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Molecular Characterization of a Functional Type VI Secretion System from a Clinical Isolate of *Aeromonas hydrophila*

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Abstract

Our laboratory recently molecularly characterized the type II secretion system (T2SS)- associated cytotoxic enterotoxin (Act) and the T3SS-secreted AexU effector from a diarrheal isolate SSU of *Aeromonas hydrophila*. The role of these toxin proteins in the pathogenesis of *A. hydrophila* infections was subsequently delineated in *in vitro* and *in vivo* models. In this study, we characterized the new type 6 secretion system (T6SS) from isolate SSU of *A. hydrophila* and demonstrated its role in bacterial virulence. Study of the role of T6SS in bacterial virulence is in its infancy, and there are, accordingly, only limited, recent reports directed toward a better understanding its role in bacterial pathogenesis. We have provided evidence that the virulence-associated secretion (*vas*) genes *vasH* (Sigma 54-dependent transcriptional regulator) and *vasK* (encoding protein of unknown function) are essential for expression of the genes encoding the T6SS and/or they constituted important components of the T6SS. Deletion of the *vasH* gene prevented expression of the potential translocon hemolysin coregulated protein (Hcp) encoding gene from bacteria, while the *vasK* gene deletion prevented secretion but not translocation of Hcp into host cells. The secretion of Hcp was independent of the T3SS and the flagellar system. We demonstrated that secreted Hcp could bind to the murine RAW 264.7 macrophages from outside, in addition to its ability to be translocated into host cells. Further, the *vasH* and *vasK* mutants were less toxic to murine macrophages and human epithelial HeLa cells, and these mutants were more efficiently phagocytosed by macrophages. We also provided evidence that the expression of the *hcp* gene in the HeLa cell resulted in apoptosis of the host cells. Finally, the *vasH* and *vasK* mutants of *A. hydrophila* were less virulent in a septicemic mouse model of infection, and animals immunized with recombinant Hcp were protected from subsequent challenge with the wild-type (WT) bacterium. In addition, mice infected with the WT *A. hydrophila* had circulating antibodies to Hcp, indicating an important role of T6SS in the pathogenesis of *A. hydrophila* infections. Taken together, we have characterized the T6SS from *Aeromonas* for the first time and provided new features of this secretion system not yet known for other pathogens.

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Keywords

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1. Introduction

The genus *Aeromonas*, which is comprised of 17 species (spp.), was recently placed into its own family, namely the *Aeromonadaceae* [1]. These Gram- negative bacteria inhabit freshwater sources and produce a wide range of virulence factors, including surface molecules [2,3]; extracellular enzymes [4–7]; adhesins, and various toxins [8].

Among the different species of *Aeromonas*, *A. hydrophila* is most commonly associated with a wide variety of human diseases, which include skin and wound infections and septicemia, that are often fatal [8–10]. Although *Aeromonas* spp. lead to gastroenteritis in young, elderly, or immunocompromised individuals [11], numerous cases of intestinal and extraintestinal infections in immunocompetent individuals have led to the suggestion that the virulence of this pathogen is not entirely dependent upon the immune status of the host [12]. As in the case of other forms of bacterial gastroenteritis, underlying factors such as liver and gastrointestinal diseases, as well as recent therapy with antimicrobials ineffective against aeromonads have been reported as relevant for the development of *Aeromonas*-associated diseases [13].

