A type VI secretion system effector protein VgrG1 from *Aeromonas hydrophila* that induces host cell toxicity by ADP-ribosylation of actin

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Running title: T6SS and *Aeromonas hydrophila* virulence

Key words: *Aeromonas hydrophila*; T6SS; proteomics; apoptosis; ADP-ribosylation

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We recently delineated the importance of a type 6 secretion system (T6SS) gene cluster in the virulence of diarrheal isolate SSU of *Aeromonas hydrophila* and showed that VasH, a sigma 54 activator and T6SS component, was involved in the production of its associated effectors, e.g., hemolysin co-regulated protein. To identify additional T6SS effectors and/or secreted proteins, we subjected culture supernatants from deletion mutants of *A. hydrophila*, namely Δact (a T2SS-associated cytotoxic enterotoxin encoding gene) and Δact/ΔvasH, to 2-dimensional gel electrophoresis and mass spectrometric analysis. Based on these approaches, we identified a member of the VgrG protein family, (VgrG1), containing a vegetative insecticidal protein (VIP-2) domain at its carboxyl-terminal end. Consequently, the vgrG1 gene was cloned in pBI-EGFP and pET-30a vectors to be expressed in HeLa Tet-Off cells and *Escherichia coli*, respectively. We assessed the ADP-ribosyltransferase (ADPRT) activity of various domains of purified recombinant VgrG1 (rVgrG1) and provided evidence that only the full-length VgrG1, as well as its carboxyl-terminal domain encoding the VIP-2 domain, showed ADPRT activity. Importantly, bacterial-host cell interaction was needed for the T6SS to induce cytotoxicity in eukaryotic cells, and we demonstrated translocation of VgrG1. Further, our data indicated that expression of the genes encoding the full-length VgrG1 and its carboxyl-terminal domain in HeLa Tet-Off cells disrupted the actin cytoskeleton, which was followed by a decrease in cell viability and an increase in apoptosis. Taken together, these findings demonstrated for the first time that VgrG1 of *A. hydrophila* possessed actin ADPRT activity associated with its VIP-2 domain, and that this domain alone was able to induce a rounded phenotype in HeLa Tet-Off cells, followed by apoptosis mediated by caspase 9 activation.
INTRODUCTION

The presence of Aeromonas species in water distribution systems and different foods indicates their potential as a food-borne pathogen, and, therefore, this organism represents a public health concern (10). Consequently, our laboratory identified and characterized multiple virulence factors from diarrheal isolate SSU of A. hydrophila. One of the most potent virulence factors of this A. hydrophila strain is the cytotoxic enterotoxin, designated as Act, which is secreted via a type II secretion system (T2SS) (14, 15, 37). The other recently identified virulence factor from this isolate is the type III secretion system (T3SS)-associated effector AexU (41-43).

Microbial toxins with ADP-ribosylating activity have been grouped into four different families based on their respective host targets (20, 45). Type I toxin proteins target heteromeric guanosine-triphosphate (GTP)-binding proteins (i.e., cholera and pertussis toxins) (16, 21); type II proteins modify elongation factor 2 (i.e., diphtheria toxin and Pseudomonas aeruginosa exotoxin A) (46, 51); type III proteins target small GTP-binding proteins (i.e., Clostridium botulinum C3 exoenzyme) (3); and type IV proteins are specific for ADP-ribosylation of G-actin. The last family of proteins includes: C. botulinum C2 toxin, C. perfringens iota-toxin, C. spiroforme toxin, C. difficile binary toxin A, and Bacillus cereus vegetative insecticidal protein (VIP) (1, 2, 5, 18, 31, 32, 47).

The type VI secretion system (T6SS) was recently characterized from several gram-negative bacteria (6, 8, 36). Most of the genes located in the T6SS cluster do not exhibit any identity with the genes of the other secretion systems; however, ortholog proteins, such as IcmF and DotU, have been reported as part of the T4SS in Legionella pneumophila (39). The T6SS system is able to export effector proteins into the extracellular milieu and/or is able to translocate...
them into eukaryotic host cell cytoplasm as T3- and T4- secretion systems do by an as yet unknown mechanism, which is currently under study (9, 24, 27, 34, 35, 44). Importantly, known proteins that are exported or translocated through this system do not have any known signal peptide, which indicates they are secreted in a Sec- or Tat-independent manner (8, 9, 29, 50).

Two T6SS-associated effectors, namely hemolysin co-regulated protein (Hcp) and valine-glycine repeat G (VgrG), have recently been molecularly and structurally characterized from *Vibrio cholerae* and *P. aeruginosa* (24, 27, 34, 35). The *V. cholerae* genome has three copies of VgrG which are located both within and outside of the T6SS gene cluster (35). Our recent analysis of the complete genome of *A. hydrophila* ATCC 7966T, an environmental isolate, also indicated the presence of three copies of the *vgrG* gene with a distribution in the genome similar to that of *V. cholerae* (44). In some bacteria, *vgr* genes are linked to the *hcp* gene (12), and the expression of the *vgrG* (*vgrG2* and *vgrG3*) genes is required for Hcp secretion. Likewise, expression of the *hcp* gene is required for secretion of VgrG proteins in *V. cholerae* (34, 35). In addition, structural analysis of Hcp and VgrG from *P. aeruginosa* and *V. cholerae* showed that these proteins independently formed channel-like structures, which could be used to transport macromolecules through them (23, 27, 34). Thus, these data suggested that VgrG and Hcp proteins could be part of the secretion apparatus.

Sequence analyses of VgrG proteins from different bacteria showed that all of them were highly conserved in their NH₂-terminal domains. The domain called VgrG or COG3501 shared similarities to the gp5 and gp27 proteins of the bacteriophage T4 tail spike (12, 34). Some VgrG proteins had different COOH-terminal extensions, which contained domains having different activities. For example, VgrG from *P. aeruginosa* carried a zinc-metalloprotease domain while VgrG1 and VgrG3 from *V. cholerae* contained a repeat in structural toxin A (RtxA) and
peptidoglycan binding domains, respectively (34). Only recently was it reported that VgrG1 from
V. cholerae is translocated into eukaryotic host cells with deleterious effects (24), a finding that
suggests VgrG proteins with an extended COOH-termini could have a role as a T6SS effector.

Recently, we reported the importance of vasH (sigma 54 activator) and vasK of A. hydrophila SSU in expression of the T6SS gene cluster, secretion and translocation of T6SS–associated effector proteins, and their crucial roles in evoking mouse lethality (44). In our previous study (44), and now in the current manuscript, we demonstrated that the vasH isogenic mutant was unable to express and produce known T6SS proteins, such as Hcp and VgrG2/3. On the contrary, the vasK isogenic mutant was able to express and translocate Hcp into the host eukaryotic cell, but unable to secrete it into the extracellular milieu (44). Since VasH is a member of the RpoN family of σ54 transcriptional factor regulators, which are associated with modulation of virulence factors in several bacteria (33), it is not surprising that the ΔvasH mutant of A. hydrophila SSU did not express genes present in the T6SS gene cluster.

The A. hydrophila SSU genome has not been sequenced; however, the proteomics analysis of the data indicated the existence of VgrG1, with its gene localized out of the T6SS gene cluster. Further, we found that this protein has a COOH-terminal extension containing a vegetative insecticidal protein-2 (VIP-2) domain, known for its actin-ADP-ribosylating activity (18). We noted that the expression of the gene encoding either the full-length VgrG1 or its COOH-terminal domain that contained only the VIP-2 domain induced apoptosis in HeLa Tet-Off cells. Overall, we demonstrated that VgrG1 is an important virulence factor of A. hydrophila, which is secreted and also translocated via the T6SS. Further, this is the first characterization of VgrG from any bacteria with actin-ADP ribosylating activity.
MATERIALS AND METHODS

Bacterial Strains. Wild-type (WT) *A. hydrophila* SSU and its various mutant strains were grown in Luria Bertani (LB) medium at 37°C with continuous shaking (180 rpm). Deletion mutants of *A. hydrophila* SSU, ∆act and ∆act/∆vasH, previously developed in our laboratory (44), were grown in LB medium supplemented with 100 µg/mL of kanamycin (Sigma St. Louis, MO) and streptomycin plus spectinomycin (100 µg/mL each; Sigma), respectively. The bacterial strains used in this study are listed in Table 1.

Vectors. The genes encoding the full-length, NH₂-terminal or the COOH-terminal domain of VgrG1 from *A. hydrophila* ATCC 7966 were cloned into a pET-30a vector (Novagen Madison, WI) for hyperexpression and purification purposes. The DNA fragments were cloned into the *Bgl*II and *Xho*I or *Sal*I restriction enzyme sites of the vector. The recombinant proteins contained at the NH₂-terminal end a histidine tag for nickel affinity chromatography. The pBI-EGFP (enhanced green fluorescent protein) vector (Clontech, Mountain View, CA) was used to express and produce recombinant proteins from a HeLa Tet-Off cell system (Clontech). The fragments encoding the full-length, NH₂-terminal or the COOH-terminal domain of VgrG1 were cloned into the *Mlu*I and *Nhe*I restriction enzyme sites.

