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Actin cross-linking domain of *Aeromonas hydrophila* repeat in toxin A (RtxA) induces host cell rounding and apoptosis

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Abstract

The repeat in toxin (Rtx) of an environmental isolate ATCC 7966 of *A. hydrophila* consists of six genes (*rtxACHBDE*) organized in an operon similar to the gene organization found for the Rtx of the *Vibrio* species. The first gene in this operon (*rtxA*) encodes an exotoxin in vibrios, while other genes code for proteins needed for proper activation of RtxA and in secretion of this toxin from *V. cholerae*. However, the RtxA of ATCC 7966, as well as from the clinical isolate SSU of *A. hydrophila*, was exclusively expressed and produced during co-infection of this pathogen with the host, e.g., HeLa cells, indicating that *rtxA* gene expression required host cell contact. Within the RtxA, an actin cross-linking domain (ACD) exists and to investigate the functionality of this domain, several truncated versions of ACD were generated to discern its minimal biological active region. Such genetically modified genes encoding ACD, which were truncated on either the NH₂- or the COOH- terminal, as well as on both ends, were expressed from a bidirectional promoter of the pBI-enhanced green fluorescent protein (EGFP) vector in a HeLa-Tet-Off cell system. We demonstrated that only the full-length ACD of RtxA from *A. hydrophila* catalyzed the covalent cross-linking of the host cellular actin, whereas the ACD truncated on the NH₂-, COOH- or both ends did not exhibit such actin cross-linking characteristics. Further, we showed that the full-length ACD of *A. hydrophila* RtxA disrupted the actin cytoskeleton of HeLa cells, resulting in their rounding phenotype. Finally, our data provided evidence that the full-length ACD of RtxA induced host cell apoptosis. Our study is the first to report that *A. hydrophila* possesses a functional RtxA having an ACD that contributes to the host cell apoptosis, and hence could represent a potential virulence factor of this emerging human pathogen.

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Keywords

A. hydrophila; Rtx; actin cross-linking domain (ACD); HeLa Tet-Off system; cell rounding; apoptosis

1. INTRODUCTION

Aeromonas hydrophila is an emerging human pathogen, and its ability to produce a wide variety of virulence factors facilitates the organism in causing both intestinal and extra-intestinal infections (Chopra et al., 1993; Merino et al., 1995; Chopra et al., 1996; Chopra and Houston, 1999; Merino et al., 1999). Importantly, *Aeromonas* species represented one of the most common pathogens associated with skin and soft tissue infections among the 2004 Tsunami survivors in Southern Thailand (Hiransuthikul et al., 2005). Also, an increased isolation rate of *Aeromonas* species was reported in the floodwater samples following Hurricane Katrina in New Orleans (Presley et al., 2006), suggesting that this microbe could pose potential public health threats during natural disasters. The role of *A. hydrophila* in causing necrotizing fasciitis was recently documented, which confirms some of the other earlier reports on the flesh-eating nature of this pathogen (Abuhammour et al., 2006; Borger van der Burg et al., 2006; Monaghan et al., 2008).

In our laboratory, we identified and characterized three enterotoxins from a diarrheal isolate SSU of *A. hydrophila* (Sha et al., 2002). Among these, the cytotoxic enterotoxin (Act) (Ferguson et al., 1997) was found to be the most potent toxin; it was secreted *via* the type 2 secretion system (T2SS) and functioned as a hemolysin, a cytotoxin, or an enterotoxin, depending on the host target (Chopra and Houston, 1999). Recently, we identified a new T3SS effector in *A. hydrophila* SSU, designated as AexU, which possessed adenosine diphosphate (ADP)-ribosyltransferase and GTPase-activating protein (GAP) activities that led to host cell apoptosis (Sierra et al., 2007). In addition, we showed that the T6SS effector protein, valine-glycine-repeat G1 (VgrG1) of *A. hydrophila*, also possessed actin ADP-ribosylating activity associated with its carboxyl-terminal vegetative insecticidal protein-2 (VIP-2) domain that induced cell rounding followed by host cell apoptosis (Suarez et al., 2010a). On the other hand, the other T6SS effector protein, hemolysin-coregulated protein (Hcp), inhibited phagocytosis of *A. hydrophila* SSU by macrophages that allowed bacterial multiplication and spread to different organs in a septicemic mouse model of infection, leading to animal mortality (Suarez et al., 2010b).