Evidence of the pathogenicity of *Aeromonas* spp. was recently noted in southern Thailand tsunami survivors, as 22% of all wounds in these patients were infected with this bacterium [14]. Furthermore, the floodwater samples collected after hurricane Katrina in New Orleans had elevated numbers of a variety of *Aeromonas* spp. [15]. In addition, the worldwide isolation rate of *Aeromonas* from diarrheic stool has been reported to be as high as 10.8%, compared to only 2.1% from the stools of healthy control subjects [16]. In a separate study, although it was noted that in the majority of the patients, only the small intestine seemed to be affected by this pathogen; up to one-third of the infected patient population also showed colitis by endoscopy [17,18]. It has been documented that *A. hydrophila* is present in a wide variety of foods (introduced from water, animal feces containing organisms, or food handlers), and, thereby, it has the potential to be a significant food-borne pathogen and hence represents a serious public health concern [16]. With the high resistance of this organism to both water chlorination and multiple antibiotics [19], *A. hydrophila* has been categorized as an emerging human pathogen, and consequently, it has been placed on the Environmental Protection Agency's (EPA) "Contaminant Candidate List" [20].

Our laboratory characterized two of the most potent virulence factors from a diarrheal isolate SSU of *A. hydrophila*, namely Act (*Aeromonas* cytotoxic enterotoxin) and a type III secretion system (T3SS) secreted effector protein, AexU [21,22]. Act is secreted by the T2SS and possesses several biological activities, including its ability to lyse erythrocytes, inhibit phagocytosis by professional phagocytes, induce cytotoxicity in eukaryotic cells, and to evoke fluid secretory responses in the ligated ileal loops of animals [22,23]. At sub-lethal doses, Act induces the production of pro-inflammatory cytokines, prostaglandins, and reactive oxygen species (ROS) from murine and human macrophages and human colonic epithelial cells by activating various kinase pathways [8,9,22,24–28]. In addition, Act leads to mouse mortality when injected by the intravenous route with an LD_{50} dose of 27.5 ng [25].

AexU, on the other hand, leads to ADP-ribosylation of host cell proteins and actin reorganization resulting in HeLa cell rounding phenotype and eventual cell death *via* apoptosis [29]. AexU also inhibits phagocytosis, as AexU null mutant was phagocytosed more efficently

by murine RAW 264.7 macrophages [21]. Likewise, both *Δact* and *ΔaexU* isogenic mutants caused less mortality (40–60%) in mice when injected *via* the intraperitoneal (i.p.) route, with Δ*act* and Δ*aopB* (*Aeromonas* outer membrane protein B; an essential component of the T3SS) double knockout mutant causing only 10–20% mouse mortality in a septicemic model of *A. hydrophila* infection [21,30]. Although our recent studies suggested that AexU is most likely the T3SS effector that leads to cell toxicity [21], we noted that the Δ*act*/Δ*aexU* mutant of *A. hydrophila* SSU still caused the release of lactate dehydrogenase (LDH) enzyme from murine RAW 264.7 macrophages, albeit at a significantly lower level compared to that in the Δ*act* background strain of the wild-type (WT) *A. hydrophila* for up to 5.5 hr of infection. Importantly after 6 hr of infection of macrophages with the Δ*act*/Δ*aexU* mutant and the corresponding WT (parental) *A. hydrophila* SSU, no difference in LDH release was observed [21]. These data suggested the presence of some as yet unknown factor(s) that contributed to this cell toxicity associated with the Δ*act*/Δ*aexU* mutant of *A. hydrophila*.

Protein secretion in Gram-negative bacteria presents a challenge because the secreted proteins must pass through at least two membranes before they can reach the extracellular milieu. To date, five secretion systems have been molecularly well-characterized in Gram-negative bacteria. These systems are highly conserved across different bacteria with unique characteristics which permit their differentiation from one another [31]. Recently, a new protein secretion mechanism called virulence-associated secretion (VAS), or type VI secretion system (T6SS), was described in *Vibrio cholerae* [32]. Previously, a gene cluster encompassing the T6SS was identified by bioinformatics analysis as being highly conserved among several gram-negative pathogens, and yet it exhibited differences in its organization in various bacteria [33]. It is believed that the primary function of the T6SS is to mediate the extracellular export of virulence factors [32]. This mechanism of secretion is different from T3- and T4- secretion systems because the T6SS represents an assembly of genes with a novel linkage that secretes proteins lacking the classical Sec-dependent signal sequences [34]. Several studies indicated participation of this cluster in the pathogenicity of different bacteria, such as *Pseudomonas aeruginosa* in which the role of T6SS-associated effector, hemolysin co-regulated protein (Hcp), has been demonstrated in cystic fibrosis [35]. Likewise, in *Francisella tularensis* and *Salmonella enterica*, these gene clusters are necessary for intracellular growth in eukaryotic cells [36,37]. In *Burkholderia mallei*, it was found that the T6SS is required for virulence in the hamster model of glanders infection [38]. Most important, however, is that the role of this secretion system in the virulence of bacterial pathogens in general is still largely unknown.

