delta aexU$ null mutant was complemented with the mutated *aexU* gene devoid of ADPRT and GAP activities, a higher mortality rate in mice with concomitant increase in the production of proinflammatory cytokines/chemokines was noted. These data indicated that either such a mutated AexU is a potent inducer of them or that AexU possesses yet another unknown activity that is modulated by ADPRT and GAP activities and results in this aberrant cytokine/chemokine production responsible for increased animal death.

Keywords

Aeromonas hydrophila; T3SS; AexU; site-directed mutagenesis; ADP-ribosyltransferase activity; GAP activity; HeLa Tet-Off cells; apoptosis; septicemic mouse model of infection

1. INTRODUCTION

Aeromonas hydrophila is a Gram-negative bacterium responsible for multiple human diseases, such as gastroenteritis, cellulitis, bacteremia, soft-tissue infection, peritonitis and hemolytic-uremic syndrome [1–3]. Human infections can be acquired by contact with contaminated water

© 2010 Elsevier Ltd. All rights reserved

*Corresponding author A.K. Chopra Department of Microbiology & Immunology 301 University Blvd., UTMB, Galveston, TX 77555
Tel: 409-747-0356; Fax: 409-747-6869 achopra@utmb.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

or soil, and this organism has also been isolated from food samples, which has suggested its importance as a food-borne pathogen [4–7].

The ability of *A. hydrophila* to cause various diseases is associated with the production of virulence factors [7–9], the most potent of which is the cytotoxic enterotoxin referred to as Act. This toxin is secreted via the type 2 secretion system (T2SS) and has various biological activities, including hemolysis, cytotoxicity, and enterotoxicity. Moreover, Act was found to be lethal when injected intravenously in mice [9,10]. Act, when used in sub-lethal doses, was also able to induce the production of pro-inflammatory cytokines, prostaglandins, and reactive oxygen species from murine macrophages and human colonic epithelial cells by activating various kinase pathways [9,11–13].

Previously, we characterized the T3SS-associated effector AexU (with 512 amino acid [aa] residues), that possessed ADP-ribosyltransferase (ADPRT) activity [14]. AexU inhibited phagocytosis of bacteria by macrophages, and it eventually led to host cell death by apoptosis [14,15]. We also demonstrated that AexU played an important role during the infection process, as 60% of mice infected with the $\Delta aexU$ isogenic mutant survived a minimal lethal challenge dose, which killed 90–100% of animals infected with the wild-type (WT) *A. hydrophila* SSU [15].

Studies on the T3SS-secreted effector proteins (e.g., ExoS/T and AexT) from other bacteria, such as *Pseudomonas aeruginosa* and a fish pathogen *Aeromonas salmonicida*, respectively, indicated that they were bifunctional toxins, with the NH₂-terminal domain having GTPase activating protein (GAP) activity, while the COOH-terminal domain had ADPRT activity [16–20]. Importantly, however, was our finding that although the NH₂-terminal domain of AexU (aa residues 1–231) shared 54–67% homology in the corresponding domains of *P. aeruginosa* ExoS (aa residues 1–233) and *A. salmonicida* AexT (aa residues 1–255), respectively, the COOH-terminal domain of AexU (aa residues 232–512) was unique with no homology to any functional protein in the NCBI database. Because of this novel domain, the overall homology of AexU with ExoS/T and AexT dropped to 31 and 40%, respectively. Further, while AexU is 512 aa residues in length, ExoS and AexT are comprised of 453 and 475 aa residues, respectively [14,21,22].

Within the NH₂-terminal domain of AexU, we identified an arginine-finger motif (GALRSLA), similar to that of ExoS (GALRSLS) and AexT (GPLRSLC). Earlier studies based on site-directed mutagenesis indicated an essential role of an arginine residue in the GAP activity of these bacterial toxins [17,23]; however, whether AexU possesses a similar activity is unknown.

In this study, we evaluated the GAP activity of native and mutated versions of AexU from *A. hydrophila* SSU and examined various signaling pathways mediated by AexU in the host cell. Further, we studied the ability of the $\Delta aexU$ null mutant strain to be detected in the peripheral organs of mice after intraperitoneal (i.p.) infection. Overall, our data indicated that AexU operated by inhibiting the activation of NF- κ B by down-regulating the phosphorylation of I κ B α and, thereby, negatively modulating the production of key cytokines/chemokines.

In addition, we provided the first evidence that AexU devoid of ADPRT and GAP activities when produced *in trans* from *A. hydrophila* SSU $\Delta aexU$ strain, was able to trigger significant production of cytokines/chemokines in spleens of infected mice when compared to animals infected with the $\Delta aexU$ strain expressing the native *aexU* gene with intact ADPRT and GAP activities. These data indicated that AexU devoid of its intrinsic activities was even more potent in activating genes for cytokines and chemokines and their overwhelming production resulted in increased mortality in mice in a septicemic mouse model. To our knowledge, this is the first detailed mechanistic study unraveling the mechanism of action of AexU.

2. RESULTS

2.1. AexU functions as a GTPase-activating protein (GAP) for RhoA, Rac1 and Cdc42, and this activity is dependent on the arginine residue located at position 145

Multiple bacterial toxins target GTP-binding proteins, and a particular subset of toxins mimic the action of eukaryotic GAPs by promoting the hydrolysis of bound GTP [24,25]. Sequence analysis and site-directed mutagenesis studies have shown the importance of an essential arginine residue in all of the bacterial GAPs, and this residue is believed to function as the arginine finger already described for GAP activity of eukaryotic proteins [26]. By sequence analysis of AexU, we identified a region in the NH₂-terminal domain that exhibited homology to other bacterial proteins with GAP activity, and this region is referred to as arginine finger sequence motif [27]. In particular, the arginine residue located at position 145 in AexU could serve as the catalytic aa residue mediating the GAP activity (Fig. 1A).

Consequently, we performed an *in vitro* GTP-hydrolysis assay of the small GTPases, such as RhoA, Rac1, Cdc42, and Ras. The GAP activity of native recombinant AexU (rAexU) towards RhoA, Rac1, and Cdc42 was confirmed by this assay (Fig. 1C); however, no activity was detected towards Ras (data not shown). Additionally, we over-produced and purified the mutated version of rAexU (GAP⁻), and its GAP activity evaluated. Importantly, rAexU_{GAP⁻} showed a significant reduction in its capacity to hydrolyze GTP, when we compared it with the native form of rAexU and the positive control p50 Rho GAP that was used in this assay (Fig. 1C), using the same substrates. These data confirmed that arginine 145 indeed was responsible for the GAP activity of rAexU. The stability of the mutated versus the native form of rAexU based on Coomassie blue staining of the gel is shown in Fig. 1B.

2.2. Alterations in actin cytoskeleton of HeLa Tet-Off cells transfected with the *aexU* gene are associated with GAP activity

Several bacterial toxins target GTP-binding proteins to alter host cellular functions, such as chemotaxis, phagocytosis, and secretion and proliferation necessary to eliminate the pathogen from the host [24,26]. One particular strategy is the inhibition of small GTPases by bacterial toxins having a GAP-like activity. Small GTPases, in particular the Rho GTPases, are involved in regulating host cell polarity, cell adhesion, and the contractile actin/myosin stress fiber formation [24,25]. Since rAexU possesses GAP activity, we investigated whether alterations in the cellular cytoskeleton induced by native AexU were related to GAP activity. The actin filament architecture was evaluated by immunofluorescence and flow cytometry using Alexa-Fluor 568-conjugated phalloidin.

The rounded cell morphology was evident in HeLa Tet-Off cells transfected with the gene encoding native AexU (Fig. 2A-II); the actin cytoskeleton was almost completely disrupted (**at arrow**). In contrast, HeLa cells transfected with the gene encoding the mutated version of AexU (GAP⁻) (Fig. 2A-III) exhibited an actin filament network which was much improved compared to in HeLa Tet-Off cells that were transfected with the native *aexU* gene, although not reaching to the level seen in HeLa cells transfected with the vector alone (Figs. 2A-I and 2B&C). HeLa cells expressing the gene encoding GFP were clearly seen in these three panels. These data indicated that GAP activity contributed to the alteration of the actin cytoskeleton of the host cells.

We then quantified the reorganization of actin by flow cytometry using fluorescently labeled phalloidin, and for this analysis, we used only the GFP⁺ population. The phalloidin mean fluorescence intensity (MFI) of the HeLa Tet-Off cells transfected with the gene encoding GAP⁻ AexU was significantly higher (doubled) compared to the MFI of the host cells transfected with the gene encoding native AexU (Figs. 2B&C). The expression and production

of native AexU as well its mutated version after transfection into HeLa Tet-Off cells was evaluated by Western blot analysis that used anti-AexU antibodies to ensure that the effects observed in the actin cytoskeleton were not influenced by their expression and production levels. As shown in Fig. 2D, the production levels of AexU were comparable between the native and the mutated version.