Several studies reported that each Rtx (repeat in toxin) is a member of a protein family that is produced by a wide range of Gram-negative bacteria (Lee et al., 2008a; Lee et al., 2008b; Li et al., 2008; Kwak et al., 2011) and functions as an important virulence factor. The Rtx of the *Vibrio* species is well characterized, and the *rtx* operon consists of six genes (*rtxACHBDE*) in which *rtxA* encodes an exotoxin, *rtxC* codes for an RtxA activator, *rtxH* encodes a conserved hypothetical protein and *rtxBDE* genes code for an ABC transporter (Li et al., 2008). Some important characteristics of this toxin include: i) it requires post-translational modification, i.e., acylation to become biologically active; ii) has a COOH-terminal calcium-binding domain with tandem glycine/aspartic acid-rich repeats; iii) it has a high molecular mass of usually 100 to > 400 kDa; and iv) it is delivered to the extracellular milieu through the T1SS (Boardman and Satchell, 2004; Li et al., 2008).

The Rtx was first detected in *V. cholerae* (Lin et al., 1999), and later, a similar toxin was identified in several other pathogens such as *V. vulnificus* (Lee et al., 2008a), *V. anguillarum* (Li et al., 2008), *Actinobacillus actinomycetemcomitans* (Kraig et al., 1990), *A. pleuropneumoniae* (Devenish et al., 1989), *Morganella morganii* (Eberspacher et al.,

1990), and *Kingella kingae* (Kehl-Fie and St Geme, 2007). Recent studies indicated that RtxA of *V. cholerae* harbored an actin cross-linking domain (ACD) that played a crucial role in the pathogenesis of this organism (Sheahan et al., 2004; Cordero et al., 2006; Cordero et al., 2007). Other domains, such as a Rho-GTPase inactivation domain (RID), an autocatalytic cysteine protease domain (CPD), and an α/β -hydrolase domain, were also identified in the RtxA of several pathogens, including *V. cholerae* (Satchell, 2007). Later, RtxA of *V. cholerae* was re-designated as a multifunctional autoprocessing repeat in toxin (MARTX) (Satchell, 2007) by virtue of its highly conserved structural and catalytic activity domains, which were assembled as mosaics. In addition, it was shown that the expression of the *rtxA* gene from *V. vulnificus* was induced after host cell contact (Kim et al., 2008), the RtxA enhanced the survival of this pathogen during infection by evading phagocytosis (Lo et al., 2011), and, finally, the toxin assisted in the invasion process, i.e., translocation of bacteria from the intestinal tract to the bloodstream (Kim et al., 2008). An earlier study reported that the *rtxA* mutant of *V. vulnificus* was less virulent in a mouse model of infection when compared to the parental strain, and *in vitro* data also showed that the *rtxA* mutant had lower cytotoxicity when tested on different host cells and compared to the parental bacterium (Lee et al., 2008a).

In this study, we sought to characterize the biological functions of ACD located within the RtxA of the reference *A. hydrophila* strain ATCC 7966. Although RtxA is also found in the diarrheal isolate SSU of *A. hydrophila*, since the genome sequence of ATCC 7966 strain was available (Seshadri et al., 2006), emphasis was placed on characterizing ACD of RtxA from the latter strain. We showed that the ACD of RtxA from *A. hydrophila* induced actin cross-linking of the host cells. In addition, we demonstrated by using the pBI-EGFP vector that ACD was associated with cell rounding and apoptosis when expressed and produced from the HeLa Tet-Off cells. Our study is the first to show that RtxA of *A. hydrophila* possesses a functionally active ACD, which could contribute to the pathogenesis during the infection process.

2. MATERIALS AND METHODS

2.1. Bacterial strains and vectors

A. hydrophila ATCC 7966 and SSU strains were grown in Luria Bertani (LB) medium at 27°C or 37°C with continuous shaking (180 rpm). The DNA fragment encoding the full-length ACD (Fig. 1) of RtxA from *A. hydrophila* ATCC 7966 was cloned into a pET-30a vector (Novagen Madison, WI) for hyperexpression and purification purposes. The DNA fragment was cloned into the *Bgl*II and *Xho*I or *Sa*I restriction enzyme sites of the vector. The primers used to amplify the DNA fragment are listed in Table 1. The recombinant protein contained at the NH₂-terminal end a histidine tag for nickel affinity chromatography. The recombinant plasmid was propagated and maintained in *E. coli* DH5 α , and recombinant protein was produced in *E. coli* HMS174-DE3 cells (Novagen). Both the *E. coli* strains were grown in LB medium supplemented with 100 μ g/mL of kanamycin. The pBI-EGFP vector (Clontech, Mountain View, CA) was used to express and produce recombinant proteins in the HeLa Tet-Off cell system (Clontech).