In this paper, we report, for the first time, the presence of a functional T6SS gene cluster in a clinical isolate SSU of *A. hydrophila*. Our characterization of the T6SS showed that this cluster is able to secrete and translocate effector proteins into eukaryotic host cells and that mice immunized with a secreted component of this system (Hcp) were protected from a lethal challenge dose of the WT bacterium. Thus, the components of the T6SS in *A. hydrophila* SSU constitute exciting candidates for the potential development of preventative or therapeutic vaccines, as well as targets for antimicrobial drug development.

2. RESULTS

2.1. Type VI secretion system gene cluster is present in *Aeromonas hydrophila* SSU

DNA sequence analysis demonstrated the presence of a T6SS gene cluster in the genome of diarrheal isolate SSU of *A. hydrophila* (DQ667172) (Fig. 1A). We first noted the possible presence of the T6SS in this isolate by polymerase chain reaction (PCR)-amplification of the portion of *vasH*-, *vasK*-, and *hcp*-encoding genes based on the sequence of *V. cholerae* T6SS [32]. Our subsequent genome sequencing and annotation of the sequence from *A. hydrophila* ATCC 7966 [39] further provided evidence for the presence of T6SS in *A. hydrophila* SSU. We then designed primers based on the sequences of the corresponding T6SS gene cluster from

A. hydrophila ATCC 7966 and *V. cholerae* to cover the entire T6SS gene cluster in isolate SSU of *A. hydrophila.* Most interesting are our findings that although the T6SS cluster is present in both *A. hydrophila* SSU and ATCC 7966 strains, the T3SS encoding genes are found only in the clinical isolate SSU, but not in the environmental isolate ATCC 7966 [39].

This T6SS gene cluster in *A. hydrophila* SSU contained 20 open reading frames (ORFs), out of which 18 genes had high identity (>80%) with genes present in a similar cluster that was identified in *A. hydrophila* ATCC 7966 [39]. We also noted that 10 ORFs had considerable identity (>50%) with genes present in the *V. cholerae* N16961 strain T6SS gene cluster (Fig. 1A), with the remaining 10 genes showing significant diversity in the sequence. Table 1 depicts the list of genes linked to the T6SS gene cluster present in both *A. hydrophila* strains (SSU and ATCC 7966) and in *V. cholerae*, the identity/homology between common genes/proteins, and the gene product description based on *A. hydrophila* SSU conserved domain analysis.

Previous studies in several Gram-negative bacteria have demonstrated the secretion of effector proteins *via* the T6SS [32,35,38,40]. For example, *V. cholerae* secretes four proteins *via* the T6SS; one is a homolog of hemolysin-coregulated protein (Hcp) and the other three are members of the Vgr family of proteins [32]. *A. hydrophila* SSU T6SS gene cluster contains ORFs with a high identity to *hcp* (ORF 1, 71% identity,) as well as to two members of the *vgr* family (COG3501), VgrG2 (ORF 2; 51% identity with VCA0018) and VgrG3 (ORF 20; 48% identity with VCA0123) from *V. cholerae* (Table 1).