For translocation studies, the gene encoding either the full-length or the NH₂-terminal domain of VgrG1 from *A. hydrophila* ATCC 7966 without the stop codon was cloned in the pGEN222 vector (11), between the *Cla*I and *Mlu*I restriction enzyme sites. Downstream of the above-mentioned vgrG1 genes, the β-lactamase gene (*blaM*, without the region encoding the signal peptide) was cloned between the *Mlu*I and *Sal*I restriction enzyme sites to produce in-frame fusions. These recombinant plasmids were electroporated into *A. hydrophila* SSU ∆act and ∆act/∆vasH mutant strains. The production of fusion proteins from these bacterial strains was
tested by Western blot analysis using antibodies against Bla (Abcam, Cambridge, MA), the VIP-2 domain of VgrG1, and VgrG2 after 2 h of cultivation at 37°C in Dulbecco’s modified eagle medium (DMEM) containing 75 mM NaCl to induce the target gene expression. Table 1 lists the plasmids used in this study. The primers having different restriction enzyme sites, and used to clone various DNA fragments were synthesized by Integrated DNA Technologies (Cedar Rapids, IA) and are shown in Table 2 along with their sequences.

Cell lines. HeLa Tet-Off cells (Clontech), a human cervical epithelial cell line, were grown in DMEM with high glucose (Invitrogen/Gibco Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS)-Tet approved (Clontech) and 100 μg/mL of G418 (Cellgro, Hemdon, VA) under standard tissue culture conditions at 37°C and 5% CO₂ in a humid atmosphere. The HeLa Tet-Off cells were transfected by electroporation with the recombinant pBI-EGFP plasmids containing different vgrG1-encoding regions. Briefly, single-cell suspensions (~5 x 10⁶ cells/mL) were transfected with different plasmid constructs in 4 mm cuvettes (Bio-Rad, Hercules, CA) by using an exponential protocol (300 V, 950 μF, and ∞Ω) in a Gene Pulser Xcell (Bio-Rad). The cells were then recovered in complete medium, plated and grown under standard tissue culture conditions.

Normal HeLa cells (without the Tet-off system) were used for assays that did not require transfection. These cells were grown in DMEM-high glucose supplemented with 10% FBS.

Recombinant protein production. E. coli HMS174-DE3 cells containing pET-30a recombinant plasmids (i.e., coding for VgrG2 and VIP-2 domain of VgrG1) were grown in LB medium and induced with 1 mM of IPTG (Sigma) for 4 h at 37°C. Recombinant proteins were purified by using the ProBond purification system (Invitrogen) and following the non-denaturing protocol as described by the manufacturer. One-mL fractions were collected and subjected to
sodium-dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), followed by Coomassie blue staining. Fractions containing the protein of interest were mixed and dialyzed overnight against phosphate-buffered saline (PBS) at 4°C. Protein concentration was measured by using a Bradford assay (Bio-Rad).

**Antibody production.** Female Swiss Webster mice (n=5; Taconic Farms, Germantown, NY) were immunized via the intraperitoneal route with 10 µg of purified recombinant protein mixed with complete Freund’s adjuvant (Sigma) and boosted on day 15 by using incomplete Freund’s adjuvant (Sigma). Sera were obtained from mice at weeks 2 and 4 after immunization. The antibody specificity was determined by Western blot analysis by using whole *E. coli* lysates containing recombinant proteins, as well as the respective purified recombinant protein as the source of antigen. Antibodies against rHcp were previously developed in our laboratory (44). Since we used rVgrG2 for antibody production, the sera obtained did not differentiate between VgrG2 and VgrG3 proteins of *A. hydrophila* SSU due to the high homology (~90%) between them and their similar sizes (~77 kDa) on Western blots. As a result, we referred to VgrG2 as VgrG2/3 in our Western blot analysis data.

**Western blot analysis.** HeLa Tet-Off cells were lysed in Tris-Glycine Buffer after 24 h of transfection and subjected to SDS-PAGE. The proteins from the gels were then transferred to hybond-ECL nitrocellulose membranes (GE Healthcare, Piscataway, NJ), which were blocked and then treated with specific primary and secondary antibodies, as we previously described (44). Super signal west pico or femto chemiluminescence substrate (Thermo Scientific, Rockford, IL) was used to develop the blots followed by X-ray film exposure.

**2-dimensional (2-D) gel electrophoresis and mass spectrometry.** The LB cultures of the *A. hydrophila* SSU mutants, Δact and Δact/ΔvasH, were grown for 2 h to a cell density of 5x
10^6 cells/mL in 10 mL of DMEM with high glucose and without the FBS at 37°C. The supernatants obtained after centrifugation (6000 x g for 10 min) were filtered through 0.2-μm filters. Next, the proteins in the supernatants were precipitated with trichloroacetic acid (TCA) (Sigma; 10% v/v final concentration), and the pellet was collected by centrifugation at 14000 x g for 20 min at 4°C. The protein pellets were washed 3 times with cold acetone, dried, and resuspended in 200 μL of DeStreak rehydration solution (GE Healthcare).

For the first dimension, the proteins were separated in 13-cm, 3-10 pH non-linear strips (GE Healthcare) by using the following protocol: rehydration 50V, 10 h; 250V, 1 h; 500V, 1 h; 1000V, 2 h; and 8000V, 7 h, in a IPphor apparatus (GE Healthcare) at 75 μA/strip and 20°C. Subsequently, the strips were incubated in equilibrium buffer (GE Healthcare) for 10 min followed by SDS-(4-20%) PAGE. The gels were stained with Sypro-Ruby (Bio-Rad), and images for analysis were acquired in a Gel-Doc system (Bio-Rad).

As an analysis strategy for the 2-D gels, the proteins present in supernatants of the Δact mutant of *A. hydrophila* SSU, but not in the supernatants of the Δact/ΔvasH mutant, were considered prime candidates for future study. For this analysis, we used 3 gels per bacterial mutant strain, and the differential spots from the 2-D gels were identified by using Progenesis Samespot v.2.0.2733.19819 software (Nonlinear Dynamics, Durham, NC). Different spots were manually picked, trypsin digested, and analyzed by mass spectrometry (MALDI-TOF). The peptide sequences then were matched against the proteobacteria database, and results with high homologies (low expectation numbers [Table 3]) were examined.

**ADP-ribosylation assay.** HeLa cells in suspension were lysed by sonication in a buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 5 mM MgCl2 and protease inhibitors. The whole lysate was centrifuged at 14000 x g for 10 min at 4°C, and the supernatant
was used for the ADP-ribosylation assay (19). Briefly, 50 µg of normal HeLa cell lysate proteins or 1 µg of recombinant non-muscle actin (Cytoskeleton, Denver, CO) as the target protein for ADP-ribosylation was incubated with 1 µg of different purified rVgrG1 proteins and 10 µM of NAD conjugated with Biotin (R&D Systems, Minneapolis, MN) for 30 min at 37°C. The reaction was stopped by adding SDS-sample buffer, the proteins separated by SDS-PAGE, transferred to nitrocellulose membranes (GE Healthcare), and incubated with streptavidin-horseradish peroxidase (HRP) after blocking of non-specific sites with 1% bovine serum albumin (BSA). Super signal west pico chemiluminescence substrate was used to develop the blot, followed by X-ray film exposure.

**Translocation of VgrG1.** For studying translocation of VgrG1::Bla fusion proteins in HeLa cells, we used CCF4-AM as a substrate for Bla. CCF4-AM, a fluorescence resonance energy transfer (FRET)-based substrate for β-lactamase, contains a cephalosporin core linked to coumarin and fluorescein. CCF4 requires an excitation of 408 nm and produces emissions of 460 nm (blue from coumarin) and 530 nm (green from fluorescein). In the presence of β-lactamase activity, the substrate is cleaved, resulting in the breakage of FRET and loss of 530-nm emission (green).

For translocation proposes, the full-length VgrG1::Bla or the NH₂-terminal VgrG1::Bla from the pGEN222 vector was produced from *A. hydrophila* SSU Δact and Δact/ΔvasH mutant strains. The mutant strains harboring only the vector were used as a negative control. HeLa cells in suspension were placed in flow cytometry tubes in DMEM medium without serum at a concentration of 1x10⁶ cells/mL. These cells were infected at a multiplicity of infection (MOI) of 10, with *A. hydrophila* SSU Δact and Δact/ΔvasH mutant strains producing different forms of VgrG1::Bla fusion proteins. After 15 min of infection, CCF4-AM working solution (6X)
(Invitrogen) was added and the tubes incubated for 45 min. The cells were then acquired in a Becton Dickinson FACS Aria flow cytometer in which a violet laser diode (408 nm) was used for excitation and which detected emissions at 450/40 (blue) and 530/30 (green). Ratios between percentages of blue HeLa cells and percentages of green cells were calculated for analysis.