To characterize the biological activity associated with ACD of *A. hydrophila* RtxA, we generated several truncated versions of the DNA fragment encoding ACD, which were deleted either at the 5' - or the 3' - end region, as well as a fragment, which had deletions at both the termini. In total, seven DNA fragments were generated which were truncated for the corresponding number of aa residues: 62 (Δ N62), 121 (Δ N121), and 241 (Δ N241) from the NH₂-terminal end, 61 (Δ C61), 120 (Δ C120), and 220 (Δ C220) from the COOH-terminal end, and finally one fragment that had a truncation of 62 aa residues from the NH₂- and 61 aa residues from the COOH- terminal end (Δ N62/ Δ C61) (Fig. 1). The DNA

fragments encoding the full-length, NH₂-terminal or the COOH-terminal truncated version of ACD were cloned into the *Mlu*I and *Nhe*I restriction enzyme sites of the pBI-EGFP plasmid for ectopic expression and production of ACD of various lengths from the HeLa-Tet-Off system (Fig. 1). This vector was propagated and maintained in *E. coli* DH5 α and grown in LB medium supplemented with 100 μ g/mL of ampicillin (Sigma-Aldrich, St Louis, MO).

2.2. 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 system approved; Clontech) (Sierra et al., 2010; Suarez et al., 2010a).

During transfection, HeLa Tet-Off cells were electroporated with the pBI-EGFP plasmid (5 μ g/mL) containing full-length and truncated fragments of ACD by using 4-mm cuvettes in a Gene Pulser Xcell as described in our previous studies (Sierra et al., 2010; Suarez et al., 2010a). Normal HeLa cells were obtained from the American Type Culture Collection (Manassas, VA) and grown according to standard tissue culture techniques (Sierra et al., 2010; Suarez et al., 2010a).

2.3. Recombinant protein production

E. coli HMS174-DE3 cells containing the pET-30a recombinant plasmid-encoding ACD of RtxA from *A. hydrophila* were grown in 10 mL of LB medium supplemented with kanamycin (100 μ g/mL) overnight. The bacterial culture was diluted, induced with 1 mM of IPTG (Sigma), and the recombinant proteins purified by using the ProBond purification system (Invitrogen) according to the procedure described in our previous studies (Sierra et al., 2010; Suarez et al., 2010a). Protein concentration was measured by using a Bradford assay (Bio-Rad).

2.4. Antibody production

Female Swiss Webster mice (n=5; Taconic Farms, Germantown, NY) were immunized *via* the intraperitoneal route with 10 μ g of purified recombinant protein (ACD of RtxA) mixed with complete Freund's adjuvant (Sigma) and boosted with the rACD antigen on day 15 by using incomplete Freund's adjuvant (Sigma). Sera were obtained from mice after bleeding them at weeks 2 and 4 after immunization. The antibody specificity was determined through Western blot analysis by using whole *E. coli* lysates expressing and producing the recombinant protein, as well as the purified recombinant protein (rACD) as the source of antigen.

2.5. Western blot analysis

HeLa cells were infected with *A. hydrophila* ATCC 7966 and SSU strains (multiplicity of infection [MOI] of 5) for 2 h at different temperatures (27°C and 37°C). Then, HeLa cells were lysed in Tris-Glycine Buffer, subjected to SDS-PAGE, and proteins were transferred to hybond-ECL nitrocellulose membranes (GE Healthcare, Piscataway, NJ). Western blot analysis was performed by using anti-ACD antibodies as described earlier (Sierra et al., 2010; Suarez et al., 2010a). For determination of actin cross-linking, HeLa Tet-off cells expressing and producing different fragments of ACD were lysed after 24 h of transfection, and the proteins were electrophoresed on a SDS-6% PAGE and then transferred to hybond-ECL nitrocellulose membranes. Western blot analysis was performed by using an anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz CA).

2.6. Host cell morphology

The morphology of HeLa Tet-Off cells was examined after 24 h of transfection with the pBI-EGFP vector containing genes encoding either the native or the truncated versions of ACD by fluorescence microscopy and flow cytometry by using Alexa-fluor 568-conjugated phalloidin (Invitrogen) as described in our previous studies (Sierra et al., 2010; Suarez et al., 2010a). 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 by using BD FACSDiva software. For fluorescence microscopy, the cells were placed on glass slides and the images acquired on a fluorescence microscope (Olympus BX51/DPMManager v.1.2.1.107/DPCController v.1.2.1.108, Olympus Optical CO. LTD).

2.7. Host cell viability

Incorporation of 7-amino actinomycin D (7-AAD) (Becton Dickinson) was used to determine the HeLa Tet-Off cell viability according to established procedures, as described earlier (Sierra et al., 2007). HeLa Tet-Off cells expressing different ACD fragments were detached from the tissue culture plate with 0.25% trypsin-EDTA, washed and then incubated for 10 min with 7-AAD (5 μ L per tube). Immediately after staining, the cells were examined in a FACScan flow cytometer to determine the percentage of EGFP and 7-AAD double-positive cells.