The *clpB* gene (VCA0116 [ORF 13]), present in the T6SS gene cluster of *A. hydrophila* SSU, is a member of the AAA+ (ATPase associated with diverse cellular activities) protein family and is a chaperon protein associated with thermotolerance and translocation of aggregated proteins in an energy-dependent manner [41]. Other genes in the T6SS gene cluster of *A. hydrophila* include homologs of the genes in *V. cholerae*, such as *vasA, vasF, vasK* and *vasH* (ORFs 7, 12, 17 and 14, respectively).

Recently, the function of 3 genes present in the *P. aeruginosa* T6SS gene cluster was described. These genes include *ppkA,* which has kinase activity; *pppA,* which has phosphatase activity; and *fha1,* a scaffold protein with a forkhead-associated (FHA) domain [42]. The T6SS gene cluster of *A. hydrophila* SSU has an ORF which contains an FHA domain (ORF 9) and could have a function similar to that of its homolog in *P. aeruginosa*. However, neither significant similarities nor kinase- or phosphatase-conserved domains were found after sequence alignments of *ppkA* and *pppA* genes from *P. aeruginosa*, with any of the 20 genes found in *A. hydrophila* SSU T6SS gene cluster (Table 1).

Other members of this T6SS gene cluster in *A. hydrophila* SSU include two ImpA N-terminal-related domain proteins (ORFs 16 and 18 [COG3515]) which could be associated with the export of proteins [43]; a putative lipoprotein (ORF 10 [COG3521]), and a PAAR motif protein (ORF 19 [COG4104]).

2.2. Hcp is secreted and translocated into human colonic epithelial cells *via* the T6SS in *A. hydrophila* SSU

Previous reports have shown that inactivation of the *vasK* gene in *V. cholerae* or its homologs in other bacteria blocks secretion of Hcp or other proteins associated with the T6SS gene cluster [32,35,40]. In a similar way, *vasH*, a sigma-54 dependent transcriptional regulator, in *V. cholerae* controls the expression of the *hcp* gene [32]. Mass spectrometry analysis, after 2-dimensional (2D)-gel electrophoresis, of culture supernatants from Luria-Bertani (LB)-grown WT *A. hydrophila* SSU revealed the presence of a protein which is highly homologous to Hcp from *V. cholerae* (Fig. 1B). These findings indicated the functionality of the T6SS gene cluster

of *A. hydrophila* SSU under normal culture conditions, which consisted of overnight incubation in LB medium at 37°C with shaking (150 rpm).

Based on above-mentioned studies, we decided to generate deletion mutants for *vasH* and *vasK* genes in *A. hydrophila* SSU to evaluate the effects of these mutations on the expression and secretion of Hcp. For these experiments, HT-29 human colonic epithelial cells were infected with the WT *A. hydrophila* SSU and its corresponding mutants, and Hcp was detected in various cellular fractions by Western blot analysis using Hcp-specific antibodies. As shown in Fig. 2A, deletion of the *vasH* gene abrogated the expression and secretion of Hcp (lane 4), whereas deletion of the *vasK* gene eliminated the secretion without affecting the expression and translocation of Hcp (lane 6). After complementation of both of these genes, *vasH* and *vasK*, in their respective mutants, the secretion and expression was restored, although the secretion was not fully complemented (lanes 3 versus 5 and 7). We demonstrated complementation of these mutants *in vivo* as well (section 2.7) indicating no potential polar effects of the mutation.

Since it has been reported that cytotoxicity induced by *V. cholerae* in murine J774.1 macrophages requires direct cell-cell contact, and deletion of the *hcp* gene abrogates these morphological changes [32], we decided to evaluate the ability of *A. hydrophila* SSU WT and its various mutants to translocate Hcp into HT-29 cells. The above-mentioned bacteria were co-cultured with HT-29 cells, and, after 2 hr of infection, the supernatants were removed, and the cells were fractionated to obtain cytoplasmic proteins, eukaryotic host membrane proteins and the remaining pellet containing the bacterial cells [21]. To ensure that there was no cross-contamination of various cellular fractions, each one of the fractions was tested for the presence of actin, calnexin and DnaK as markers for host cytoplasmic proteins, host membrane proteins and bacterial intactness, respectively. We were able to detect the translocation of Hcp into the host cell cytoplasm by Western blot analysis of the HT-29 cells after infection with WT *A. hydrophila* SSU (Fig. 2A, lane 2). However, as expected, no translocated Hcp was seen in HT-29 cells infected with the Δ*vasH* strain (lane 4). In addition, although the Δ*vasK* mutant was unable to secrete Hcp into the extracellular media, translocation of Hcp was not affected (lane 6). We did not observe any cross-contamination of cellular fractions, and the bacterial integrity was intact, when antibodies to actin, calnexin, and DnaK were used for Western blot analysis (Fig. 2A)