**Host cell morphology.** HeLa cells plated in 6-well plates were co-cultured with different mutant strains of *A. hydrophila* SSU at an MOI of 5 in DMEM without FBS. Changes in morphology were evaluated by phase contrast microscopy over a 90-120 min period. In parallel, to test the importance of bacterial-host cell contact in morphological changes of the HeLa cells, we performed co-cultures of bacteria and host cells by using transwell inserts having a 0.4-µm pore size (Costar, Corning, NY).

HeLa Tet-Off cells were electroporated with various vgrG1 constructs and, after 24 h of incubation, they were stained with Alexa-fluor 568-conjugated phalloidin (Invitrogen-Gibco) following the manufacturer’s instructions. Briefly, the cells were fixed and permeabilized in situ with Cytofix/Cytoperm (Becton Dickinson, San Jose, CA) for 20 min at 4°C. Then, the cells were scraped and stained with Alexa fluor 568-conjugated phalloidin for 1 h at room temperature and washed with Perm/Wash solution (Becton Dickinson). The cells were acquired in a FACScan flow cytometer and analyzed with FACSDiva software (Becton Dickinson). Additionally, the stained cells were placed on glass slides by using a Shandon cytocentrifuge (Thermo Scientific Waltham, MA) at 1500 rpm for 5 min. The cover slips were mounted with medium containing DAPI (Vector, Burlingame CA), and the images were then acquired and analyzed under fluorescence microscopy (Olympus BX51/DPManager v.1.2.1.107/DPController v.1.2.1.108, Olympus Optical CO. LTD).
Quantification of G- and F-actin. The G-actin/F-actin In Vivo Assay Kit (Cytoskeleton) was used to quantify the amounts of G- and F-actin present in HeLa Tet-Off cells transfected with the pBI-EGFP vector expressing and producing various domains of VgrG1. Likewise, G-actin/F-actin ratios were determined in HeLa cells co-cultured with different mutant strains of A. hydrophila SSU, by following the manufacturer’s instructions. Briefly, HeLa cells plated in 12-well plates were lysed with 300 µL of lysis buffer. The cells were then scraped, and passed through a 25-gauge needle. The total cell lysates were centrifuged at 100,000 xg for 1 h at 37°C and the supernatants collected. The pellets were resuspended in the same volume (300 µL) of the SDS-PAGE loading buffer. Supernatants containing G-actin and pellets containing F-actin were subjected to SDS-10% PAGE and Western blot analysis by using an anti-actin antibody (Cytoskeleton). The density of each band was calculated by using AlphaEasyFC software (Alpha Innotech, San Leandro, CA). The results were reported as a percentage of F- and G-actin per sample. The sum total of G- and F-actin corresponded to 100%.

Host cell viability. Incorporation of 7-amino actinomycin D (7-AAD) (Becton Dickinson) was used to determine the HeLa Tet-Off cell viability. HeLa Tet-Off cells expressing different vgrG1 fragments were detached, washed and then incubated for 10 min with 7-AAD (5 µL per tube). Immediately after staining, the cells were analyzed by using flow cytometry.

Host cell apoptosis. We evaluated the extent of apoptosis of HeLa Tet-Off cells expressing different vgrG1 fragments by detection of cytoplasmic nucleosomes and measurement of caspase 3 and 9 activation, as we previously described (43).

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

*A. hydrophila* SSU secretes VgrG1 via the T6SS.

We analyzed supernatants from *A. hydrophila* SSU Δact and Δact/ΔvasH mutant strains by 2-D gel electrophoresis and identified differentially expressed and produced protein spots by mass spectrometry. We chose to use *A. hydrophila* SSU Δact mutant as the parental strain to avoid any interference of T6SS-associated cytotoxicity with that contributed by Act. It has been shown that deletion of the *vasH* gene prevented the transcription, translocation and secretion of effector proteins via the T6SS (i.e., Hcp) (35, 44). The approach used in this analysis was to identify protein spots present in the Δact mutant strain, but not in the Δact/ΔvasH mutant, thus allowing us to specifically target T6SS-secreted proteins. Of the 32 differentially expressed and produced spots (Table 3), we detected homology to the known T6SS proteins, such as Hcp-1, Hcp-2, VgrG1, VgrG2, and VgrG3. These differentially produced proteins related to the T6SS gene cluster showed low expectation scores (high homology) compared to those proteins not related to the T6SS gene cluster. However, these proteins with high expectation scores (low homology) need to be analyzed carefully (Table 3).

We identified well-defined clusters by 2-D gel electrophoresis in the culture supernatant of the Δact mutant strain that were absent from the Δact/ΔvasH mutant strain. One of these clusters contained various isoforms of Hcp, while the other contained VgrG homologs. From the VgrG cluster (Figure 1A, highlighted in green), we identified proteins (isoforms) with high homology (expected score: 1.2e-032) to VgrG1 (gi:117619461) of the environmental isolate ATCC 7966 of *A. hydrophila*, whose genome sequence was recently annotated (38). Figure 1B depicts the alignment of sequences of 12 peptides (highlighted in red) obtained by mass spectrometric analysis of VgrG1 from *A. hydrophila* SSU with that of the sequence of *A.
hydrophila ATCC 7966 VgrG1 and they were exactly identical (38). Since the genome of A. hydrophila SSU is not totally sequenced yet, and we only have sequenced T6SS and T3SS gene clusters (41, 44), we were unable to detect, based on computer alignment programs, matches of VgrG1 of A. hydrophila SSU with that of VgrG1 of A. hydrophila ATCC 7966. This is because the vgrG1 gene is located on the bacterial chromosome outside of the T6SS gene cluster in contrast to vgrG2 and vgrG3 genes which reside within the T6SS gene cluster.

Importantly, we identified the presence of a VIP-2 domain in the COOH-terminal portion (amino acid residues 702-927) of VgrG1 of A. hydrophila (both SSU and ATCC 7966 strains) (E-value 5e-10) by using a specialized BLAST search of conserved domains (Figure 1B [indicated by underlining]).

Figure 1 (supplementary data) depicts amino acid residues in red that are conserved between the reference sequence (cd00233) and the VIP-2 domain of A. hydrophila ATCC 7966 (25). To confirm the secretion of VgrG proteins by A. hydrophila SSU, we performed Western blot analysis on supernatants from both Δact and Δact/ΔvasH mutant strains of A. hydrophila SSU by using antibodies specific to the VIP-2 domain of VgrG1, as well as antibodies against VgrG2 (which recognize all members of the VgrG family) (Figure 1C).

We were able to detect VgrG1 (~103 kDa) and VgrG2/3 (exhibiting similar molecular masses; ~77 kDa) in the supernatant and pellet fractions of A. hydrophila SSU Δact mutant (Figure 1C, lanes 1 and 3). However, the Δact/ΔvasH mutant of A. hydrophila SSU was unable to secrete any member of the VgrG family of proteins (Figure 1C, lane 4). Importantly, we could detect the presence of VgrG1, but not of VgrG2/3, in the pellet fraction of A. hydrophila SSU Δact/ΔvasH mutant (Figure 1C, lane 2), which signified that, since the vgrG1 gene is out of the T6SS gene cluster, its regulation is possibly independent of VasH. However, the inability
of VgrG1 to be secreted by the ∆act/∆vasH mutant (Figure 1C, lane 4) indicated that a T6SS apparatus was needed for the secretion of VgrG1.

We noted that the NH$_2$-terminal domain of VgrG1 (amino acid residues 1-701) harboring the VgrG domain is present in all the members of VgrG proteins (Figure IIA [supplementary data]). Interestingly, within the VgrG domain, there is a phage GPD superfamily region (c|01392) which is present in the bacteriophage T4 tail spike, and the DUF586 (pfam04524) region, which has no known function (Figure IIA [supplementary data]). As noted in Figure IIB (supplementary data), the COOH-terminal extension of V. cholerae VgrG1 has an RtxA domain, unlike that of the VIP-2 domain (cd00233/pfam01129) found in VgrG1 of A. hydrophila SSU and ATCC 7966 strains (Figure IIA [supplementary data]). Recent reports have shown structural similarities between the VgrG domain and gp27 and gp5, as well as with Hcp-hexameric rings and gp25 of bacteriophage T4 (23, 34) (Figure IIB; supplementary data).

These findings highlighted the similarity of the T6SS apparatus to the bacteriophage T4 needle-like structure and suggested there are similar functions of T6SS and bacteriophage in the translocation of proteins through host cell membranes.

VgrG1 from A. hydrophila possesses actin-ADP-ribosyltransferase (ADPRT) activity.