2.8. Host cell apoptosis

The extent of apoptosis of HeLa Tet-Off cells expressing different ACD fragments was determined through detection of cytoplasmic nucleosomes by using the cell death ELISA kit (Roche, Indianapolis, IN) and following the manufacturer's instructions (Sierra et al., 2010; Suarez et al., 2010a). Briefly, the HeLa Tet-Off cell lysates, anti-DNA peroxidase, and anti-histone biotinylated antibodies were incubated in streptavidin-coated, 96-well plates for 2 h at room temperature. Subsequently, the plates were washed three times and incubated with ABTS (2, 2'-Azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]) as the substrate. The color reaction was measured in a microplate reader at a 405-nm wavelength. Since the efficiency of transfection was different for each of the recombinant ACD fragment encoding gene, the optical density values at 405 nm were normalized to the percentage of transfection for each of the recombinant fragment.

2.9. Statistical analysis

A two-way ANOVA and Bonferoni post-test were used for statistical analysis of the data by using GraphPad Prism V 4.02 for Windows (Software MacKiev, San Diego, CA).

3. RESULTS

3.1. Characterization of RtxA ACD from *A. hydrophila* ATCC 7966

By genome sequence analysis of ATCC 7966 (Seshadri et al., 2006), we found that as with *V. cholerae* (Lin et al., 1999; Roig et al., 2011), the *rtx* operon of *A. hydrophila* also contained six genes (*rtxACHBDE*). The RtxA, as well as its ACD from *A. hydrophila* (AHA_1359), exhibited 52% and 65% identity, respectively, with the corresponding full-length RtxA and its ACD of *V. cholerae* (VC1451) (Supplemental Fig. S1). The full-length RtxA of the ATCC 7966 strain has 4685 aa residues, and the ACD is encoded by aa residues 1928–2388 (Fig. 1).

3.2. Expression of the *rtxA* gene in *A. hydrophila* ATCC 7966 and SSU strains requires host cell contact

To investigate whether the RtxA of *A. hydrophila* is functional, we performed Western blot analysis on samples by using anti-ACD antibodies after co-infection of HeLa cells with either the environmental isolate ATCC 7966 or the diarrheal isolate SSU of *A. hydrophila*. These antibodies to ACD of RtxA were developed in the laboratory (section 2.4) and could detect full-length RtxA. We used temperatures of 27° and 37°C (as they represent ambient vs. human body temperature) to grow the bacteria and to infect HeLa cells. As noted from Fig. 2, the full-length RtxA (~520 kDa) was detected on Western blots in the co-culture samples of HeLa cells infected with either SSU (27°C; lane 1 and 37°C; lane 2) or ATCC 7966 strain (27°C; lane 3 and 37°C; lane 4). Because of the crude nature of the hyper-immune sera to ACD, these antibodies exhibited some non-specific reaction with the HeLa cell proteins (Fig. 2, lanes 1–6).

Importantly, RtxA was neither detected in the supernatant fractions of the co-cultures nor in the culture filtrates (Supplemental Fig. S2) or cell lysates (Supplemental Fig. S3) of bacteria at either temperature when grown in the absence of the host cells. A non-specific band of 60–65 kDa was detected in the trichloroacetic acid (TCA) precipitated bacterial culture filtrates in all the lanes, inclusive of uninfected HeLa cells (Supplemental Fig. S2, lanes 1–5); however, no RtxA band was observed. The antibodies to ACD reacted with the correct size rACD (lane 6), which served as a positive control.

Likewise, in the whole cell lysates of either SSU or ATCC 7966 when grown at 27°C, no RtxA band was detected on the Western blot (Supplemental Fig. S3; lanes 1 and 2). Although a band of approximately 60–65 kDa was observed in the cell lysates of these two isolates (lanes 3 and 4) when grown at 37°C, these sizes did not match either with the molecular mass of full-length RtxA or its ACD domain, which should be approximately 520 kDa (Fig. 2) and 50 kDa, respectively (Supplemental Fig. S3; lane 6). Therefore, the identity of this band is currently unknown. Uninfected HeLa cell samples (grown either at 27°C or 37°C) were used as controls, and no band corresponding to the size of RtxA was detected (Supplemental Fig. S3; lane 5). We did notice some non-specific cross-reacting eukaryotic protein bands that interacted with antibodies to ACD of *A. hydrophila* on the Western blots upon prolonged exposure. These data demonstrated that RtxA of *A. hydrophila* was not secreted into the medium and that the expression of the *rtxA* gene in *A. hydrophila* required host cell contact.