2.3. Induction of apoptosis by AexU in transfected HeLa Tet-Off cells is dependent on GAP activity

We previously reported an increase in the apoptosis of HeLa Tet-Off cells expressing and producing AexU [14]. We measured cytoplasmic histone-associated DNA fragments (nucleosomes) by ELISA in HeLa cells after 24 h of transfection with genes encoding native AexU and the GAP mutant (GAP⁻). The apoptotic rates of HeLa Tet-off cells transfected with genes encoding AexU_{GAP⁻} were significantly lower compared to those in HeLa cells transfected with the gene encoding native AexU (Fig. 3A). Additionally, we evaluated the activation of caspase 3 in HeLa Tet-Off cells in response to the mutated version of AexU. As shown in Fig. 3B, there was a significant decrease in the activation of caspase 3 in the HeLa cells transfected with gene encoding AexU_{GAP⁻} compared to HeLa cells transfected with the native version of AexU. As a positive control, we used HeLa Tet-Off cells transfected with vector alone and treated with camptothecin and it showed similar levels of cytoplasmic nucleosomes and caspase 3 activation as in HeLa Tet-Off cells transfected with the native *aexU* gene.

2.4. AexU interferes with the phosphorylation of c-Jun and IκBα in normal HeLa cells co-cultured with *A. hydrophila* SSU

In addition to controlling the assembly and disassembly of the host actin cytoskeleton, members of the Rho family of small GTPases activate nuclear factor κB (NF-κB) [28,29]. There is evidence that constitutively activated forms of Rac1 and Cdc42 are able to induce the activation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) [30,31]. Given that AexU possesses GAP activity towards RhoA, Rac1, and Cdc42, we evaluated the effects of either WT *A. hydrophila* or its *ΔaexU* null mutant on the signaling cascades leading to the activation of transcription factors c-Jun and NF-κB.

We observed an increase in the phosphorylation of c-Jun, IκBα, and JNK in HeLa cells infected with the *ΔaexU* mutant of *A. hydrophila* at different time points when compared to HeLa cells infected with the WT strain (Fig. 4), with statistically significant differences observed at 60 min for phospho c-Jun, at 30 and 60 min for phospho IκBα, and at 60 and 90 min for phospho JNK. These results were confirmed by Western blot analysis using antibodies specific for the phosphorylated form of c-Jun and densitometric analysis of the Western blot (Figs. 5A&B). For data presented in Fig. 5, we used *A. hydrophila* with an *Δact* background to eliminate the strong biological effects associated with this toxin. As shown in Fig. 5B, the intensity value of the bands (represented as fold increase) for phosphorylated c-Jun was lower in HeLa cells infected with the *Δact* mutant of *A. hydrophila*, when compared with findings in HeLa cells infected with the *Δact/ΔaexU* double mutant. Specifically between time points 30–120 min (approximately 4–5 fold increase), we observed statistically significant increases in phosphorylation of c-Jun when the *aexU* gene was deleted from *A. hydrophila*. These results supported our earlier observation (Fig. 4) of AexU's involvement in down regulation of the JNK pathway.

Additionally, we measured IκBα degradation pattern in HeLa cells infected with the *Δact* and *Δact/ΔaexU* mutant strains of *A. hydrophila* SSU (Figs. 5C&D). After 40–80 min of co-culture, the intensity of the bands (represented as fold change and % decrease) for IκBα was approximately 70–80% lower in HeLa cells infected with the *Δact/ΔaexU* mutant compared

to that in host cells infected with the parental strain ($p < 0.01$). These data indicated higher degradation of I κ B α in $\Delta act/\Delta aexU$ mutant-infected HeLa cells and therefore correlating with a higher activation of NF- κ B (Fig. 5D).

2.5. AexU inhibits secretion of IL-6 and IL-8 by HeLa cells co-cultured with *A. hydrophila* SSU

Non-immune cells, like epithelial cells, produce cytokines/chemokines and are the first line of defense against infections [32,33]. Pathogens have developed strategies to interfere with the NF- κ B signal transduction pathway, thus reducing the secretion of inflammatory cytokines/chemokines, such as IL-6, IL-8 and IL-1 β [32]. Based on the ability of AexU to delay the degradation of I κ B α in HeLa cells (Figs. 5C&D), we decided to measure the secretion of IL-6 and IL-8 by HeLa cells co-cultured with either the Δact or the $\Delta act/\Delta aexU$ mutant strain of *A. hydrophila*. As can be seen from Fig. 6, IL-6 and IL-8 secretion was reduced in the HeLa cells co-cultured with the parental *A. hydrophila* (act -minus background) when compared to the secretion of these cytokines/chemokines by host cells infected with the double mutant $\Delta act/\Delta aexU$. These differences were statistically significant at 24 h after addition of the bacteria.

2.6. Detection of *A. hydrophila* SSU in peripheral organs of mice resulting in tissue injury is mediated by AexU

To evaluate the role of AexU during *in vivo* infection, we challenged mice with a sub-lethal dose of either the Δact or the $\Delta act/\Delta aexU$ double mutant of *A. hydrophila*. Additionally, a group of mice that did not receive any bacteria (but given phosphate buffered saline [PBS]) was used as a negative control. Our earlier data indicated that the LD₅₀ of the Δact mutant bacteria was almost two logarithmic doses higher than the LD₅₀ of WT *A. hydrophila* [34]. After 24 h and 48 h of infection, lungs, livers and spleens were collected, homogenized, and plated to measure the bacterial load. As seen in Fig. 7, at the 48 h time point, there were no bacteria present in the organs of the animals infected with the $\Delta act/\Delta aexU$ mutant of *A. hydrophila*. In contrast, we recovered bacteria from the lungs, livers and spleens of mice infected with the parental strain. The number of parental strain bacteria recovered from different organs at 48 h was higher than the ones detected at 24 h post infection (data not shown).

Tissues from the animals infected with either the parental or the $\Delta act/\Delta aexU$ mutant of *A. hydrophila* were also collected for histopathological analysis. The control group of mice (uninfected) exhibited normal pathology of liver, lung, and spleen (Fig. 8, A–C). Various manifestations of tissue pathology were noted in the livers of mice infected with the parental (Δact) strain including minimal necrosis (Fig. 8A, **middle panel arrow**) or inflammation of the capsule, minimal parenchymal inflammation and granuloma formation. In the lungs of mice infected with the parental strain, the most predominant findings were minimal to mild inflammation in the interstitium with infiltrates of polymorphonuclear cells (Fig. 8B **middle panel arrow**). Bacteria were also observed in the perivascular region of the lungs. In the spleens, mild to moderate lymphoid necrosis of the white pulp and mild myeloid hyperplasia in the red pulp was noted in mice infected with the parental *A. hydrophila* strain (Fig. 8C **middle panel arrow**). The group of mice infected with the $\Delta act/\Delta aexU$ mutant presented significantly much reduced histopathological lesions in the liver, lung, and spleen when compared with the tissues from animals infected with the parental strain.

2.7. *A. hydrophila* $\Delta act/\Delta aexU$ isogenic mutant complemented with the ADPRT[−]/GAP[−] mutated version of the *aexU* gene is more virulent than the one producing the native form of the *aexU* gene

Previously, we reported the ability of AexU to ADP ribosylate eukaryotic proteins [14]. One of the key structural features of ADPRTs is the Q/E-X-E motif, in which the catalytic glutamate (E) is located two residues downstream of a conserved glutamine (Q) or glutamate (E) residue

[35,36]. It was shown that substituting catalytic glutamate to alanine significantly reduced the activity of multiple ADPRTs [17,37]. However, our studies with AexU indicated the presence of three Q-X-E motifs, one in the NH₂-terminal domain (aa residues 28–30) and two in the COOH-terminal domain (aa residues 261–263 and 419–421) (Fig. 1A) [14]. Based on this sequence analysis, we generated by site directed mutagenesis a mutated version of AexU in which all of the three glutamates were replaced by alanine. The recombinant protein with these three substitutions (AexU ADPRT123⁻) was tested by an *in vitro* ADPRT assay, as we previously described [38]. No ADPRT activity was found to be associated with this mutated version of rAexU (data not shown). The stability of the mutated rAexU devoid of ADPRT activity compared to that of the native protein was confirmed by Coomassie blue staining of the gel (Fig. 1B).