Since the COOH-terminal domains of VgrG1 were highly conserved between A. hydrophila ATCC 7966 and A. hydrophila SSU and because the NH$_2$-terminal domains of all known VgrGs have significant homologies, we tested the ADPRT activity associated with the VgrG1 of A. hydrophila ATCC 7966. We cloned the full-length vgrG1 gene and its NH$_2$-terminal domain-encoding DNA fragment (1-2106 bp [1-701 amino acid residues]) and the COOH-terminal end-encoding DNA fragment (2107-2784 bp [702-927 amino acid residues], which harbors only the VIP-2 domain) in pET-30a vector and produced the recombinant proteins.
in E. coli. The purified rVgrG1 full-length and rVgrG1 COOH-terminal domain proteins were able to catalyze the incorporation of Biotin-ADP (from 6-Biotin-17-NAD) into an approximately 45-kDa target protein of HeLa cells lysate (Figure 2A, lanes 5 and 6) or into recombinant non-muscle actin (Figure 2B, lanes 5 and 6). In contrast, the purified rVgrG1 NH\textsubscript{2}-terminal domain protein was not able to catalyze this reaction (Figures 2A and 2B, lane 4). The appropriate negative controls, which included HeLa cell lysate or rActin alone (Figures 2A and 2B, lane 1), or in conjunction with NAD (lane 2) as well as NAD alone (lane 3), did not exhibit any ADPRT activity.

**A. hydrophila SSU \Delta act mutant strain induces a rounded phenotype in HeLa cells which is cell contact-dependent.**

To test the cytotoxic effect of the T6SS effector proteins of *A. hydrophila* on eukaryotic cells, changes in the morphology of the latter were evaluated in co-cultures of normal HeLa cells infected with either *A. hydrophila* SSU \Delta act mutant (with functional T6SS) or the \Delta act/\Delta vasH mutant strain (with non-functional T6SS). We noted that HeLa cells co-cultured with *A. hydrophila* SSU \Delta act mutant showed a host-bacterial, contact-dependent, rounded morphology after 90 min of co-culture (Figure 3A-II, column 1). In contrast, this phenotype was minimally observed in co-cultures of HeLa cells with the *A. hydrophila* SSU \Delta act/\Delta vasH mutant (Figure 3A-II, column 2). Importantly, we could demonstrate reversal of this rounding phenotype of HeLa cells when the *A. hydrophila* SSU \Delta act/\Delta vasH mutant was complemented with the vasH gene *in trans* by using the pBR322 vector (Figure IIIA [supplementary data]).

In parallel, co-cultures using transwell inserts were employed to test whether host-bacterial cell contact was, indeed, needed for the induction of the cell-rounding phenotype. Consequently, *A. hydrophila* SSU mutant strains (\Delta act or \Delta act/\Delta vasH) were placed in the upper
chamber, while the HeLa cells were placed in the bottom chamber to avoid direct host-bacterial cell contact. Importantly, in contrast to direct-contact co-cultures, HeLa cells co-cultured by using transwell inserts with different *A. hydrophila* SSU mutant strains had a cell morphology similar to that of the control cells without the bacteria (Figure 3A-III, columns 1 and 2 versus 3).

Subsequently, supernatants of co-cultures of HeLa cells with different strains of *A. hydrophila* SSU were removed after 90 min, filtered through a 0.2-µm membrane, and used as pre-conditioned media on fresh HeLa cells. Host cells exposed to these pre-conditioned media did not show any changes in their morphology even after 2 h of exposure (Figure 3A-IV, columns 1 and 2 versus control HeLa cells, column 3). These results indicated that induction of the cell-rounding phenotype of HeLa cells required direct bacterial-host cell contact and that the protein effector(s) was translocated directly into the eukaryotic cell cytoplasm via the T6SS.

Figure IIIB [Supplementary data] shows the presence of Hcp and VgrG2/3 in conditioned medium before (Pre) and after (Post) incubation (for 90 min) with fresh HeLa cells based on Western blot analysis. It is unclear as to why we detected lesser amounts of Hcp and VgrG2/3 in the post conditioned media. The decrease in Hcp was more prominent than that of VgrG2/3, and it could possibly be related to either degradation of the proteins or their binding to the host cells (44).

To evaluate a relationship between the rounded phenotype and actin ADP-ribosylation, we quantified the ratios of G- and F- actin by Western blot analysis in HeLa cells co-cultured with different strains of *A. hydrophila* SSU. As seen in Figure 3B, HeLa cells co-cultured with *A. hydrophila* SSU ∆act mutant showed a significant change in the ratio of G/F-actin (70%/30%), when compared to HeLa cells co-cultured with *A. hydrophila* ∆act/∆vasH mutant.
and control HeLa cells (51%/49%). These results correlated with findings regarding the rounded phenotype of HeLa cells co-cultured with the parental (Δact) A. hydrophila SSU strain (Figure 3A-II, column 1).

*A. hydrophila SSU is able to translocate VgrG1 into the eukaryotic host cell cytoplasm via the T6SS.*

We examined the importance of cell-to-cell contact for the induction of the rounded phenotype of HeLa cells by evaluating the translocation of VgrG1 into the host cell cytoplasm. For these studies, we first showed by Western blot analysis using antibodies to β-lactamase, the presence of fusion proteins (full-length VgrG1::Bla and VgrG1-NH₂::Bla) in the bacterial pellet, as well as in the supernatant of the parental A. hydrophila SSU Δact strain (Figure IV, lanes 2 and 3 [Supplementary Data]). On the contrary, by using the Δact/ΔvasH mutant strain, we could detect the fusion proteins in the bacterial pellet, but not in the supernatant (Figure IV, lanes 5 and 6). Appropriate negative controls did not show the presence of fusion proteins either in the pellet or the supernatant fraction of the above-mentioned mutant bacteria (Figure IV, lanes 1 and 4). Similarly sized fusion proteins were detected on Western blots when antibodies to VgrG2 and VIP-2 domain of VgrG1 were used (data not shown).

These bacterial strains were then used to infect HeLa cells and we tracked the β-lactamase activity associated with VgrG1 fusion proteins in the host cell cytoplasm by using the CCF4 FRET-based substrate. By flow cytometry, we showed that the A. hydrophila SSU Δact mutant strain expressing and producing either the full-length VgrG1::Bla or the VgrG1-NH₂::Bla was able to cleave the substrate turning HeLa cells from green to blue (Figure 4A, panel I [15% blue/85% green] versus Panel II [60% blue/40% green] and panel III [55% blue/45% green]). Panel I shows HeLa cells transfected with the A. hydrophila SSU Δact mutant strain with vector
alone, which served as a negative control. On the contrary, when we compared it to the controls, A. hydrophila SSU Δact/ΔvasH mutant strains expressing and producing the same fusion proteins were found to not have induced any significant changes in the proportion of blue/green HeLa cells (Figure 4A, panel IV [16% blue/84% green] versus panel V [17% blue/83% green] and panel VI [15% blue/85% green]). Figure 4B shows the fold increase in blue cells (with cleaved substrate) over green cells (with uncleaved substrate) in a representative experiment from 5 independent experiments.

**Expression of the vgrG1 gene in HeLa Tet-Off cells induces a rounded phenotype.**

To determine the functionality of the COOH-terminal VIP-2 domain present in VgrG1, the full-length gene and its NH₂- and the COOH-terminal encoding gene segments from A. hydrophila ATCC 7966 were cloned into a pBI-EGFP vector and then expressed in HeLa Tet-Off cells. The pBI-EGFP vector allowed co-expression of the gene of interest together with the expression of the gene encoding EGFP to differentiate the transfected from untransfected host cells. The transfected HeLa Tet-Off cells were then evaluated by Western blot analysis by using antibodies specific for VgrG2 and the VIP-2 domain of VgrG1 (Figure 5A).

Since VgrG2 is highly homologous to its family members, antibodies to VgrG2 recognized the full-length (~103 kDa) and the NH₂-terminal domain (~77 kDa) of VgrG1 in HeLa Tet-Off cell lysates (Figure 5A, lanes 2 and 3) but not the COOH-terminal domain (~25 kDa) of VgrG1, as VgrG2 does not possess the VIP-2 domain (Figure 5A, lane 1). As expected, antibodies to the VIP-2 domain reacted with only the full-length and the COOH-terminal domain of VgrG1 (Figure 5A, lanes 5 and 7) but not with the NH₂-terminal domain of VgrG1 (Figure 5A, lane 6). Lanes 4 and 8 in Figure 5 represented the cell lysates from HeLa Tef-Off cells expressing only the pBI-EGFP vector, which served as a negative control.
After 24 h of transfection, the HeLa-Tet-Off cells expressing either the full-length \textit{vgrG1} (Figure 5B-III) or its COOH-terminal domain (Figure 5B-IV) showed a rounded morphology, in contrast to that of the HeLa Tet-Off cells expressing only the NH\textsubscript{2}-terminal domain of \textit{vgrG1} (Figure 5B-II), the vector alone (pB1-EGFP) (Figure 5B-I), or the full-length \textit{vgrG2} (Figure V [supplementary data]) which maintained normal morphology. In order to examine alterations in the actin cytoskeleton of the HeLa Tet-Off cells expressing and producing different forms of the VgrG1, we evaluated ratios of G/F-actin by Western blot analysis. In addition, the host cells were also stained with Alexa fluor-568-Phalloidin (red stain) and analyzed by fluorescent microscopy and flow cytometry.