3.3. Full-length ACD of RtxA from *A. hydrophila* ATCC 7966 induces actin cross-linking

To characterize the function of ACD within the RtxA of *A. hydrophila*, we first confirmed that all of the DNA fragments encoding truncated versions of ACD were correctly produced from the HeLa Tet-Off cells by Western blot analysis using anti-ACD antibodies of RtxA after 24 h of infection (Fig. 3A). A representative blot, which shows full-length ACD (lane 2), Δ N121 (lane 3), Δ N241 (lane 4), and Δ C220 (lane 5), is depicted in Fig. 3A. Ectopic expression of the pBI-EGFP empty plasmid in HeLa-Tet-Off cells (lane 1), untransfected cells (lane 7), and cells transfected with the VIP-2 domain of VgrG1 (lane 6) served as non-actin cross-linking negative controls. Multiple bands observed in lanes 2 and 3 could represent either degradation products or differently spliced forms of ACD.

To further examine the functionality of ACD, we determined actin cross-linking after expressing and producing these native and truncated versions of ACD in the HeLa Tet-off cells after 24 h of infection by Western blot analysis by using anti-actin antibodies. Interestingly, we found that actin cross-linking was only detected (high molecular weight multiple bands) when the full-length gene encoding ACD was expressed from the HeLa Tet-

Off system (Fig. 3B, lane 2) and not with the NH₂- and/or -COOH terminal truncated proteins (Fig. 3B, lanes 3–5). The band at 45 kDa in all the lanes represented monomeric form of the cellular actin. These results indicated that full-length ACD of RtxA was required for the induction of actin cross-linking.

Since our truncations were based on the study on ACD of *V. cholerae* RtxA (Sheahan et al., 2004), which exhibits 65% sequence identity with the ACD of *A. hydrophila* ATCC 7966 (Seshadri et al., 2006) (Supplemental Fig. S1), we believe these ACD domains to have similar secondary structures with α -helices and β strands. Consequently, the loss in the actin cross-linking activity in the truncated versions of ACD of *A. hydrophila* we observed might not be related to the differential folding of the molecule. However, in the future, we will develop site-directed mutations within the ACD of *A. hydrophila* SSU and show which aa residues are key players that contribute to this activity.

3.4. ACD of RtxA in *A. hydrophila* ATCC 7966 induces host cell rounding by disrupting actin cytoskeleton

To examine the role of ACD of RtxA in inducing host cell toxicity, a pBI-EGFP vector expressing DNA fragments encoding various truncated versions of ACD was electroporated into HeLa Tet-Off cells. After 24 h of transfection, a rounded morphology was observed only in the HeLa-Tet-Off cells expressing and producing the full-length ACD (Fig. 4A, panel III). All of the HeLa Tet-Off cells successfully transfected with the pBI-EGFP plasmid were green due to the production of GFP. The actin cytoskeleton of the cells was stained red by the Alexa 568 conjugated phalloidin. As noted in panel III, the actin cytoskeleton was completely disrupted in the HeLa-Tet-Off cells, which were green, indicating that the full-length ACD led to host cell killing and rounding due to actin cross-linking (Fig. 3B). In contrast, none of the HeLa-Tet-Off cells expressing and producing various truncated versions of ACD showed disruption of the actin-cytoskeleton architecture, and a specific example of HeLa-Tet-Off cells producing ACD Δ N62/ Δ C61 is shown in panel II. The data presented in panel II is similar to the one shown in panel I in terms of normal morphology of the HeLa-Tet-Off cells that expressed the empty vector. The VIP-2 domain of VgrG1 (a T6SS effector) of *A. hydrophila* when produced from the HeLa-Tet-Off cells exhibited typical rounding of the host cells, and served as a positive control. However, the VIP-2 domain of VgrG1 induced cell-rounded morphology via actin-ADP-ribosylation (Fig. 4A, panel IV) (Suarez et al., 2010a) rather than actin cross-linking, as observed with ACD of *A. hydrophila*. These differences in activities are showed in Fig. 3B, lanes 2 and 6, as no actin cross-linking was noted when the VIP-2 domain of VrgG1 encoding gene was expressed in the HeLa cells (lane 6) while ACD caused cellular actin cross-linking (lane 2).

In addition, we quantified by flow cytometry the disruption of the actin cytoskeleton of HeLa-Tet-Off cells expressing and producing various truncated versions of ACD by using Alexa 568-conjugated phalloidin for staining of the actin cytoskeleton on GFP-positive cells. A much reduced percentage of phalloidin-positive HeLa Tet-Off cells (40%) was detected when the full-length ACD was expressed and produced (Fig. 4B, panel III) when compared to HeLa-Tet-Off cells producing various truncated versions of ACD (more than 85%). We have representative data with ACD Δ N62/ Δ C61 in panel II in which 88% of the GFP-positive cells had intact actin filaments. This number of 88% phalloidin-positive cells was similar to that of phalloidin-positive HeLa-Tet-Off cells (89%) expressing an empty vector (Fig. 4B, panel I). These data lead us to conclude that the full-length ACD of RtxA in *A. hydrophila* was necessary to disrupt the actin cytoskeleton, resulting in host cell rounding.