In order to assess the contribution of ADPRT and GAP activity of AexU to the virulence of *A. hydrophila* *in vivo*, we expressed and produced the native and mutated versions of AexU *in trans* from *A. hydrophila* $\Delta act/\Delta aexU$ mutant by using the pBR322 vector. Mice were infected *via* the i.p. route with different strains of parental *A. hydrophila* that were complemented with either the native or the mutated form of AexU. As can be seen from Fig. 9A, infection with bacteria producing the native form of AexU resulted in 90% survival of mice at a dose of 3×10^7 cfu. In contrast, only 20% ($p=0.0025$) survival of mice was observed in the group infected with *A. hydrophila* expressing the *aexU* gene that was devoid of ADPRT and GAP activities. Mice infected with the *A. hydrophila* strain producing AexU without ADPRT or GAP activity exhibited 60% ($p=0.13$) and 50% ($p=0.06$) survival, respectively. Although these percentages did not reach statistical significance, increased virulence of both ADPRT⁻ and GAP⁻ mutants compared to that of the parental bacteria was noted in at least three independent experiments with 10 animals per group.

Since our results in Fig. 7 indicated that AexU mediated spreading of *A. hydrophila* to peripheral organs (possibly by inhibiting bacterial phagocytosis and killing) [15], we decided to investigate the ability of *A. hydrophila* $\Delta act/\Delta aexU$ mutant producing the mutated form of AexU (ADPRT123⁻/GAP⁻) to be detected in the spleens of infected mice. After 48 h of infection, $\Delta act/\Delta aexU$ parental strain producing either the native or the mutated form of AexU could be detected in the spleen with latter in more numbers than the former, although the data did not reach statistical significance (Fig. 9B).

Finally, we assessed cytokine/chemokine profiles in the spleens of mice infected with $\Delta act/\Delta aexU$ strain of *A. hydrophila* expressing gene for the native or mutated version of AexU (ADPRT123⁻/GAP⁻) by a multiplex bead array. As shown in Fig. 9C, we detected significant increases in amounts of IL-6, KC (human equivalent of IL-8), macrophage- inflammatory protein (MIP-2), IL-1 α , macrophage-colony stimulating factor (M-CSF), regulated upon activation, normal T cell expressed and secreted (RANTES), macrophage-chemoattractant protein (MCP-1), and MIP-1 α in spleens of animals infected with *A. hydrophila* $\Delta act/\Delta aexU$ mutant that produced AexU from pBR322 but devoid of ADPRT and GAP activities compared to animals that were infected with the $\Delta act/\Delta aexU$ strain producing the native version of AexU from the vector pBR322. The levels of cytokines/chemokines in the latter group of animals were minimal and similar to the levels detected in animals which were uninfected and served as a negative control (data not shown). These data indicated that AexU devoid of intrinsic ADPRT and GAP activities triggered expression of several cytokine/chemokine genes.

3. DISCUSSION

In this study, we showed that the T3SS effector AexU from *A. hydrophila* SSU has GAP activity towards RhoA, Rac1, and Cdc42, and that this activity was dependent on the arginine residue 145 located in a conserved motif shared by multiple GAPs.

Bacteria have developed strategies to alter the activation of host Rho GTPases, and, in the case of toxins with GAP activity, the inhibition of the small GTPases lead to alterations in the assembly and disassembly of the host actin cytoskeleton that is necessary for cell migration, phagocytosis or cell contraction [39,40]. Additionally, it has been reported that Rho GTPases control other cellular functions, such as JNK and p38 MAPK cascades and the transcription factors NF- κ B and SRF (serum-response factor) [41]. Our data indicated that the downregulation in the activity of this GTPase led to alterations in the actin cytoskeleton of HeLa Tet-Off cells transfected with the gene for AexU (Fig. 2). These cells were rounded and detached from the culture plates.

When the HeLa Tet-Off cells were transfected with the gene encoding AexU_{GAP⁻} mutant, the disruption of the actin cytoskeleton was significantly reduced, indicating that the GAP activity of AexU mediated the alterations in cell morphology. However, the MFI of the phalloidin staining did not reach the same level of the cells transfected with the empty vector (Fig. 2), suggesting that AexU devoid of GAP activity possibly could trigger some other cell signaling events (possibly *via* ADPRT or other activity) in the host cells which might be contributing to actin reorganization as well. We will pursue such studies in the future. Indeed studies have shown that although the ADPRT activity associated with ExoS of *P. aeruginosa* contributed to host cell apoptosis (*via* caspase 3 activation) [42], mutant of ExoS devoid of this activity still induced apoptosis in host cells through a different pathway of IL-1 β maturation and secretion [43].

Our previous studies showed that the expression of the *aexU* gene in HeLa Tet-Off cells led to apoptosis mediated by caspase 3 and 9 [14]. Here, we observed that HeLa Tet-Off cells expressing the gene encoding the mutated version of AexU (GAP⁻) had a significant decrease in the cytoplasmic nucleosomes as well as caspase 3 activation compared to HeLa Tet-Off cells expressing the gene encoding native AexU (Fig. 3). Based on these results, we deduced that GAP activity contributed to the induction of apoptosis, although the residual activity might be contributed by the ADPRT activity or other yet unidentified activity of AexU and will be tested in the future. Indeed, it has been reported that inhibition of the Rho function in different cell types induces apoptosis, as evidenced by the reduced expression of anti-apoptotic Bcl-2, increased levels of proapoptotic Bid, and the activation of caspase 3 [44–47]. Since AexU increases the intrinsic rate of GTP hydrolysis of Rho GTPases, specifically inactivating RhoA, Rac1, and Cdc42, our results supported the correlation between Rho protein inactivation and induction of apoptosis.

In addition to their important roles in the assembly and disassembly of the host actin cytoskeleton, Rho GTPases also regulate multiple biochemical pathways like JNK/SAPK and transcription factors, such as SRF, activator protein 1 (AP-1), and NF- κ B [28–31]. We provided evidence that AexU interfered with the phosphorylation of c-Jun, I κ B α and JNK, increasing them in HeLa cells co-cultured with the Δ *aexU* null mutant of *A. hydrophila*, when compared to the phosphorylation levels in host cells co-cultured with the parental bacteria (Figs. 4&5). The ability to interfere with the degradation of I κ B α is an important strategy used by bacterial pathogens to inhibit the activation of NF- κ B [48]. Additionally, alterations in the activation state of RhoA, Rac1, and Cdc42 can also inhibit the activity of NF- κ B [29].

Here we reported the ability of AexU from *A. hydrophila* to inhibit the activation of NF- κ B through inactivation of Rho GTPases, as well as through the inhibition of I κ B α degradation. This is an important tool for bacteria because NF- κ B regulates the transcription of a large number of genes involved in immune and inflammatory responses. In agreement with this, we observed a significant decrease in the secretion of IL-6 and IL-8 from HeLa cells co-cultured with the parental bacteria, when compared to findings with the mutant strain lacking the *aexU* gene (Fig. 6). One of the functional consequences of this repression in the secretion of

proinflammatory cytokines/chemokines could be a delay in the recruitment of phagocytic cells to the infection site and the development of a protective immune response allowing bacteria to multiply and subsequently to invade other tissues. This scenario is supported by our previous studies showing a significant increase in the survival of mice infected with an $\Delta aexU$ isogenic mutant strain of *A. hydrophila*, when compared to that of animals infected with the WT strain [15].

Histopathological analysis and bacterial load of the tissues from mice infected with either the parental or the $\Delta aexU$ mutant of *A. hydrophila* showed significant differences (Figs. 7&8), with rapid clearing of the mutant from mouse organs and exhibiting minimal histological lesions. On the other hand, organs from the parental bacteria-infected animals showed inflammation of the interstitium of the lungs and necrosis of the spleens and livers, as we reported earlier in mice infected with WT *A. hydrophila* [49,50]. Our findings supported an important role of AexU in bacterial survival during *in vivo* infection with *A. hydrophila*.

By using the $\Delta act/\Delta aexU$ mutant strain of *A. hydrophila* complemented with the native or the mutated form of AexU, we were able to show that elimination of ADPRT and GAP activities from AexU resulted in increased virulence of the bacteria (Fig. 9A). These data suggested there could be an additional activity(ies) that is modulated by the two known intrinsic activities of AexU (i.e., ADPRT and GAP) and needs to be further studied. We were intrigued by our data showing that animals infected with the $\Delta act/\Delta aexU$ mutant complemented with the *aexU* gene devoid of ADPRT and GAP activities could be detected in the spleens (Fig. 9B).