2.3. T6SS in *A. hydrophila* SSU is independent of the Type III Secretion System (T3SS) and the flagellar system

The WT *A. hydrophila* SSU harbors different secretion systems, including the T3SS [30] and the flagellar system [44,45], which are involved in the secretion as well as in the direct translocation of proteins into host eukaryotic cells. Therefore, we decided to test if shutting down these secretion systems would impact the secretion of Hcp. For these experiments, cellular fractions of HT-29 cells after infection with WT *A. hydrophila* SSU and its mutants for the T3SS (Δ*ascV*) and for the flagellar system (Δ*flhA*) were used. The Δ*ascV* mutant is able to express the *aexU* gene (encoding a T3SS effector protein) but unable to secrete the expressed protein [21], and the *flhA* gene codes for a protein that is believed to be part of the export apparatus for lateral flagellar assembly, as the *flhA* mutant of *A. hydrophila* showed reduced adherence and biofilm formation [46].

Our data indicated that translocation and secretion of Hcp was not affected in the Δ*ascV* and Δ*flhA* mutants, indicating that, indeed, the T6SS is independent of the T3SS and the flagellar secretion system in mediating the secretion and translocation of Hcp (Fig. 2A, lanes 8 and 9). Furthermore, translocation of AexU (T3SS effector) was not affected in the Δ*vasK* and Δ*vasH* mutants, as shown in Fig. 2B (lanes 3 and 4). As positive controls, we infected HT-29 cells with Δ*aexU* and Δ*ascV* mutants, and no translocation of AexU was noted (lanes 2 and 5).

As expected, we noted the expression of the *aexU* gene in WT, *vasH*, *vasK*, and *ascV* mutants of *A. hydrophila* SSU (pellet fraction, lanes 1, 3, 4, and 5).

2.4. Binding of secreted Hcp to murine macrophages

Previous studies in *P. aeruginosa* indicated the ability of Hcp to form hexameric rings after secretion, and the findings suggested that these rings could be inserted into the eukaryotic membrane as a part of the "translocon" [35]. Since Hcp was detected in the culture supernatant of WT *A. hydrophila* SSU in abundant amounts (Fig. 1B), we decided to confirm if secreted Hcp was able to bind the cell membrane of RAW 264.7 murine macrophages to possibly initiate cell signaling from outside, in addition to the ability of Hcp to affect host cell signaling as a result of a translocated effector. For this experiment, a double mutant, Δ*act*/Δ*vasH*, was generated to avoid the cytotoxic effects induced by Act present in the culture supernatants. Culture supernatants of *A. hydrophila* SSU Δ*act* and Δ*act*/Δ*vasH* mutants were collected after 2 hr of inoculation, filtered, and added to RAW 264.7 cells for 2 hr. After washing the host cells, cytoplasmic and membrane fractions were obtained. As shown in Fig. 2C, Western blot analysis demonstrated that Hcp present in the supernatant of the Δ*act A. hydrophila* SSU mutant was able to bind to the cell membrane of RAW 264.7 cells (lane 4). In contrast, Hcp was not detected in the cytoplasmic fraction of these cells (lane 3), indicating no cross-contamination of the cytosolic and membrane fractions of the host cells. As expected, we did not observe Hcp binding in uninfected RAW 264.7 cells (lanes 1 and 2) and in host cells infected with the Δ*act*/Δ*vasH* mutant (lanes 5 and 6). The latter mutant is unable to express the *hcp* gene. Lanes 7–9 show the presence or absence of Hcp in the culture medium of Δ*act* and Δ*act*/Δ*vasH* mutants of *A. hydrophila* SSU. Similar assays were performed using recombinant Hcp (rHcp) obtaining essentially the same results (data not shown).