As shown in Figures 5B-III and 5B-IV, HeLa Tet-Off cells expressing and producing full-length VgrG1 and the COOH-terminal domain (EGFP-positive cells) showed a rounded phenotype, and the actin cytoskeleton was severely disrupted, as evidenced by little or no phalloidin staining. On the other hand, HeLa Tet-Off cells expressing/producing only the vector (Figure 5B-I) or the VgrG1 NH\textsubscript{2}-terminal domain (Figure 5B-II) exhibited a normal phenotype with an intact actin cytoskeleton (red staining) having a morphology similar to adjacent un-transfected cells (no EGFP, but phalloidin-positive cells).

Changes in the actin cytoskeleton were quantified by flow cytometry after phalloidin staining of the transfected cells expressing and producing EGFP. We found a lower percentage of phalloidin-positive HeLa Tet-Off cells producing full-length VgrG1 (29%) and its COOH-terminal domain (25%), when these were compared to HeLa Tet-Off cells that produced the VgrG1 NH\textsubscript{2}-terminal domain (86%) or expressed the vector alone (82%) (Figure 5C, left panel). In addition, we analyzed the mean fluorescence intensity (MFI) of the actin cytoskeleton staining in the transfected HeLa Tet-Off cells. As shown in Figure 5C, right panel, after 24 and
72 h of transfection, the MFI of HeLa Tet-Off cells expressing and producing the full-length VgrG1, and the VgrG1 COOH-terminal domain was significantly lower than that of HeLa Tet-Off cells producing the VgrG1 NH\textsubscript{2}-terminal domain or expressing the vector alone (p<0.001).

To confirm phalloidin staining data, we quantified the amount of G- and F-actin present in these samples by Western blot analysis and densitometric scanning of the blots (Figure 5D). HeLa cells expressing genes encoding either the full-length VgrG1 or its COOH-terminal domain showed increased amounts of G-actin (63% and 67%, respectively) in contrast to cells expressing the gene encoding the NH\textsubscript{2} terminal domain or HeLa cells transfected with the vector alone (49% and 48%, respectively). Together, these results showed the importance of the VIP-2 domain of VgrG1 in the induction of the rounded phenotype of host cells via ADP-ribosylation of actin.

Expression of the \textit{vgrG1} gene in HeLa Tet-Off cells induces apoptosis.

The viability of the HeLa cells expressing and producing VgrG1 was evaluated by the incorporation of 7-AAD. At 72 h, the percentage of 7-AAD-positive cells was significantly higher in HeLa Tet-Off cells producing the full-length (44.7 ± 7.7%) and the COOH-terminal domain (46.3 ± 5.5%) of VgrG1 compared to that of HeLa Tet-Off cells expressing and producing the NH\textsubscript{2}-terminal domain of VgrG1 (14 ± 4.2%) (p<0.01) or the vector alone (13.6 ± 5.7%) (p<0.001) (Figure 6).

We then evaluated the rate of apoptosis induced by \textit{vgrG1} expression in HeLa Tet-Off cells. We found a significant increase (p<0.001) in the cytoplasmic nucleosomes of the HeLa Tet-Off cells expressing and producing the full-length and the COOH-terminal domain of VgrG1 which we compared to HeLa Tet-Off cells producing the NH\textsubscript{2}-terminal domain of VgrG1 or expressing the vector alone after 24 h of transfection (Figure 7A). Accordingly, the activation of
caspase 3 and 9 was significantly higher (p<0.001) in the HeLa Tet-Off cells producing the full-
length VgrG1 and its COOH-terminal domain than in cells producing the NH2-terminal domain
of VgrG1 or expressing the vector alone (Figure 7B).
DISCUSSION

In this study, we reported that the VgrG1 protein from *A. hydrophila* carries a VIP-2 domain, which has ADPRT activity with deleterious effects on the host cells. Thus far, this is the only VgrG member of the family known that has the VIP-2 domain and is present in tested *A. hydrophila* strains, namely SSU and ATCC 7966. Since we do not have the full genome sequence of *A. hydrophila* SSU, the precise location of the vgrG1 gene on its chromosome is currently unknown. Consequently, we preferred to conduct studies with rVgrG1 of *A. hydrophila* ATCC 7966. The VgrG protein family was initially associated with the Rhs (Recombination hot spot) family, and, although VgrG and RhsG proteins are not homologous, they do share some common characteristics, such as their hydrophilic nature, large size and regularly repeated peptide motifs (12, 48). Based on the similarity between the RhsG and VgrG family proteins, it was hypothesized that VgrG could have acquired the multiple COOH-terminal extensions by horizontal transfer (6, 12).

It is important to note that the presence of VgrG1 was detected in the bacterial culture supernatants by Western blot analysis only when antibodies against VgrG2 were combined with antibodies against the VIP-2 domain of VgrG1 and by using a highly sensitive, west femto chemiluminescence substrate (Figure 1C). These data might suggest the availability of a limited number of immuno-reactive epitopes in the VIP-2 domain to which antibodies could be generated. This, in combination with the low concentration of VgrG1 present in the bacterial culture supernatant, might cause difficulties in its detection.

Interestingly, VgrG1 could be detected and identified in the concentrated supernatants of bacterial cultures through highly sensitive fluorescent Sypro-Ruby staining used for 2-D gels. On the other hand, VgrG2/3 could be easily detected in bacterial culture supernatants (Figure 1C,
lane 3) and in tissue culture medium of bacterial-host cell co-cultures by Western blot analysis by using only antibodies against VgrG2 (Figure IIB [supplementary data]). We believe that this difference in detection of VgrG2/3 and VgrG1 by Western blot analysis could be due to varying amounts of VgrGs present in the culture supernatants. We could easily detect VgrG2 and -3 because of possible higher expression of their genes and more secretion of the corresponding proteins and/or their possible lesser degradation. Further, accumulation of both of these proteins in a single band due to their similar molecular sizes could result in a band which is much stronger in intensity. In HeLa Tet-Off cell lysates, we could also easily detect the presence of VgrG1 on Western blots by employing either VgrG2 or VIP-2-specific antibodies (Figure 5A) because highly concentrated protein preparations were used for the assay, and the gene was hyperexpressed using the pBI vector.

By bioinformatic and structural analyses, VgrG proteins were shown to share structural features of a bacteriophage T4 tail spike (7, 34). During phage infection, the tail spike is inserted into the bacterial outer membrane, and, hence, VgrG could act similarly, i.e., as a needle tip that, in conjunction with the tube-like structure formed by Hcp, could puncture the host cellular membrane and translocate effector protein(s) (7, 12, 23, 24, 30). In these models, the COOH terminal extensions from some VgrG proteins, such as VgrG1 from *V. cholerae* with actin cross-linking activity (Figure IIB [supplementary data]) and VgrG1 from *A. hydrophila* with actin-ADPRT activity, which impedes actin polymerization (Figure IIA [supplementary data]), might be introduced directly into the host cellular cytoplasm where they exhibit their action. Likewise, *V. cholerae* VgrG3 with a peptidoglycan binding domain, *P. aeruginosa* VgrG with a zinc metalloprotease domain, and *Y. pestis* VgrGs with a tropomyosin-like domain, and with a YadA-like and a pertactin-like domain (34), could induce alteration in host cells.
Importantly, although VgrG1s from *V. cholerae* and *A. hydrophila* were able to induce a cell-rounded phenotype, its induction was by a different mechanism. Finally, sequence alignment of VgrG1 from *V. cholerae* and *A. hydrophila* ATCC 7966 showed a high homology in the VgrG domains (NH₂-terminal) (55%), but a low homology (8%) in the COOH-terminal regions where the RtxA or the VIP-2 domain is respectively located (Figures IIA and B [supplementary data]).

In our earlier study, we characterized a T3SS effector protein AexU, which also has ADPRT activity. This effector induced a rounded phenotype when its corresponding gene was expressed in HeLa Tet-off cells (43). In addition, our previous study indicated that the production and translocation of AexU was not altered when the *vasH* gene was deleted from the parental *A. hydrophila* strain; however, this *vasH* mutant showed delayed cytotoxicity in HeLa cells as well as in RAW 264.7 murine macrophages (44). Together, these data suggested that even though AexU and VgrG1 could induce a rounded phenotype in the host cell via the ADPRT activity, their mechanisms of regulation could be different and their corresponding genes might be activated or repressed under different stimuli. Further, unidentified other T3SS effectors and proteins secreted by other mechanisms might also lead to host cell toxicity.

Recently published data showed the translocation of VgrG1 from *V. cholerae* into the eukaryotic cell cytoplasm (24). Similarly, we showed translocation of VgrG1 from *A. hydrophila* SSU Δact mutant strain but not from the Δact/ΔvasH mutant in HeLa cells. These data indicated an absolute need for the T6SS to translocate this effector.