These were surprising results, considering our earlier data showing that the $\Delta act/\Delta aexU$ mutant of *A. hydrophila* was rapidly cleared from mouse organs (Fig. 7). Generally speaking, this $\Delta act/\Delta aexU$ mutant should behave similar to the complemented strain in which the *aexU* gene devoid of ADPRT and GAP activities was expressed *in trans*. However, it must be kept in mind that in the $\Delta act/\Delta aexU$ null mutant, AexU synthesis is abrogated, while in the complemented strain, AexU, albeit missing ADPRT and GAP activities, was still present. Since, we previously showed that AexU inhibits the phagocytic activity of murine macrophages [15], it is conceivable that mutated AexU without its intrinsic ADPRT and GAP activities still retains its antiphagocytic ability, resulting in spreading of the complemented strain to the spleen (Fig. 9B).

At the same time, those mice infected with the ADPRT¹²³⁻/GAP⁻ mutant of AexU producing strain of *A. hydrophila* had increased production of IL-1 α , IL-6, KC, MCP-1, M-CSF, MIP-1 α , MIP-2, and RANTES in the spleen when compared to mice infected with the strain expressing and producing the native form of AexU. However, this increased cytokine/chemokine production did not have a beneficial effect during the infection in terms of increasing the survivability of animals but had a detrimental effect resulting in high mortality in mice.

It is known that the pro-inflammatory cytokine IL-1 increases the ability of endothelial cells, macrophages, and fibroblast to secrete chemokines like IL-8 and MCP-1 that enhance the migration of macrophages and granulocytes to the site of infection [51]. Likewise, RANTES is a chemoattractant and mediates the trafficking and homing of T cells, granulocytes, NK cells and monocytes, and MIP-1 α promotes the local influx of neutrophils [52,53]. Although, the induction of pro-inflammatory responses mediated by cytokines/chemokines is one of the strategies used by the host to overcome infections, when this kind of response is excessive and uncontrolled, it could lead to detrimental effects [51].

In particular, the systemic inflammatory syndrome is characterized by excessive production of pro-inflammatory mediators that include IL-6, MIP-2, MCP-1, KC, eotaxin and tumor necrosis factor [54]. During sepsis, an increased synthesis and secretion of these mediators can

cause an elevated systemic inflammation resulting in tissue injury and multiple organ failure leading to septic shock and finally death [55]. As we previously mentioned, AexU is able to down-regulate the secretion of cytokines *in vitro* and also hinders the activation of NF- κ B. Consequently, by eliminating the two known activities associated with AexU (ADPRT and GAP) there is an increased production of pro-inflammatory cytokines/chemokines, resulting in increased mortality of the mice.

Another example of the activation of pro-inflammatory cytokines, like IL-1 β , mediated by the T3SS effector devoid of ADPRT activity is ExoS produced by *P. aeruginosa* [43]. It has been shown that ExoS negatively regulates the maturation of IL-1 β mediated by Caspase 1, and this effect is dependent on the presence of its ADPRT activity. Consequently, mature IL-1 β could be detected in the bronchoalveolar lavage fluid (BALF) samples of mice infected with *P. aeruginosa* Δ exoS mutant and in samples from mice infected with the Δ exoS mutant of *P. aeruginosa* that was complemented with ExoS defective in ADPRT activity [43]. In this context, caspase 1 is activated through the Nod-like receptors that are part of a multiprotein complex called “inflammasomes”. Activation of caspase 1 leads to pyroptosis, which is a cell death program characterized by cell membrane permeabilization and IL-1 β secretion [56]. Importantly, AexU devoid of ADPRT and GAP activities of *A. hydrophila* behaved differently compared to that of ExoS without the ADPRT activity. The former upregulated the production of several cytokines/chemokines with no effect on IL-1 β secretion.

In summary, we observed a reduction in apoptosis and in the alterations of the actin cytoskeleton of HeLa cells in response to inactivation of GAP activity of AexU. We provided first evidence that loss in ADPRT and GAP activities of AexU resulted in increased virulence of the Δ aexU mutant of *A. hydrophila* that expressed this mutated version of the *aexU* gene, by triggering production of several cytokines/chemokines in mice.

4. MATERIALS AND METHODS

4.1. Bacterial cultures and vectors

Escherichia coli HMS174(DE3) cells were obtained from Novagen (Madison, WI). After transformation with the pET-30a vector (Novagen, Gibbstown, NJ) containing the *aexU* gene under the control of bacteriophage T7 promoter, these *E. coli* cells were grown in Luria Bertani (LB) medium supplemented with 100 μ g/ml kanamycin (Sigma St. Louis, MO) at 37°C with continuous shaking. *E. coli* DH5 α cells transformed with pET-30a or eukaryotic pBI-EGFP (enhanced green fluorescent protein) recombinant plasmid containing the *aexU* gene were used for plasmid isolation and grown in LB medium containing 100 μ g/ml ampicillin. The genes encoding native, as well the mutated, versions of the *aexU* gene were cloned at the XhoI and BglII restriction enzyme sites of the pET-30a vector. On the other hand, for the pBI-EGFP vector, we used NheI and MluI restriction enzyme sites for cloning purposes. *E. coli* cells were transformed by electroporation with various plasmids by using a Gene Pulser Xcell (BioRad, Hercules, CA) in 2-mm cuvettes.

A. hydrophila SSU, a diarrheal isolate, was grown in LB medium supplemented with 100 μ g/ml rifamicin (a spontaneous antibiotic-resistant strain) [15]. The single deletion isogenic mutant strains, namely Δ act and Δ aexU, were grown in the presence of 100 μ g/ml kanamycin, while the Δ act/ Δ aexU double isogenic mutant was cultivated in the presence of both kanamycin and 40 μ g/ml each of spectinomycin and streptomycin [15].

4.2. Site-directed mutagenesis of the *aexU* gene

To obtain different mutated versions of the *aexU* gene, we used the Altered Sites *in vitro* Mutagenesis System (Promega, Madison, WI). The encoding region of the gene for native

AexU was amplified by polymerase chain reaction (PCR) and using the primers listed in Table 1. This gene was then cloned into the pALTER-1 vector, which is Tetracycline resistant (Tc^R) and Ampicillin sensitive (Ap^S), and used to transform *E. coli* JM109 cells, which subsequently were infected with the R408 helper phage. The phagemic, single-stranded DNA was isolated by following the manufacturer's instructions. This DNA was used as a template for the mutagenesis reaction with different mutagenic primers (Table 1). For generating the ADPRT123⁻ mutant, consecutive rounds of mutations were performed using the three mutagenic primers. Each mutagenesis reaction generated a different mutated form of the *aexU* gene in pALTER-1, which was then transformed into *E. coli* ES1301 *mutS*-competent cells. Plasmid DNA was isolated from *E. coli* ES1301 cells and then transformed into *E. coli* JM109 cells which were screened for Tc^S and Ap^R phenotype. All of the mutations were confirmed by DNA sequence analysis.

4.3. Complementation of the *A. hydrophila* SSU $\Delta act/\Delta aexU$ isogenic mutant *in trans* with the native *aexU* or *aexU* devoid of ADPRT or GAP and/or both activities

The genes encoding the native and mutated versions of AexU (e.g., ADPRT123⁻, GAP⁻ and ADPRT123⁻/GAP⁻) were PCR amplified from the pALTER plasmids prepared for the site directed mutagenesis using primers listed in Table 1. These DNA fragments were cloned in the pBR322 vector at Sall and EcoRI sites and transformed into *E. coli* DH5 α cells. The pBR322-*aexU*_{native}, pBR322-*aexU*_{ADPRT123⁻}, pBR322-*aexU*_{GAP⁻}, and pBR322-*aexU*_{ADPRT123⁻/GAP⁻} recombinant plasmids (Tc^S and Ap^R) were isolated from the corresponding *E. coli* strains and electroporated in *A. hydrophila* $\Delta act/\Delta aexU$ mutant. The presence of different plasmids in *A. hydrophila* $\Delta act/\Delta aexU$ mutant was confirmed by digestion of the isolated plasmid DNA using appropriate restriction endonucleases. The production of various forms of AexU from the pBR322 vector in *A. hydrophila* $\Delta act/\Delta aexU$ mutant was confirmed by Western blot analysis by using antibodies to AexU, which were previously developed in the laboratory [15].

4.4. Cell lines and transfections

HeLa Tet-Off cells were obtained from Clontech and grown in Dulbecco's modified eagle medium (DMEM) with high glucose (Invitrogen-Gibco, Carlsbad, CA), supplemented with 100 μ g/ml G-418 (Cellgro, Herndon, VA) and 10% fetal bovine serum (FBS) Tet-approved (Clontech).