2.5. *A. hydrophila* T6SS inhibits phagocytic activity and mediates cytotoxicity

Since infection by *A. hydrophila* SSU is not intracellular, and mutations in the *vasH* and *vasK* genes are able to alter expression and secretion of proteins (e.g., Hcp) associated with the T6SS, we decided to evaluate the effects on phagocytosis caused by mutation in those two genes, namely *vasH* and *vasK* of *A. hydrophila* SSU.

To test for phagocytic activity, RAW 264.7 macrophages were infected with WT *A. hydrophila* SSU and Δ*vasH* and Δ*vasK* mutants, and the intracellular colony forming units (cfu) were estimated as an indicator of phagocytosis. As noted from Fig. 3A, phagocytosis was significantly increased in RAW 264.7 macrophages infected with Δ*vasH* and Δ*vasK* mutants when compared to host cells infected with the WT *A. hydrophila*.

The cytotoxic effect of *A. hydrophila* SSU Δ*vasH* and Δ*vasK* mutants was then tested in RAW 264.7 macrophages and HeLa cells by measuring the release of lactate dehydrogenase (LDH) enzyme. RAW 264.7 and HeLa cells were incubated with either the *A. hydrophila* mutant deleted for the *act* gene (as a control) or the Δ*vasH* and Δ*vasK* mutants at a multiplicity of infection (MOI) of 0.5 for different time points, and supernatants were collected for measuring the LDH release. Significant differences in cytotoxicity between the Δ*act* and Δ*act*/Δ*vasK* or Δ*act*/Δ*vasH* strains were detected in RAW 264.7 and HeLa cells after 3–4.5 hr of infection (Fig. 3B). Although the Δ*act* background strain was used in this experiment as a control to remove the strong cytotoxic effects associated with this protein, five hours after infection, the percentage of cytotoxicity induced by the different bacterial mutant strains was similar to that of the control strain (Δ*act* of *A. hydrophila* SSU) as a consequence of other virulence factors produced (i.e., AexU). These data indicated that mutations in the *vasH* and *vasK* genes were able to alter the biological effects associated with the T6SS of *A. hydrophila*. Although differences in cytotoxicity induced by the parental versus mutant strains may appear small, we

believe they are biologically meaningful, as there are other *A. hydrophila* virulence factors that also lead to cell toxicity.

2.6. Expression of the *hcp* gene in HeLa Tet-Off cells and induction of apoptosis

Since we showed that Hcp is translocated into eukaryotic cells, we decided to express the *hcp* gene into HeLa cells from pBI-EGFP vector using the HeLa cell Tet-Off system. HeLa Tet-Off cells were transfected by electroporation with the pBI-EGFP vector containing the *hcp* gene (519 bp), and the transfection efficiency was measured by flow cytometry as the percentage of EGFP (enhanced green fluorescent protein)-positive cells. Expression and production of Hcp were evaluated by Western blot and flow cytometry analyses. A band of ~20 kDa was observed by Western blot analysis in the HeLa Tet-Off cells transfected with pBI-EGFP-*hcp* plasmid (Fig. 4A, panel I). The lower band probably represented a degradation product of Hcp. These results were confirmed by flow cytometry by performing intracellular staining of cells transfected with pBI-EGFP-*hcp* plasmid (Fig. 4A, panel II). As negative controls, we used host cells transfected with pBI-EGFP vector alone and isotype antibody.