Yu *et al.* (53) reported the presence of a sequence containing vgrG, vsdC, and a type III secretion-related protein gene (AY376445) in *A. hydrophila* strain PPD134/91. Blast-conserved domain analysis of this sequence showed that VgrG (GI:60328264) corresponded to a protein
without the COOH-terminal extension, and that VsdC (GI:60328266) has a VIP-2 motif.

Alignment of a similar sequence found in *A. hydrophila* ATCC 7966 with that of *A. hydrophila*
PPD134/91 showed high identity/homology with two open reading frames corresponding to
VgrG1 (GI:117619461) and a type III secretion-related protein (GI:117621298). Although vgrG
and vsdC represented two, independent, open reading frames in *A. hydrophila* strain PPD134/91,
a similar sequence in *A. hydrophila* ATCC 7966 was represented by only one vgrG1 gene
(GI:117619461). Likewise, in *A. hydrophila* SSU, based on our proteomics and Western blot
analysis data, it is apparent that the VIP-2 domain is linked to the VgrG core in a single open
reading frame. In contrast, the type III secretion-related protein encoding open reading frame
was present in both *A. hydrophila* ATCC 7966 and in *A. hydrophila* PPD134/91.

It is known that surface epithelial cells become apoptotic after detaching from their
underlying basement membrane by a process called anoikis (13), which is characterized by the
loss of mitochondrial membrane potential through the action of Bax/Baf that results in caspase
activation and DNA fragmentation (17, 22, 28). After depolymerization of actin filaments
induced by latrunculin-A or cytochalasin-D, Bax protein is localized in the outer mitochondrial
membrane, where it interacts and keeps open the voltage-dependent anion channels, and/or forms
oligomeric pores in the outer membrane which allow the loss of membrane potential, and the
release of both cytochrome *c* and other pro-apoptotic factors (26, 49, 52). Apoptosis induced by
VgrG1 of *A. hydrophila* could be mediated by perturbation of the mitochondrial function. Thus,
after the alteration of the actin cytoskeleton by VgrG1-ADPRT activity, the release of
cytochrome *c* could activate caspase 9 and, subsequently, caspase 3, as our results indicated.

Gastrointestinal infections with *A. hydrophila* induce severe diarrhea, and we previously
reported the participation of three different enterotoxins produced by *A. hydrophila* SSU as
players in inducing bloody and non-bloody diarrhea (4, 40). The studies described in this paper have introduced another possible player, namely VgrG1 which, by actin-ADPRT activity, could trigger actin depolymerization with fatal effects on the intestinal epithelial barrier, thus allowing entry of other virulence factors associated with *A. hydrophila* SSU pathogenesis. Our future studies are targeted at developing *vgrG1* or *vgrG2/3* null mutants, as well as at deleting the *ascV* gene from the ∆*act*∆*vasH* mutant of *A. hydrophila* SSU so that the contribution of T3- and T6-secretion system effectors on host cell toxicity could be fully investigated.
ACKNOWLEDGEMENTS

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 acknowledge Biomolecular Resource Facility Cores at UTMB for providing their expertise in performing 2-D gel electrophoresis image analysis, and mass spectrometry analysis. We thank Mark Griffin for providing his proficiency in the flow cytometry analysis. We thank Ms. Mardelle Susman for editorial assistance.


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FIGURES LEGENDS

Figure 1: Identification of proteins secreted via T6SS in the supernatant of *A. hydrophila* SSU.

A. Comparison of 2-D gels containing proteins from the supernatants of the *A. hydrophila* SSU ∆act mutant (left) and the ∆act/∆vasH mutant strain (right). Highlighted spots (in green) represent a cluster of proteins secreted via the T6SS, which were identified by mass spectrometric analysis. B. Alignment of VgrG1 from *A. hydrophila* ATCC 7966 (gil117619461) with peptides identified via mass spectrometry (red) on one of the secreted proteins (VgrG1) from *A. hydrophila* SSU. Bold-underlined sequence represents VIP-2 domain. C. Western blot analysis on pellet and supernatant fractions of *A. hydrophila* SSU ∆act (lanes 1 and 3) and ∆act/∆vasH (lanes 2 and 4) mutant strains using specific antibodies to the VIP-2 domain of VgrG1 in combination with specific antibodies against VgrG2.

Figure 2: VgrG1 has ADP-ribosyltransferase (ADPRT) activity which is associated with the presence of VIP-2 domain. ADPRT assays were performed by using 6-Biotin-17-NAD, purified rVgrGs proteins, and HeLa cell lysates (A) or recombinant non-muscle actin (B) as a source of target protein. The reaction mixtures were separated on SDS-12% PAGE, electro-blotted to nitrocellulose membranes, and the incorporation of Biotin-ADP by target protein was detected by using streptavidin-HRP. The nature of samples loaded in each lane of the gel is depicted in a table underneath panel B.

Figure 3: A. Induction of HeLa cell-rounded phenotype in co-cultures with different strains of *A. hydrophila* SSU. HeLa cells were co-cultured for 90 min with *A. hydrophila* SSU ∆act mutant (column 1) and ∆act/∆vasH mutant (column 2), as well as uninfected HeLa cells (column 3), in
direct bacterial-host contact (panel II) or by using trans-well inserts (panel III). Supernatants from co-cultures in direct cell-to-cell contact were collected after 90 min, and used as pre-conditioned media on fresh HeLa cells cultures (panel IV). Initial morphology of HeLa cells at 0 min is shown in panel I and non-infected HeLa cells (control) in column 3. Magnification 40X.

B. Quantification of G- and F-actin by Western Blot and densitometric analyses. HeLa cells in direct contact with different strains of *A. hydrophila* SSU were harvested after 90 min of co-culture. Cells were lysed and processed as is indicated in the materials and methods section. The bar graph represents data on percentages of G- and F-actin of HeLa cells infected with different mutant strains from three independent experiments, and the Western blot image is representative of all of them. * indicates statistically significant difference.

**Figure 4.** Translocation of VgrG1 into HeLa cell cytoplasm. HeLa cells were infected with *A. hydrophila* SSU ∆*act* or ∆*act*/∆*vasH* mutant strain expressing and producing full-length VgrG1::Bla (referred to as VgrG1::Bla) or VgrG1-NH2::Bla. As a control, HeLa cells were infected with bacteria containing the empty vector. A. Flow cytometric density plots showing disruption of CCF4 FRET (from green to blue) due to translocation of Bla into HeLa cell cytoplasm infected with *A. hydrophila* ∆*act* parental strain (panel II and III) compared to host cells infected with *A. hydrophila* SSU containing the empty vector (panels I and IV). HeLa cells infected with *A. hydrophila* ∆*act*/∆*vasH* mutant expressing and producing the fusion proteins were not able to translocate Bla and did not disrupt the CCF4 FRET (panels V and VI). For analysis, 2 x 10^5 HeLa cells were acquired and gated in side forward/side scatter to avoid aggregates. B. Fold increase in percentage of blue HeLa cells against percentage of green HeLa cells (after infection with *A. hydrophila* SSU ∆*act* and ∆*act*/∆*vasH* mutant strains) compared to
percentage of HeLa cells infected with bacteria carrying the empty vector. The graph shows data from a representative experiment.

Figure 5: A. Western blot analysis of HeLa Tet-off cell lysates expressing and producing different fragments of VgrG1. The various forms of VgrG1 were detected by using two types of sera. We used sera from mice immunized with rVgrG2 of *A. hydrophila* SSU which cross-reacted with the NH$_2$-terminal portion of VgrG1 (left panel) and from mice immunized with the rVIP-2 domain of VgrG1 of *A. hydrophila* ATCC 7966 which recognized only VgrG1 and its COOH-terminal domain. The samples loaded in each of the lanes 1-8 are depicted on the top. B. Morphological changes of HeLa Tet-off cells induced by the expression of different VgrG1-encoding fragments of *A. hydrophila* ATCC 7966. Host cells were stained for actin-cytoskeleton by using Alexa fluor 568-phalloidin (red), and expression of enhanced fluorescent green protein (EGFP) encoding gene was detected in cells successfully transfected with the pBI-EGFP vector alone (I) or by those containing genes encoding an NH$_2$-terminal (II), full-length (III), and COOH-terminal (IV) fragments of VgrG1. Magnification 40X. C. Quantification of actin-cytoskeleton (F-actin) as measured by fluorescent phalloidin staining of HeLa Tet-off expressing and producing different VgrG1 fragments. Flow cytometry dot plots showing HeLa Tet-off cells stained with Alexa fluor 568-phalloidin and expressing different encoding fragments of VgrG1. The analysis was performed on EGFP-positives cells. Percentage of positives cells from a representative experiment (72 h) is shown in the plotted quadrant (Left panel), and mean fluorescent intensity values (MFI) from three different assays at 24 h and 72 h were potted (Right panel). Statistical differences at 24 h (p<0.01) and 72 h (p<0.001) were noted between cells expressing vector alone (pBI-EGFP) versus cells expressing and producing full-length
VgrG1 and the COOH-terminal fragment and between cells expressing and producing NH$_2$-terminal fragment versus cells expressing full-length VgrG1 and COOH-terminal fragment. 