3.5. ACD of RtxA in *A. hydrophila* ATCC 7966 induces apoptosis of HeLa Tet-Off cells

The viability of HeLa Tet-Off cells expressing and producing various truncated versions of ACD was measured by incorporating 7-amino actinomycin D (AAD). The percentage of 7-AAD-positive cells was significantly higher (Fig. 5) in HeLa Tet-Off cells expressing and producing the full-length ACD than that of HeLa cells expressing various truncated versions of ACD (Fig. 5). Representative data with NH₂-terminal truncation of ACD are shown here; however, similar results were seen with the COOH-terminal truncation of ACD or when ACD was truncated from both the NH₂- and COOH- termini (data not shown). HeLa-Tet-Off cells expressing and producing either the empty vector or the VIP-2 domain of VgrG1 of *A. hydrophila* served as a negative and positive control, respectively.

We then measured the rate of apoptosis induced by various truncated versions of ACD in HeLa Tet-Off cells by determining the level of cytoplasmic nucleosomes. A significant increase in the cytoplasmic nucleosomes (Fig. 6) was noted in HeLa Tet-Off cells expressing and producing the full-length ACD, which was similar to HeLa cells producing the VIP-2 domain of VgrG1 (Fig. 6). The level of cytoplasmic nucleosomes in HeLa-Tet-Off cells producing various truncated versions of ACD was similar (only 4 shown here) to HeLa cells expressing the empty vector. These results indicated that ACD of RtxA of *A. hydrophila* was able to induce apoptosis in host cells.

4. DISCUSSION

The ability of *A. hydrophila* to produce numerous virulence factors underlines its potential to cause a wide range of human diseases from mild diarrhea to severe forms of infections, such as necrotizing fasciitis and hemolytic uremic syndrome (HUS) (Abuhammour et al., 2006; Figueras et al., 2007; Janda and Abbott, 2010). Secreted/effector proteins that are delivered through different secretion systems have been identified in various species of *Aeromonas* that contributed to host cell cytotoxicity during infection (Ferguson et al., 1997; Sierra et al., 2007; Suarez et al., 2010a; Suarez et al., 2010b). Earlier, RtxA was shown to be secreted through the T1SS of *V. cholerae* and contributed to pathogenic sequelae in the host (Boardman and Satchell, 2004; Sheahan et al., 2004; Cordero et al., 2006). In this study we demonstrated that RtxA of *A. hydrophila* ATCC 7966 possessed a functional ACD that induced host cell rounding and apoptosis.

A recent study on comparative sequence analysis of different *Vibrio* species (e.g., *V. cholerae*, *V. vulnificus*, and *V. anguillarum*) and that of *A. hydrophila* ATCC 7966 revealed a similar genomic organization of Rtx operon (Roig et al., 2011). In addition, in this comparative analysis it was noticed that ACD was only present in RtxA of *V. vulnificus* isolates belonging to biotype 2, while RtxA of all the tested strains of *V. cholerae* had ACD. Importantly, ACD was not found in RtxA of other tested Gram-negative organisms, including other *Vibrio* species (Roig et al., 2011). From these data, we hypothesized that the RtxA of *A. hydrophila* would have similar biological functions to those of *V. cholerae* and biotype 2 strains of *V. vulnificus*. We provided evidence that expression of the *rtxA* gene in *A. hydrophila* required host cell contact, which was in accordance with a previous study performed on *V. vulnificus* (Kim et al., 2008). However, unlike *V. cholerae* RtxA (Fullner and Mekalanos, 2000), we found that *A. hydrophila* RtxA was not secreted into the medium, indicating that although the genomic organization of Rtx operons is similar in these pathogens, they possess subtle differences as well.

We noted that the full-length ACD of RtxA in *A. hydrophila* possessed actin cross-linking activity that resulted in a cell-rounding phenotype of HeLa Tet-Off cells. An earlier study has shown that in *V. cholerae*, the cell rounding was not completely eliminated when ACD was deleted from RtxA. It was later discovered that cell rounding was also associated with

another domain of RtxA of *Vibrio*, referred to as a Rho-GTPase inactivation domain (RID) (Sheahan and Satchell, 2007). Therefore, in *V. cholerae*, cell rounding was induced by two domains of RtxA (ACD and RID); however, sequence annotation of *A. hydrophila* ATCC 7966 RtxA revealed that it did not contain an RID domain, possibly meaning that in *A. hydrophila* the ACD domain might primarily be associated with the induction of a host cell-rounding phenotype. Additionally, it is important to mention that *A. hydrophila* produces VgrG1 with ADP-ribosyltransferase activity that induces host cell-rounding phenotype. This activity prevents the formation of F-actin by the ADP-ribosylation of G-actin leading to apoptosis (Suarez, 2010a).