Normal HeLa cells were obtained from the American Type Culture Collection (Manassas, VA) and grown as earlier described for Tet-Off cells without G-418. HeLa Tet-Off cells were electroporated with the pBI-EGFP plasmid containing the *aexU* gene (either native or mutated) by using 4-mm cuvettes in a Gene Pulser Xcell, as previously described [38].

4.5. Recombinant protein purification

Native and mutated forms of AexU were hyper-produced from *E. coli* HMS174-DE3 cells by using the pET-30a vector with histidine-tags at their NH₂-terminal ends. The term "native" referred to rAexU that did not have any mutations in the sequence. A probond purification system (Invitrogen-Gibco) was used by following the manufacturer's protocol for purification of the recombinant proteins. One ml fractions were collected from the nickel column (100 mM-1 M imidazole), and an aliquot of each was subjected to sodium dodecyl sulfate (SDS)-10% polyacrylamide gel electrophoresis (PAGE) to identify, by Coomassie blue staining of the gels, the fractions containing the rAexU. Fractions containing rAexU were mixed and dialyzed overnight at 4°C against Tris-buffered saline (TBS) for the *in vitro* GAP and ADPRT assays and the protein concentration of the samples was measured by using the Bradford assay (BioRad). The stability of the native and mutated forms of AexU was evaluated by Coomassie blue staining of the gels.

4.5.1. *In vitro* GAP assay—GAP activity of purified native and mutated rAexU was evaluated by using the RhoGAP Assay Biochem Kit (Cytoskeleton, Denver, CO). Small GTPase proteins (Ras, RhoA, Rac1, and Cdc42) were incubated with either 4 µg of rAexU (native and GAP mutant) or 6 mg of p50 Rho GAP as a positive control, reaction buffer, and 200 µM of GTP in a 96-well plate. Additionally, we evaluated the intrinsic activity of the small GTPase proteins and detected very low absorbance in these samples (absorbance in the range of 0.01) and served as a negative control for the assay. The reaction was incubated for 20 min at 37°C, and then 120 µl of the CytoPhos reagent was added to each well. The color reaction was measured at 650 nm in a microplate reader.

4.5.2. ADP-ribosyltransferase (ADPRT) assay—Briefly, HeLa cell lysates were used as a source of target protein and were incubated with rAexU (native and ADPRT123⁻) and NAD conjugated with biotin for 30 min at 37°C. The reaction was stopped and the proteins separated by SDS-PAGE, transferred to nitrocellulose membranes which were then incubated with streptavidin-horseradish-peroxidase (HRP), as we previously described [38].

4.6. Western blot analysis

After 24 h of transfection, HeLa Tet-Off cells were lysed in SDSTris-Glycine buffer and subjected to SDS-PAGE and Western blot analysis using anti-AexU antibodies [14]. The phosphorylation status of c-Jun and IκBα degradation was also confirmed by Western blot analysis. Briefly, HeLa cells were infected (multiplicity of infection [MOI] of 2) with either the Δact or the $\Delta act/\Delta aexU$ mutant strain of *A. hydrophila* SSU for different time points. Cells were washed with PBS and then resuspended in RIPA buffer (Pierce, Rockford, IL). Samples were subjected to SDS-4–20% PAGE and Western blot analysis [14]. Specific antibodies to β -tubulin, c-Jun, phospho c-Jun (Ser63), and IκBα were used in these assays.

4.7. Host cell morphology

We evaluated the morphology of HeLa Tet-Off cells transfected with the pBI-EGFP vector containing genes encoding either the native or the mutated versions of AexU by fluorescence microscopy and flow cytometry using phalloidin staining [14]. Briefly, the cells were fixed in plates after electroporation with Cytofix/Cytoperm (Becton Dickinson, San Diego, CA) for 20 min in the dark. The cells were scraped and stained with Alexa-fluor 568-conjugated phalloidin, and, after washing, they were acquired in a FACScan flow cytometer (Becton Dickinson) and analyzed using BD FACSDiva software. For fluorescence microscopy, the cells were placed in glass slides and the images were acquired in a fluorescence microscope (Olympus BX51/DPManager v.1.2.1.107/DPController v.1.2.1.108, Olympus Optical CO. LTD).

4.8. Host cell apoptosis

Induction of apoptosis in HeLa Tet-Off cells expressing the native or the mutated version of the *aexU* gene was evaluated by detection of cytoplasmic nucleosomes and caspase 3 activation. HeLa Tet-Off cells transfected with the empty vector were treated overnight with 10 µM camptothecin as a positive control for both the assays. For the detection of nucleosomes, we used the cell death detection ELISA kit (Roche, Indianapolis, IN). Lysates (1×10^5 cells) from the HeLa cells were prepared after 24 h of transfection by following the manufacturer's instructions. The color reaction was measured in a microplate reader at 405 nm.

The caspase 3 activation was evaluated with a colorimetric assay from BioVision Inc. Mountain View, CA). After 24 h of transfection, whole lysates (200 µg) of HeLa Tet-Off cells were incubated with 50 µl of 2X reaction buffer containing 10 mM DTT and 5 ml of peptide substrate (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-*p*-nitroalanine [DEVD-pNA]) for 2–24 h at 37°C and read at 405 nm in a microplate reader.

4.9. Phospho-protein detection

Bio-Plex Phosphoprotein Testing Assay (BioRad) was employed for the detection of phosphorylated proteins in HeLa cells infected with various strains of *A. hydrophila*. Briefly, HeLa cells were infected with the WT *A. hydrophila* SSU and its $\Delta aexU$ mutant strain at an MOI of 2 for 30, 60 and 90 min. At these time points, the monolayers were intact with minimal number of floating cells. After centrifugation to recover cells in suspension, the supernatants were aspirated. Then the host cells (including the ones in the culture supernatant) were washed with cell wash buffer (BioRad), resuspended in lysis buffer, and processed as described by the manufacturer. The protein concentration of each sample was measured by Lowry's method [57] and same concentration of proteins (25 μ L of a 500 μ g/mL cell lysate) was used for each sample in the assay.

4.10. Cytokine/chemokine detection

Normal HeLa cells were infected with either the Δact or the $\Delta act/\Delta aexU$ mutant strain of *A. hydrophila* SSU at an MOI of 2 at 37°C. After 1 h of incubation, 100 μ g/ml of gentamicin (Cellgro, Manassas, VA) was added to the culture, and the supernatants recovered at 4, 6 and 24 h after the initial infection. IL-6 and IL-8 secretion was measured with a BioPlex Cytokine detection kit (BioRad). On the other hand, the homogenates of spleens from control and infected mice were centrifuged and cytokines/chemokines were measured by using a multiplex bead array (Millipore, Billerica, MA).

4.11. Animal infections and detection of bacteria in peripheral tissues

Groups of 15 Swiss Webster mice were infected *via* the i.p. route with either the Δact or the $\Delta act/\Delta aexU$ mutant strain of *A. hydrophila* SSU at a dose of 8×10^5 (representing approximately 1 LD₅₀) colony forming units (cfu) [34]. After 24 h and 48 h of infection, five mice from each group were euthanized and a portion of the lungs, livers and the spleens were weighed and homogenized in 1 mL of sterile water for evaluating bacterial load. Serial dilutions of the homogenized tissues were plated on LB agar plates, and after overnight incubation at 37°C, bacteria were enumerated and the results presented as cfu per gram of tissue.

For the animal survival experiments, groups of 10 mice were infected *via* the i.p. route with 3×10^7 cfu of *A. hydrophila* $\Delta act/\Delta aexU$ mutant complemented *in trans* with either pBR322-*aexU*_{native}, pBR322-*aexU*_{ADPRT123⁻}, pBR322-*aexU*_{GAP⁻} or pBR322-*aexU*_{ADPRT123⁻/GAP⁻} plasmids. Deaths were recorded for 15 days post-infection. In another experiment, animals (n=15) were infected with 3×10^7 cfu of *A. hydrophila* $\Delta act/\Delta aexU$ mutant complemented with either the native or the ADPRT123⁻/GAP⁻ mutant of AexU. After 48 h of infection, bacterial load was evaluated in the spleens. An aliquot of the homogenate was also used for the detection of cytokines/chemokines by a multiplex bead array (Millipore).

4.12. Histopathology

In parallel with the detection of the Δact or the $\Delta act/\Delta aexU$ mutant strain of *A. hydrophila* SSU in peripheral tissues, portions of the tissues were fixed in 10% buffered formalin, mounted on glass slides and stained with hematoxylin and eosin for light microscopic analysis. The lesions were scored based on a scale correlating with the extent of lesion severity: minimal (2–10%), mild (>10–20%), moderate (>20–50%), and severe (>50%).