Quantification of F-actin and G-actin present in HeLa Tet-off cells expressing and producing different VgrG1 fragments. The percentages of F- and G-actin per sample (30 µL) were analyzed by Western blot analysis and by using antibodies to actin followed by densitometric scanning of the blots. A densitometric quantification from three different assays was plotted and a representative Western blot image is shown. Statistical differences at 24 h (p<0.001) were noted between fractions containing F- and G-actin in HeLa cells expressing full-length VgrG1 and the COOH-terminal encoding fragments. Asterisks indicate statistically significant differences. The designation ter refers to NH$_2$- or COOH-terminal domains.

**Figure 6:** Viability of HeLa Tet-off cells expressing genes encoding different fragments of VgrG1 from *A. hydrophila* ATCC 7966. Percentage of dead and/or dying cells were quantified by incorporation of 7-AAD and flow cytometry on HeLa Tet-off cells expressing vector alone (pBI-EGFP) or producing different fragments of VgrG1 (NH$_2$- and COOH-terminal, and full-length) after 24 h and 72 h of transfection. Results from three different assays were plotted, and statistical significance is indicated by upper horizontal lines.

**Figure 7:** Apoptosis of HeLa Tet-off cells expressing genes encoding different fragments of VgrG1. Apoptosis rates were measured by quantification of (A) cytoplasmic nucleosomes and (B) caspase 3 and caspase 9 activity in lysates of HeLa Tet-off cells expressing vector alone (pBI-EGFP), or expressing genes encoding NH$_2$- and COOH-terminal fragments, and the full-length VgrG1 from *A. hydrophila* ATCC 7966. Results from three different assays were plotted.
and statistical significance ($p<0.001$) is indicated by upper horizontal lines. The designation ter
refers to NH$_2$- or COOH- terminal domains.

**SUPPLEMENTARY FIGURES**

**Figure I:** Alignment of the VIP-2 reference sequence (cd00233) with the sequence present in the COOH-terminal fragment of *A. hydrophila* ATCC 7966 VgrG1. In red are conserved amino-acid residues. CD Length: 201; Bit Score: 62.27; E-value: 5e-10 (taken from BLAST-Conserved Domains report). Amino acid residues in open boxes constitute a conserved Ser-Thr-Ser motif, which plays an important role in catalysis and structure as well as in NAD binding. Underlined sequences denote ADP-ribosylating toxin turn-turn motif, and an orange arrow shows conformational flexibility of ligand binding pocket.

**Figure II:** Schematic representation of the conserved domains present in VgrG1. **A.** *A. hydrophila* ATCC 7966 (gi|117619461) and **B.** *V. cholerae* N16961 (gi|15641427). The gp27- and gp5-like motifs are represented by red and blue lines, respectively (modified from Pukatzki, et al., 2007) (34). The encoding fragments representing the NH$_2$-terminal (gray), COOH-terminal (cyan) and the full-length (gray and cyan) of VgrG1 from *A. hydrophila* ATCC 7966 were cloned into a pET-30a vector to produce recombinant proteins, and into pB1-EGFP vector for expression in the HeLa Tet-off cells. The positions of VIP-2 and RtxA domains in VgrG1 of *A. hydrophila* and *V. cholerae* are also shown.

**Figure III:** A. Induction of HeLa cell-rounded phenotype in co-cultures with different strains of *A. hydrophila* SSU. HeLa cells were co-cultured in direct bacterial-host cell contact for 90 min...
# Table 1. Bacterial strains and vectors used in this study.

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<th>Strain and Plasmids</th>
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<tr>
<td><strong>A. hydrophila</strong></td>
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<tr>
<td>SSU</td>
<td>Diarrheal isolate of <em>A. hydrophila</em></td>
<td>CDC&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>Rrifampin (Rrif) Strain.</td>
<td>Lab Stock</td>
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<td>This study</td>
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<td>Isogenic vasH gene mutant of SSU-R Δact expressing the vgrG1-NH&lt;sub&gt;2&lt;/sub&gt;::bla gene in pGEN222, Rif&lt;sup&gt;b&lt;/sup&gt;, Km&lt;sup&gt;c&lt;/sup&gt;, Sm&lt;sup&gt;d&lt;/sup&gt;/Sp&lt;sup&gt;e&lt;/sup&gt;, Ap&lt;sup&gt;f&lt;/sup&gt;.</td>
<td>This study</td>
</tr>
<tr>
<td>ATCC 7966</td>
<td>Environmental isolated strain</td>
<td>ATCC&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>Production of recombinant plasmids, recA, gyrA.</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>HMS174-DE3</td>
<td>Production of recombinant proteins cloned in pET-30a. Carries a T7 RNA polymerase copy under the control of lacUV5 promoter.</td>
<td>Novagen</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pET-30a</td>
<td>pBR322-derived expression vector with T7 lac promoter. Expression and production of recombinant proteins with 6XHis tag. Km&lt;sup&gt;c&lt;/sup&gt;.</td>
<td>Novagen</td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;, Tc&lt;sup&gt;f&lt;/sup&gt;.</td>
<td>GE Healthcare</td>
</tr>
<tr>
<td>pGEN222</td>
<td>Ap&lt;sup&gt;f&lt;/sup&gt;.</td>
<td>(11)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Centers for Disease Control and Prevention.

<sup>b</sup> American Type Culture Collection

Compl: Complemented
Table 2. Primer sequences used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>vgrG1-Full-MluI</td>
<td>5’-TCATCCGCCTATGGCAGACAGCAGAGG-3’</td>
<td>PCR amplification of full-length vgrG1 encoding region for cloning in pBI-EGFP</td>
</tr>
<tr>
<td>vgrG1-Full-NheI</td>
<td>5’-GGTCACTAGCTTAAATACCGAAGACCTC-3’</td>
<td>PCR amplification of full-length vgrG1 encoding region for cloning in pBI-EGFP</td>
</tr>
<tr>
<td>vgrG1-NH2-MluI</td>
<td>5’-TCATCCGCCTATGGCAGACAGCAGAGG-3’</td>
<td>PCR amplification of vgrG1-NH2-ter encoding region for cloning in pBI-EGFP</td>
</tr>
<tr>
<td>vgrG1-NH2-ter-NheI</td>
<td>5’-GGTCACTAGCTTAAATACCGAAGACCTC-3’</td>
<td>PCR amplification of vgrG1-NH2-ter encoding region for cloning in pBI-EGFP</td>
</tr>
<tr>
<td>vgrG1-COOH-ter-AflII</td>
<td>5’-TCATCCGCCTATGGCAGACAGCAGAGG-3’</td>
<td>PCR amplification of vgrG1-COOH-ter encoding region for cloning in pBI-EGFP</td>
</tr>
<tr>
<td>vgrG1-COOH-ter-XhoI</td>
<td>5’-GGTCACTAGCTTAAATACCGAAGACCTC-3’</td>
<td>PCR amplification of vgrG1-COOH-ter encoding region for cloning in pBI-EGFP</td>
</tr>
<tr>
<td>vgrG1-Full-BglII</td>
<td>5’-TCATCCGCCTATGGCAGACAGCAGAGG-3’</td>
<td>PCR amplification of full-length vgrG1 encoding region for cloning in pET-30a</td>
</tr>
<tr>
<td>vgrG1-Full-XhoI</td>
<td>5’-GGTCACTAGCTTAAATACCGAAGACCTC-3’</td>
<td>PCR amplification of full-length vgrG1 encoding region for cloning in pET-30a</td>
</tr>
<tr>
<td>vgrG1-NH2-ter-BglII</td>
<td>5’-TCATCCGCCTATGGCAGACAGCAGAGG-3’</td>
<td>PCR amplification of vgrG1-NH2-ter encoding region for cloning in pET-30a</td>
</tr>
<tr>
<td>vgrG1-NH2-ter-XhoI</td>
<td>5’-GGTCACTAGCTTAAATACCGAAGACCTC-3’</td>
<td>PCR amplification of vgrG1-NH2-ter encoding region for cloning in pET-30a</td>
</tr>
<tr>
<td>vgrG1-COOH-ter-BglII</td>
<td>5’-TCATCCGCCTATGGCAGACAGCAGAGG-3’</td>
<td>PCR amplification of vgrG1-COOH-ter encoding region for cloning in pET-30a</td>
</tr>
<tr>
<td>vgrG1-COOH-ter-XhoI</td>
<td>5’-GGTCACTAGCTTAAATACCGAAGACCTC-3’</td>
<td>PCR amplification of vgrG1-COOH-ter encoding region for cloning in pET-30a</td>
</tr>
<tr>
<td>vgrG1-MluI</td>
<td>5’-TCATCCGCCTATGGCAGACAGCAGAGG-3’</td>
<td>PCR amplification of full-length vgrG1 encoding region for cloning in pET-30a</td>
</tr>
<tr>
<td>vgrG1-NheI</td>
<td>5’-GGTCACTAGCTTAAATACCGAAGACCTC-3’</td>
<td>PCR amplification of full-length vgrG1 encoding region for cloning in pET-30a</td>
</tr>
<tr>
<td>vgrG1-BglII</td>
<td>5’-TCATCCGCCTATGGCAGACAGCAGAGG-3’</td>
<td>PCR amplification of full-length vgrG1 encoding region for cloning in pET-30a</td>
</tr>
<tr>
<td>vgrG1-XhoI</td>
<td>5’-GGTCACTAGCTTAAATACCGAAGACCTC-3’</td>
<td>PCR amplification of full-length vgrG1 encoding region for cloning in pET-30a</td>
</tr>
<tr>
<td>vgrG1-Full-ClaI</td>
<td>5’-TCATCCGCCTATGGCAGACAGCAGAGG-3’</td>
<td>PCR amplification of full-length vgrG1 without the stop codon for cloning in pGEN222</td>
</tr>
<tr>
<td>vgrG1-Full-MluI</td>
<td>5’-GGTCACTAGCTTAAATACCGAAGACCTC-3’</td>
<td>PCR amplification of full-length vgrG1 without the stop codon for cloning in pGEN222</td>
</tr>
<tr>
<td>vgrG1-NH2-ClaI</td>
<td>5’-TCATCCGCCTATGGCAGACAGCAGAGG-3’</td>
<td>PCR amplification of vgrG1-NH2-ter without the stop codon for cloning in pGEN222</td>
</tr>
<tr>
<td>vgrG1-NH2-MluI</td>
<td>5’-GGTCACTAGCTTAAATACCGAAGACCTC-3’</td>
<td>PCR amplification of vgrG1-NH2-ter without the stop codon for cloning in pGEN222</td>
</tr>
<tr>
<td>bla-MluI</td>
<td>5’-GGTCACTAGCTTAAATACCGAAGACCTC-3’</td>
<td>PCR amplification of blaM without the signal sequence for cloning in pGEN222-vgrG1s</td>
</tr>
</tbody>
</table>