In agreement with the present study, it was reported that *V. vulnificus* RtxA played an important role in the apoptotic death of human epithelial cells, as it was noticed that the RtxA mutant induced significantly lower levels of apoptotic death when compared to the parental strain (Lee et al., 2008a). In addition, these investigators further demonstrated that *V. vulnificus* RtxA induced apoptotic death through a mitochondria-dependent pathway (Lee et al., 2008a). In future studies, we plan to explore in detail the mechanism(s) as to how *A. hydrophila* RtxA of both ATCC 7966 and SSU induces apoptosis in host cells.

Taken together, our data showed that *A. hydrophila* possesses a functional RtxA that leads to host cell rounding and apoptotic death. It appears that *A. hydrophila* produces several toxins and/or effector proteins (Act, AexU, VgrG1, RtxA) that have identical functions, such as apoptotic cell death. From these observations, it can be speculated that redundancy in toxin functions of *A. hydrophila* may be necessary to cause a successful infection in the host. In the future, we plan to investigate the detailed mechanisms of regulation of expression and production of these different toxins and /or effector proteins in *A. hydrophila* both *in vitro* and *in vivo* models of infection. Likewise, we will delineate the function of RtxA in bacterial virulence *in vivo* by generating a knockout mutant. In the long run, such studies would be beneficial for discovering the appropriate target for the development of novel therapeutics. Together, the Rtx operon of *A. hydrophila* has some unique characteristics as well as similarities with the Rtx operon of *Vibrio* species. The actin cross-linking domain of *A. hydrophila* has not been characterized previously, and our current study shed light on how this domain leads to cytotoxic effects on the host cells.

Supplementary Material

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Abbreviations

Rtx	repeat in toxin
ACD	actin cross-linking domain
EGFP	enhanced green fluorescent protein
Act	<i>Aeromonas</i> cytotoxic enterotoxin
T1SS	type 1 secretion system
T2SS	type 2 secretion system

T3SS	type 3 secretion system
T6SS	type 6 secretion system
ADP	adenosine diphosphate
GAP	GTPase-activating protein
VgrG1	valine-glycine-repeat G1 protein family
VIP-2	vegetative insecticidal protein-2 domain
Hcp	hemolysin-coregulated protein
RID	Rho-GTPase inactivation domain
CPD	autocatalytic cysteine protease domain
MARTX	multifunctional autoprocessing repeat in toxin
IPTG	isopropyl-1-thiogalactopyranoside
rACD	recombinant ACD
MOI	multiplicity of infection
SDS	sodium-dodecyl-sulfate
PAGE	polyacrylamide gel electrophoresis
ECL	enhanced chemiluminescence
7-AAD	7-amino actinomycin D
EDTA	ethylenediaminetetraacetic acid
ABTS	2,2'-Azino-bis[3-ethylbenzthiazoline-6-sulfonic acid]
aa	amino acid
TCA	trichloroacetic acid
HUS	hemolytic uremic syndrome

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Highlights

- RtxA of *A. hydrophila* possesses a functional actin cross-linking domain (ACD).
- The *rtxA* gene is expressed only after bacterial-host cell contact.
- RtxA of *A. hydrophila* is not secreted, unlike that of *V. cholerae*.
- Full-length ACD is required for the functionality of RtxA.
- ACD of RtxA leads to host cell apoptosis.

Fig. 1.

Schematic representation of the full-length and truncated versions of ACD of RtxA from *A. hydrophila* ATCC 7966. Total numbers of amino acid (aa) residues of the RtxA, as well as the aa residues that constitute ACD are indicated. The figure is not drawn to scale.

Fig. 2.

Western blot analysis showing expression of the *rtxA* gene after co-infection of HeLa cells with *A. hydrophila* ATCC and SSU strains. **Lanes 1 & 2:** expression and production of RtxA in the SSU strain after infection with HeLa cells at 27°C (lane 1) and 37°C (lane 2). **Lanes 3 & 4:** expression and production of RtxA in the ATCC 7966 strain after infection with HeLa cells at 27°C (lane 3) and 37°C (lane 4). **Lanes 5 & 6:** control uninfected HeLa cells grown at 27°C and 37°C, respectively. MW=markers (kDa).

Fig. 3.