4.13. Statistics

The Tukey's multiple comparison test and Bonferroni post-test were used for statistical analyses of the data by using GraphPad Prism (Software MacKiev, San Diego, CA). For animal studies, we used Kaplan-Meier survival estimates for data analysis. Three independent experiments

were performed unless otherwise stated, and arithmetic means with standard deviations were plotted.

Acknowledgments

This work was supported by the grants from NIH/NIAID (AI041611) and the Environmental Protection Agency. Johanna C. Sierra and Giovanni Suarez were supported by the J.W. McLaughlin Endowment Pre-doctoral Fellowships, University of Texas Medical Branch at Galveston. We thank Ms. Mardelle Susman for editorial assistance.

References

- [1]. Kuhn I, Albert MJ, Ansaruzzaman M, Bhuiyan NA, Alabi SA, Islam MS, et al. Characterization of *Aeromonas* spp. isolated from humans with diarrhea, from healthy controls, and from surface water in Bangladesh. *J Clin Microbiol* 1997;35:369–73. [PubMed: 9003598]
- [2]. Janda JM, Guthertz LS, Kokka RP, Shimada T. *Aeromonas* species in septicemia: laboratory characteristics and clinical observations. *Clin Infect Dis* 1994;19:77–83. [PubMed: 7948561]
- [3]. Brouqui P, Raoult D. Endocarditis due to rare and fastidious bacteria. *Clin Microbiol Rev* 2001;14:177–207. [PubMed: 11148009]
- [4]. Palu AP, Gomes LM, Miguel MA, Balassiano IT, Queiroz ML, Freitas-Almeida AC, et al. Antimicrobial resistance in food and clinical *Aeromonas* isolates. *Food Microbiol* 2006;23:504–9. [PubMed: 16943044]
- [5]. Altwegg M, Martinetti Lucchini G, Luthy-Hottenstein J, Rohrbach M. *Aeromonas*-associated gastroenteritis after consumption of contaminated shrimp. *Eur J Clin Microbiol Infect Dis* 1991;10:44–5. [PubMed: 2009882]
- [6]. Kirov SM. The public health significance of *Aeromonas* spp. in foods. *Int J Food Microbiol* 1993;20:179–98. [PubMed: 8110597]
- [7]. Edberg SC, Browne FA, Allen MJ. Issues for microbial regulation: *Aeromonas* as a model. *Crit Rev Microbiol* 2007;33:89–100. [PubMed: 17453931]
- [8]. Chopra AK, Houston CW. Enterotoxins in *Aeromonas*-associated gastroenteritis. *Microbes Infect* 1999;1:1129–37. [PubMed: 10572317]
- [9]. Chopra AK, Xu X, Ribardo D, Gonzalez M, Kuhl K, Peterson JW, et al. The cytotoxic enterotoxin of *Aeromonas hydrophila* induces proinflammatory cytokine production and activates arachidonic acid metabolism in macrophages. *Infect Immun* 2000;68:2808–18. [PubMed: 10768977]
- [10]. Ferguson MR, Xu XJ, Houston CW, Peterson JW, Copenhagen DH, Popov VL, et al. Hyperproduction, purification, and mechanism of action of the cytotoxic enterotoxin produced by *Aeromonas hydrophila*. *Infect Immun* 1997;65:4299–308. [PubMed: 9317040]
- [11]. Galindo CL, Sha J, Fadl AA, Pillai L, Chopra AK. Host immune responses to *Aeromonas* virulence factors. *Current Immunology Reviews* 2006;2:13–26.
- [12]. Ribardo DA, Kuhl KR, Boldogh I, Peterson JW, Houston CW, Chopra AK. Early cell signaling by the cytotoxic enterotoxin of *Aeromonas hydrophila* in macrophages. *Microb Pathog* 2002;32:149–63. [PubMed: 12079405]
- [13]. Galindo CL, Fadl AA, Sha J, Gutierrez C Jr. Popov VL, Boldogh I, et al. *Aeromonas hydrophila* cytotoxic enterotoxin activates mitogen-activated protein kinases and induces apoptosis in murine macrophages and human intestinal epithelial cells. *J Biol Chem* 2004;279:37597–612. [PubMed: 15215244]
- [14]. Sierra JC, Suarez G, Sha J, Foltz SM, Popov VL, Galindo CL, et al. Biological characterization of a new type III secretion system effector from a clinical isolate of *Aeromonas hydrophila*-part II. *Microb Pathog* 2007;43:147–60. [PubMed: 17582731]
- [15]. Sha J, Wang SF, Suarez G, Sierra JC, Fadl AA, Erova TE, et al. Further characterization of a type III secretion system (T3SS) and of a new effector protein from a clinical isolate of *Aeromonas hydrophila*-part I. *Microb Pathog* 2007;43:127–46. [PubMed: 17644303]
- [16]. Braun M, Stuber K, Schlatter Y, Wahli T, Kuhnert P, Frey J. Characterization of an ADP-ribosyltransferase toxin (AexT) from *Aeromonas salmonicida* subsp. *salmonicida*. *J Bacteriol* 2002;184:1851–8. [PubMed: 11889090]

- [17]. Fehr D, Burr SE, Gibert M, d'Alayer J, Frey J, Popoff MR. *Aeromonas* exoenzyme T of *Aeromonas salmonicida* is a bifunctional protein that targets the host cytoskeleton. *J Biol Chem* 2007;282:28843–52. [PubMed: 17656370]
- [18]. Henriksson ML, Sundin C, Jansson AL, Forsberg A, Palmer RH, Hallberg B. Exoenzyme S shows selective ADP-ribosylation and GTPase-activating protein (GAP) activities towards small GTPases in vivo. *Biochem J* 2002;367:617–28. [PubMed: 12132999]
- [19]. Barbieri JT, Sun J. *Pseudomonas aeruginosa* ExoS and ExoT. *Rev Physiol Biochem Pharmacol* 2004;152:79–92. [PubMed: 15375697]
- [20]. Krall R, Schmidt G, Aktories K, Barbieri JT. *Pseudomonas aeruginosa* ExoT is a Rho GTPase-activating protein. *Infect Immun* 2000;68:6066–8. [PubMed: 10992524]
- [21]. Burr SE, Stuber K, Frey J. The ADP-ribosylating toxin, AexT, from *Aeromonas salmonicida* subsp. *salmonicida* is translocated via a type III secretion pathway. *J Bacteriol* 2003;185:6583–91. [PubMed: 14594831]
- [22]. Zhang Y, Barbieri JT. A leucine-rich motif targets *Pseudomonas aeruginosa* ExoS within mammalian cells. *Infect Immun* 2005;73:7938–45. [PubMed: 16299285]
- [23]. Goehring UM, Schmidt G, Pederson KJ, Aktories K, Barbieri JT. The N-terminal domain of *Pseudomonas aeruginosa* exoenzyme S is a GTPase-activating protein for Rho GTPases. *J Biol Chem* 1999;274:36369–72. [PubMed: 10593930]
- [24]. Aktories K, Barbieri JT. Bacterial cytotoxins: targeting eukaryotic switches. *Nat Rev Microbiol* 2005;3:397–410. [PubMed: 15821726]
- [25]. Fiorentini C, Falzano L, Travaglione S, Fabbri A. Hijacking Rho GTPases by protein toxins and apoptosis: molecular strategies of pathogenic bacteria. *Cell Death Differ* 2003;10:147–52. [PubMed: 12700642]
- [26]. Aktories K, Schmidt G, Just I. Rho GTPases as targets of bacterial protein toxins. *Biol Chem* 2000;381:421–6. [PubMed: 10937872]
- [27]. Scheffzek K, Ahmadian MR, Wittinghofer A. GTPase-activating proteins: helping hands to complement an active site. *Trends Biochem Sci* 1998;23:257–62. [PubMed: 9697416]
- [28]. Montaner S, Perona R, Saniger L, Lacal JC. Multiple signalling pathways lead to the activation of the nuclear factor kappaB by the Rho family of GTPases. *J Biol Chem* 1998;273:12779–85. [PubMed: 9582304]
- [29]. Perona R, Montaner S, Saniger L, Sanchez-Perez I, Bravo R, Lacal JC. Activation of the nuclear factor-kappaB by Rho, CDC42, and Rac-1 proteins. *Genes Dev* 1997;11:463–75. [PubMed: 9042860]
- [30]. Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, et al. The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. *Cell* 1995;81:1137–46. [PubMed: 7600581]
- [31]. Minden A, Lin A, Claret FX, Abo A, Karin M. Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. *Cell* 1995;81:1147–57. [PubMed: 7600582]
- [32]. Naumann M. Nuclear factor-kappa B activation and innate immune response in microbial pathogen infection. *Biochem Pharmacol* 2000;60:1109–14. [PubMed: 11007948]
- [33]. Hedges SR, Agace WW, Svanborg C. Epithelial cytokine responses and mucosal cytokine networks. *Trends Microbiol* 1995;3:266–70. [PubMed: 7551639]
- [34]. Xu XJ, Ferguson MR, Popov VL, Houston CW, Peterson JW, Chopra AK. Role of a cytotoxic enterotoxin in *Aeromonas*-mediated infections: development of transposon and isogenic mutants. *Infect Immun* 1998;66:3501–9. [PubMed: 9673226]
- [35]. Holbourn KP, Shone CC, Acharya KR. A family of killer toxins. Exploring the mechanism of ADP-ribosylating toxins. *Febs J* 2006;273:4579–93. [PubMed: 16956368]
- [36]. Han S, Tainer JA. The ARTT motif and a unified structural understanding of substrate recognition in ADP-ribosylating bacterial toxins and eukaryotic ADP-ribosyltransferases. *Int J Med Microbiol* 2002;291:523–9. [PubMed: 11890553]
- [37]. Liu S, Kulich SM, Barbieri JT. Identification of glutamic acid 381 as a candidate active site residue of *Pseudomonas aeruginosa* exoenzyme S. *Biochemistry* 1996;35:2754–8. [PubMed: 8611582]