Underlined sequences represent restriction enzyme sites.
The designations NH2-ter and COOH-ter refer to NH2- and COOH-terminal domains of VgrG1.
Table 3. Mass spectrometric alignment of differentially produced proteins spots from supernatants of *A. hydrophila* SSU (Δact versus Δact/ΔvasH strains) based on 2-D gel electrophoresis.

<table>
<thead>
<tr>
<th>Spot</th>
<th>Rank</th>
<th>Protein Name</th>
<th>Protein Expectation Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>5_4804</td>
<td>1</td>
<td>VgrG-3 protein [Aeromonas hydrophila]</td>
<td>9.9763E-70</td>
</tr>
<tr>
<td>5_5573</td>
<td>1</td>
<td>VgrG-3 protein [Aeromonas hydrophila]</td>
<td>2.5059E-65</td>
</tr>
<tr>
<td>5_5562</td>
<td>1</td>
<td>VgrG-2 protein [Aeromonas hydrophila]</td>
<td>9.9763E-53</td>
</tr>
<tr>
<td>5_4798</td>
<td>1</td>
<td>VgrG-3 protein [Aeromonas hydrophila]</td>
<td>6.2986E-46</td>
</tr>
<tr>
<td>5_6426</td>
<td>1</td>
<td>VgrG-3 protein [Aeromonas hydrophila]</td>
<td>3.1547E-43</td>
</tr>
<tr>
<td>3_3026</td>
<td>1</td>
<td>putative hemolysin co-regulated protein [Aeromonas hydrophila]</td>
<td>1.9905E-35</td>
</tr>
<tr>
<td>5_4809</td>
<td>1</td>
<td>Rhs element Vgr family protein [Aeromonas hydrophila subsp. hydrophila ATCC 7966]</td>
<td>3.9716E-34</td>
</tr>
<tr>
<td>5_5996</td>
<td>1</td>
<td>VgrG-3 protein [Aeromonas hydrophila]</td>
<td>3.9716E-33</td>
</tr>
<tr>
<td>5_6503</td>
<td>1</td>
<td>putative vgrG protein [Aeromonas hydrophila]</td>
<td>9.9763E-26</td>
</tr>
<tr>
<td>5_6611</td>
<td>1</td>
<td>phosphatidylcholine-sterol acyltransferase [Aeromonas hydrophila subsp. hydrophila ATCC 7966]</td>
<td>9.6763E-20</td>
</tr>
<tr>
<td>5_6603</td>
<td>1</td>
<td>putative vgrG protein [Aeromonas hydrophila]</td>
<td>7.5294E-14</td>
</tr>
<tr>
<td>3_5936</td>
<td>1</td>
<td>Rhs element Vgr family protein [Aeromonas hydrophila subsp. hydrophila ATCC 7966]</td>
<td>1.2559E-13</td>
</tr>
<tr>
<td>5_5992</td>
<td>1</td>
<td>VgrG-3 protein [Aeromonas hydrophila]</td>
<td>9.9763E-10</td>
</tr>
<tr>
<td>5_4252</td>
<td>1</td>
<td>HcpA homolog [Aeromonas hydrophila subsp. hydrophila ATCC 7966]</td>
<td>9.9763E-09</td>
</tr>
<tr>
<td>3_6571</td>
<td>1</td>
<td>HcpA homolog [Aeromonas hydrophila subsp. hydrophila ATCC 7966]</td>
<td>1.9905E-08</td>
</tr>
<tr>
<td>5_5746</td>
<td>1</td>
<td>hypothetical protein AHA_3948 [Aeromonas hydrophila subsp. hydrophila ATCC 7966]</td>
<td>1.2559E-05</td>
</tr>
<tr>
<td>5_6471</td>
<td>1</td>
<td>inorganic pyrophosphatase [Aeromonas hydrophila subsp. hydrophila ATCC 7966]</td>
<td>0.00732446</td>
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<tr>
<td>5_4403</td>
<td>1</td>
<td>vgrG protein [Aeromonas salmonicida subsp. salmonicida A449]</td>
<td>0.01960339</td>
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<tr>
<td>5_539</td>
<td>1</td>
<td>type III restriction protein res subunit [Klebsiella pneumoniae Py2]</td>
<td>0.01960339</td>
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<tr>
<td>5_260</td>
<td>1</td>
<td>ribosomal large subunit pseudouridine synthase B [Aeromonas salmonicida subsp. salmonicida A449]</td>
<td>0.02916412</td>
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<tr>
<td>5_4807</td>
<td>1</td>
<td>transcriptional regulator. Gmr family [Mesorhizobium sp. BNC1]</td>
<td>0.19905359</td>
</tr>
<tr>
<td>3_6711</td>
<td>1</td>
<td>hypothetical protein ECA4275 [Environa catovoracia subsp. atroseptica SCR1043]</td>
<td>0.62942076</td>
</tr>
<tr>
<td>3_6570</td>
<td>1</td>
<td>hypothetical protein Acy_2197 [Acidiphilium cryptum JF-5]</td>
<td>1.255943216</td>
</tr>
<tr>
<td>5_6609</td>
<td>1</td>
<td>flagellar hook-associated 2 domain protein [Shewanella baltica OS155]</td>
<td>1.255943216</td>
</tr>
<tr>
<td>5_6215</td>
<td>1</td>
<td>hypothetical protein mrtF [Mesorhizobium loti MAFF303069]</td>
<td>1.255943216</td>
</tr>
<tr>
<td>5_4784</td>
<td>1</td>
<td>hypothetical protein RPC_0697 [Rhodopseudomonas palustris B18]</td>
<td>2.505936168</td>
</tr>
<tr>
<td>5_5536</td>
<td>1</td>
<td>regulatory protein [Oceanicola granulosus HTCC2516]</td>
<td>6.294627059</td>
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<tr>
<td>5_548</td>
<td>1</td>
<td>hypothetical protein HIO888 [Haemophilus influenzae Rd K12]</td>
<td>6.289427059</td>
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<tr>
<td>5_6707</td>
<td>1</td>
<td>peptidyl-prolyl cis-trans isomerase C [Yersinia pestis KIM]</td>
<td>6.289427059</td>
</tr>
<tr>
<td>5_6113</td>
<td>1</td>
<td>putative ABC-type multidrug transport system, ATPase component [Photobacterium profundum 3TCK]</td>
<td>7.529465962</td>
</tr>
<tr>
<td>5_6708</td>
<td>1</td>
<td>general secretion pathway protein L [Vibrio cholerae M20-3]</td>
<td>15.8113883</td>
</tr>
</tbody>
</table>

Highlighted areas represent protein spots matching with VgrG1 from *A. hydrophila* ATCC 7966