Expression and production of the full-length and truncated versions of ACD from the pBI-EGFP plasmid in HeLa Tet-Off cells. **A)** Western blot analysis of HeLa Tet-Off cell lysates expressing and producing different fragments of ACD of RtxA from *A. hydrophila* ATCC 7966. **Lanes 1:** control, empty vector; **2:** full length ACD; **3:** NH₂-terminal truncated ACD (ΔN121) **4:** NH₂-terminal truncated ACD (ΔN241); **5:** COOH-terminal truncated ACD (ΔC220); **6:** VIP-2 domain of VgrG1; and **7:** Untransfected HeLa cells. **B)** Western blot analysis showing actin cross-linking induced by different fragments of ACD. **Lanes 1:** control, vector only; **2:** full-length ACD; **3:** NH₂-terminal truncated ACD (ΔN121); **4:** NH₂-terminal truncated ACD (ΔN241); **5:** COOH-terminal truncated ACD (ΔC220); **6:** VIP-2 domain of VgrG1; and **7:** Untransfected HeLa cells. Abs=antibodies. MW=markers (kDa).

Fig. 4A

Fig. 4 B

Fig. 4.

Full-length ACD causes host cell rounding. **A)** Morphological changes in HeLa Tet-Off cells induced by the expression and production of different fragments of ACD of RtxA from *A. hydrophila* ATCC 7966. Host cells were stained for actin-cytoskeleton by using Alexa Fluor 568-phalloidin (red), and the expression of the enhanced green fluorescent protein (EGFP) encoding gene was detected in cells successfully transfected with the pBI-EGFP empty vector (**I**), with vector containing genes encoding the $\Delta N62/\Delta C61$ truncated version of ACD (**II**), full-length ACD (**III**), and the VIP-2 domain of VgrG1 of *A. hydrophila* used as a positive control (**IV**). Original magnification is 40X. **B)** Quantification of actin-cytoskeleton as measured by fluorescent phalloidin staining of HeLa Tet-Off expressing and producing different versions of ACD. Flow cytometry dot plots showing HeLa Tet-Off cells stained with Alexa fluor 568-phalloidin and expressing different encoding fragments of ACD of RtxA. The analysis was performed on EGFP-positive cells. The percentage of positive cells from a representative experiment is shown in the plotted quadrants. Three independent experiments were performed and similar data were obtained.

Fig. 5. Viability of HeLa Tet-off cells expressing genes encoding different fragments of ACD of *A. hydrophila* RtxA. The percentage of dead and/or dying cells was quantified by incorporation of 7-AAD and flow cytometry on HeLa Tet-off cells expressing vector alone (pBI-EGFP) or producing different fragments of ACD (as indicated) after 24 h of transfection. Results from three different assays were plotted, and statistical significance is indicated by asterisks ($p < 0.001$).

Fig. 6.

Apoptosis of HeLa Tet-off cells expressing genes encoding different fragments of ACD of *A. hydrophila* RtxA. Apoptosis rates were measured by quantification of cytoplasmic nucleosomes in lysates of HeLa Tet-off cells expressing vector alone (pBI-EGFP), or genes encoding various truncated versions (as indicated) of ACD from *A. hydrophila* RtxA. Results from three different assays were plotted, and statistical significance is indicated by asterisks ($p < 0.001$).

Table 1

Various primers used to amplify full-length and truncated versions of the gene encoding ACD of RtxA from *A. hydrophila* ATCC 7966

Name	Primers
Full-Length	5'-TCATACGCGTATGGTGGATCTCACC GG TCTG-3' 5'-GGTCGCTAGCTCACAGTCCGCTTGCATCCTG-3'
ΔN241	5'-TCATACGCGTATGACGGCCGATCTGGCCCGGG-3' 5'-GGTCGCTAGCTCACAGTCCGCTTGCATCCTG-3'
ΔC220	5'-TCATACGCGTATGGTGGATCTCACC GG TCTG-3' 5'-GGTCGCTAGCTCACTTGCCGTAGATGGAGG-3'
ΔN121	5'-TCATACGCGTATGTTCAACCAGCACATCAGC-3' 5'-GGTCGCTAGCTCACAGTCCGCTTGCATCCTG-3'
ΔC120	5'-TCATACGCGTATGGTGGATCTCACC GG TCTG-3' 5'-GGTCGCTAGCTCAGGTCGCATTGTTGATGCC-3'
ΔN62	5'-TCATACGCGTATGCTGACCAAGGACATG-3' 5'-GGTCGCTAGCTCACAGTCCGCTTGCATCCTG-3'
ΔC61	5'-TCATACGCGTATGGTGGATCTCACC GG TCTG-3' 5'-GGTCGCTAGCTCAGACCAGATTGTTACCGCC-3'
ΔN62/ΔC61	5'-TCATACGCGTATGCTGACCAAGGACATG-3' 5'-GGTCGCTAGCTCAGACCAGATTGTTACCGCC-3'

Underlines indicate restriction enzyme sites.