- [38]. Suarez G, Sierra JC, Erova TE, Sha J, Horneman AJ, Chopra AK. A type VI secretion system effector protein, VgrG1, from *Aeromonas hydrophila* that induces host cell toxicity by ADP ribosylation of actin. *J Bacteriol* 192:155–68. [PubMed: 19880608]
- [39]. Jaffe AB, Hall A. Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol* 2005;21:247–69. [PubMed: 16212495]
- [40]. Hall A. Rho GTPases and the actin cytoskeleton. *Science* 1998;279:509–14. [PubMed: 9438836]
- [41]. Hall A. Rho GTPases and the control of cell behaviour. *Biochem Soc Trans* 2005;33:891–5. [PubMed: 16246005]
- [42]. Kaufman MR, Jia J, Zeng L, Ha U, Chow M, Jin S. *Pseudomonas aeruginosa* mediated apoptosis requires the ADP-ribosylating activity of exoS. *Microbiology* 2000;146(Pt 10):2531–41. [PubMed: 11021928]
- [43]. Galle M, Schotte P, Haegman M, Wullaert A, Yang HJ, Jin S, et al. The *Pseudomonas aeruginosa* Type III secretion system plays a dual role in the regulation of caspase-1 mediated IL-1 β maturation. *J Cell Mol Med* 2008;12:1767–76. [PubMed: 18081695]
- [44]. Hippenstiel S, Schmeck B, N'Guessan PD, Seybold J, Krull M, Preissner K, et al. Rho protein inactivation induced apoptosis of cultured human endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L830–8. [PubMed: 12225960]
- [45]. Blanco-Colio LM, Villa A, Ortego M, Hernandez-Presa MA, Pascual A, Plaza JJ, et al. 3-Hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitors, atorvastatin and simvastatin, induce apoptosis of vascular smooth muscle cells by downregulation of Bcl-2 expression and Rho A prenylation. *Atherosclerosis* 2002;161:17–26. [PubMed: 11882313]
- [46]. Bobak DA. Clostridial toxins: molecular probes of Rho-dependent signaling and apoptosis. *Mol Cell Biochem* 1999;193:37–42. [PubMed: 10331636]
- [47]. Moorman JP, Bobak DA, Hahn CS. Inactivation of the small GTP binding protein Rho induces multinucleate cell formation and apoptosis in murine T lymphoma EL4. *J Immunol* 1996;156:4146–53. [PubMed: 8666781]
- [48]. Tato CM, Hunter CA. Host-pathogen interactions: subversion and utilization of the NF-kappa B pathway during infection. *Infect Immun* 2002;70:3311–7. [PubMed: 12065467]
- [49]. Sha J, Kozlova EV, Fadl AA, Olano JP, Houston CW, Peterson JW, et al. Molecular characterization of a glucose-inhibited division gene, *gidA*, that regulates cytotoxic enterotoxin of *Aeromonas hydrophila*. *Infect Immun* 2004;72:1084–95. [PubMed: 14742556]
- [50]. Sha J, Erova TE, Alyea RA, Wang S, Olano JP, Pancholi V, et al. Surface-expressed enolase contributes to the pathogenesis of clinical isolate SSU of *Aeromonas hydrophila*. *J Bacteriol* 2009;191:3095–107. [PubMed: 19270100]
- [51]. Esch T, Stefano G. Proinflammation: a common denominator or initiator of different pathophysiological disease processes. *Med Sci Monit* 2002;8:HY1–9. [PubMed: 12011758]
- [52]. Lee SC, Brummet ME, Shahabuddin S, Woodworth TG, Georas SN, Leiferman KM, et al. Cutaneous injection of human subjects with macrophage inflammatory protein-1 α induces significant recruitment of neutrophils and monocytes. *J Immunol* 2000;164:3392–401. [PubMed: 10706735]
- [53]. Cavaillon JM, Adib-Conquy M, Fitting C, Adrie C, Payen D. Cytokine cascade in sepsis. *Scand J Infect Dis* 2003;35:535–44. [PubMed: 14620132]
- [54]. Osuchowski MF, Welch K, Siddiqui J, Remick DG. Circulating cytokine/inhibitor profiles reshape the understanding of the SIRS/CARS continuum in sepsis and predict mortality. *J Immunol* 2006;177:1967–74. [PubMed: 16849510]
- [55]. Cohen J. The immunopathogenesis of sepsis. *Nature* 2002;420:885–91. [PubMed: 12490963]
- [56]. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. *Nat Rev Microbiol* 2009;7:99–109. [PubMed: 19148178]
- [57]. Coligan. Current protocols in protein science. Vol. vol. 2. John Wiley & Sons, Inc.; New York: 2002.

Figure 1.

(A) Schematic representation of AexU showing the catalytic arginine residue associated with GAP activity and the potential motifs (QXE) associated with ADPRT activity. (B) Coomassie blue staining of native and different mutated versions of rAexU, with GAP⁻ indicating the mutated version for GAP activity in which arginine residue at position 145 was replaced with alanine and ADPRT123⁻ representing the mutated version for ADPRT activity in which three glutamate residues at positions 30, 263 and 421 were replaced with alanine. (C) *In vitro* GAP activity towards RhoA, Rac1, and Cdc42 by affinity purified rAexU (native and mutated GAP⁻ version). Recombinant RhoA, Rac1, and Cdc42 (25 µg) were incubated with purified rAexU (4 µg) and GTP for 20 min at 37°C. As a positive control, recombinant p50 Rho GAP (6 µg) was added to the reaction instead of rAexU. Additionally, in order to account for the intrinsic GAP activity, each small GTPase (RhoA, Rac1 and Cdc42) was tested in the absence of the GAP protein (last column in each graph). The phosphate groups generated by GAP activity of AexU were quantified by adding the CytoPhos reagent and colorimetric analysis. The values shown are the mean absorbances ± standard deviations from three independent experiments. ANOVA with Tukey's multiple comparison test was used for statistical analyses of the data.

Figure 2.

Assessment of the actin cytoskeleton by phalloidin staining in HeLa Tet-Off cells expressing the genes encoding native AexU or its GAP⁻ mutated version. After 72 h, HeLa Tet-Off cells transfected with pBI-EGFP empty vector (control) and vector encoding gene for the native AexU or its mutated version for GAP activity (AexU_{GAP⁻}) were stained with Alexa-Fluor 568 phalloidin. **(A)** After staining, the cells were analyzed by fluorescence microscopy. Transfected cells are shown in green (EGFP positive) and red after phalloidin staining. **I:** Cells transfected with the empty vector. **II:** Cells expressing the gene encoding native AexU. Arrow indicates rounded cells with no staining for phalloidin. **III:** Cells expressing the gene encoding the mutated version of AexU which was devoid of GAP activity. **(B)** Phalloidin staining of HeLa cells transfected with native and mutated version (GAP⁻) of AexU was quantified by flow cytometry. Mean Fluorescence Intensity (MFI) of HeLa cells producing different forms of AexU (native and GAP⁻) and the empty vector (control) is shown. The figure shows an average of three independent experiments with standard deviations. ANOVA with Tukey's multiple comparison test was used for statistical analyses of the data. **(C)** Actual flow cytometry data showing MFI of HeLa Tet-Off cells expressing and producing native (Grey) and GAP⁻ mutated AexU (Dotted line), and the empty vector (Continuous line). **(D)** Western Blot analysis showing production of native and mutated version of AexU in the lysates of HeLa Tet-Off cells after 24 h of transfection. Production of β -tubulin was used as a loading control.

Figure 3.

Assessment of the apoptotic rate in HeLa Tet-Off cells expressing the genes encoding native and mutated version (GAP⁻) of AexU. HeLa Tet-Off cells transfected with the empty vector and then treated with Camptothecin were used as a positive control. **(A)** Detection of cytoplasmic nucleosomes by ELISA in HeLa Tet-Off cells after 24 h of transfection. **(B)** Caspase 3 activation measured by colorimetric assay in lysates of transfected HeLa Tet-Off cells. The values shown are mean absorbances $\pm$ standard deviations from three independent experiments. ANOVA with Tukey's multiple comparison test was used for statistical analyses of the data.

Figure 4.

Phosphorylation of c-jun, IκBα, and JNK in HeLa cells co-cultured with WT *A. hydrophila* SSU (Grey bars) or its $\Delta aexU$ mutant (Black bars) was determined by using a BioPlex phosphoprotein assay. HeLa cells were co-cultured with *A. hydrophila*, and cell lysates were obtained at different indicated time points. The values shown are the mean fluorescence intensities $\pm$ standard deviations from two independent experiments. ANOVA with Bonferroni post-test was used for statistical analyses of the data.

Figure 5.

(A) Phosphorylated and total c-Jun was determined by Western blot analysis in lysates (10 μ g) of HeLa cells co-cultured with either the Δact or the $\Delta act/\Delta aexU$ mutant strain of *A. hydrophila* SSU at different time points. Also a Western Blot for β -tubulin is shown as a loading control. (B) The numbers shown on Y-axis represent the fold increase in density values of the bands for the phosphorylated c-Jun, normalized with the density values for the total c-Jun. The grey bars represent HeLa cells co-cultured with the Δact mutant of *A. hydrophila* SSU and the black bars represent cells co-cultured with the $\Delta act/\Delta aexU$ mutant. The figure represents mean fold increases in phospho-c-jun $\pm$ standard deviations from three independent experiments. ANOVA with Bonferroni post-test was used for statistical analyses of the data. (C) IκBα degradation was assessed by Western blot analysis using specific antibodies against IκBα. HeLa cells were co-cultured with indicated strains of *A. hydrophila* SSU and lysates (10 μ g) obtained at different time points were subjected to SDS-4–15% PAGE and Western blot analysis. Western blot for β -tubulin was used as a loading control. (D) The density values of the bands for IκBα were normalized with the density for β -tubulin and then 0 min time point was used as a base line to calculate the fold changes. The Y-axis values shown on the left side are the mean fold changes $\pm$ standard deviations from three independent experiments and the Y-axis values on the right side represent percent decrease in IκBα degradation compared to the 0 min time point. ANOVA with Bonferroni post-test was used for statistical analyses of the data.

Figure 6.

Secretion of IL-6 and IL-8 was evaluated in supernatants of HeLa cells co-cultured with either the Δact mutant of *A. hydrophila* SSU or its $\Delta act/\Delta aexU$ mutant by using the BioPlex Cytokine detection kit. The figure shows mean concentrations in pg/mL of each of the cytokines/chemokines from three independent experiments $\pm$ standard deviations. ANOVA with Bonferroni post-test was used for statistical analyses of the data.

A. hydrophila parental and mutant strain

Figure 7.

Detection of either the Δact mutant of *A. hydrophila* SSU (parental strain) or its $\Delta act/\Delta aexU$ mutant in mice after 48 h of i.p. injection with 8×10^5 cfu. The tissues from five animals per group were weighed and homogenized in water, and the serial dilutions were prepared and plated on LB agar plates. After overnight incubation at 37°C, the number of bacteria was quantified and the cfu per gram of tissue calculated. The data shown represent means with error bars from 5 animals.

Figure 8.

Histopathological analysis of tissues from mice infected with 8×10^5 cfu of either the Δact mutant of *A. hydrophila* SSU (parental strain) or its $\Delta act/\Delta aexU$ mutant strain. **Panel A** shows tissue section from the liver, and the arrow indicates subcapsular necrosis (middle panel) present in animals infected with the Δact mutant strain. **Panel B** shows tissue section from the lung, and the expanded interstitium due to acute inflammatory infiltrates of mostly polymorphonuclear cells is indicated with the arrow (middle panel) in animal infected with the Δact mutant bacteria. **Panel C** shows tissue sections of the spleen, and lymphoid necrosis is indicated by the arrow in mice infected with the Δact mutant strain. No significant alterations were observed in the control group or the $\Delta act/\Delta aexU$ -infected animals. Magnification is represented by the bars (200 and 500 μm).

Figure 9.

(A) Survival curves for mice infected with *A. hydrophila* $\Delta act/\Delta aexU$ mutant strains complemented with native, ADPRT123⁻, GAP⁻ or ADPRT123⁻/GAP⁻ versions of AexU. Deaths were recorded for 15 days. For statistical analysis, survival curves for each of the mutated version of AexU complemented strain was compared with *A. hydrophila* $\Delta act/\Delta aexU$ mutant complemented with the native version of AexU using the Kaplan-Meier survival estimates. These experiments were repeated three times with n=10 in each group and a representative experiment is shown here. (B) Bacterial counts from the spleens of mice infected with *A. hydrophila* $\Delta act/\Delta aexU$ mutant strains complemented with the native or ADPRT123⁻/GAP⁻ mutant form of AexU. The tissues were homogenized, serial dilutions plated, the number of bacteria was quantified and the cfu per gram of tissue calculated. The bacterial counts in individual mouse spleen are shown and a horizontal line represents mean cfu. (C) Cytokine/chemokine profile from the spleens of mice infected with *A. hydrophila* $\Delta act/\Delta aexU$ mutant complemented with the native or ADPRT123⁻/GAP⁻ mutant form of AexU. Spleen homogenates were analyzed by a multiplex bead array for mouse cytokines/chemokines. The concentration of each cytokine/chemokine was normalized based of the weight of the tissue for each animal. All of the values are expressed as pg/mL except for IL-6

and KC that are expressed as $\mu\text{g/mL}$. Minimal cytokine production was observed in tissues from uninfected mice (data not shown). The data for each mouse was plotted and the horizontal line represents mean values. Two-way ANOVA was used for statistical analyses of the data.

Table 1

Primers used for cloning and site-directed mutagenesis.

Primer name	Sequence	Purpose
<i>aexU</i> -EcoRI	5'-CCGGAATTCATGCAGATTCAAACACATACCAGC-3'	PCR amplification of the full length <i>aexU</i> gene for cloning in pALTER
<i>aexU</i> -BamHI	5'-CGCGGATCCTTACAGATAGTCAGCCCCGACACCG-3'	
<i>aexU</i> -E30A	5'-GGCCAGCTG <u>gcc</u> GCTCGTCAGG-3'	Mutagenic oligonucleotide to replace glutamate at position 30 by alanine
<i>aexU</i> -E263A	5'-GCGCCAGACC <u>gcc</u> GTCACCGGTT-3'	Mutagenic oligonucleotide to replace glutamate at position 263 by alanine
<i>aexU</i> -E421A	5'-GCAACAGCTG <u>gcc</u> GGGGATTTTGG-3'	Mutagenic oligonucleotide to replace glutamate at position 421 by alanine
<i>aexU</i> -R145A	5'-TGGCGCCCTG <u>gcc</u> TCGCTGGCA-3'	Mutagenic oligonucleotide to replace arginine at position 145 by alanine
<i>aexU</i> -NheI	5'-GGTCGCTAGCATGCAGATTCAAACACATACCAGC-3'	PCR amplification of the full length <i>aexU</i> gene for cloning in pBR322
<i>aexU</i> -SalI	5'-GGTCGTCGACTTACAGATAGTCAGCCCCGACACCG-3'	
<i>aexU</i> -MluI	5'-TCACA <u>CGCGT</u> ATGCAGATTCAAACACATACCAGC-3'	PCR amplification of the full length <i>aexU</i> gene for cloning in pBI-EGFP
<i>aexU</i> -NheI	5'-GGTCGCTAGCTTACAGATAGTCAGCCCCGACACCG-3'	

Underlining indicates restriction enzyme sites in the primers. Lower case and underlining indicate the codon used to replace glutamine by alanine or arginine by alanine.