We then evaluated cytotoxicity and mitochondrial activity by colorimetric MTT assays in the HeLa cells transfected with the pBI-EGFP-*hcp* plasmid, and there was no significant difference between the cells transfected with the vector alone (pBI-EGFP) and the cells transfected with the vector containing the *hcp* gene after 24 hr of transfection (data not shown). Likewise, incorporation of 7-amino actinomycin D (7-ADD), which permeates the membranes of dead and dying cells and stains their DNA, was not significantly different between vector alone and the *hcp* transfected HeLa cells after 24 hr of infection (data not shown). These studies led us to examine any apoptosis of the host cells that might be associated with Hcp.

To evaluate the apoptotic rate in the HeLa Tet-Off cells transfected with the *hcp* gene for 24 hr, we measured the cytoplasmic histone-associated DNA fragments (nucleosomes) by ELISA. As shown in Fig. 4B (panel I), cells producing Hcp had a significantly higher rate of apoptosis compared to HeLa cells transfected with the vector alone ($p<0.001$). To confirm these results, we assessed caspase 3 activity in these cells by a colorimetric activity assay. The activation of caspase 3 was significantly increased in the HeLa cells producing Hcp ($p<0.001$) when compared to cells transfected with the vector alone (Fig. 4B, panel II). We could not perform similar experiments in which host cells were treated with rHcp as most of the protein produced from *E. coli* was membrane bound, thus requiring harsh conditions for its solubilization, which resulted in a loss in biological activity.

2.7. T6SS is important for the virulence of *A. hydrophila* SSU in the mouse model

To evaluate the importance of the *hcp* gene during *in vivo* infection, we challenged mice with WT *A. hydrophila* at a sub-lethal dose, and collected the sera from the surviving mice. Subsequently, the sera were probed by Western blot analysis against the purified rHcp. As shown in Fig. 5A, the surviving mice developed specific antibodies against Hcp. As a control, sera obtained before infection was also tested. The presence of anti-Hcp specific antibodies indicated that Hcp was produced during *in vivo* infection and that it highlighted the immunogenic potential of this protein. Therefore, we decided to test the ability of Hcp to protect mice from challenge with ~3 LD_{50} (5×10^7 cfu) of WT *A. hydrophila* SSU. Mice were immunized with 10 μg of rHcp, boosted after 3 weeks, and challenged with WT bacteria. Then their survival rates were monitored for 16 days. All of the mice immunized with rHcp survived after challenge, whereas all of the control (non-immunized) animals died within 48 hr (Table 2). We used only 3 LD_{50} dose initially, as in addition to the T6SS, other toxins produced *A. hydrophila* can lead to mice killing. Therefore, it is important that other virulence-associated genes should also be deleted to develop a safer vaccine.

Finally, to evaluate the role of T6SS during infection, we infected mice *via* the i.p route with WT, as well as with the Δ*vasH*, Δ*vasK*, and their respective complemented mutant strains (Δ*vasH/C* and Δ*vasK/C*) of *A. hydrophila* SSU (8×10^6 cfu), and the survival rate of the animals was recorded. After 48 hr, 80% of the animals infected with the WT and Δ*vasK/C*, and 50% of the animals infected with Δ*vasH/C* strain of *A. hydrophila* died (Fig. 5B), while, in contrast, 100% of the animals infected with Δ*vasH* and Δ*vasK* mutant strains survived 16 days postinfection (Fig. 5B). Importantly, at higher doses of the WT bacterium (5×10^7 cfu), no difference in the survival rate of mice was noted when compared to the Δ*vasH* and Δ*vasK* mutant strains. Although the Δ*vasK* mutant is still able to translocate Hcp, its virulence was attenuated in the mouse model similar to that of the Δ*vasH* mutant, which does not produce Hcp (Fig. 2A, lanes 4 and 6).

3. DISCUSSION

Although the presence of a T6SS was recently described in *V. cholerae* [32], bioinformatic analysis demonstrated that many gram-negative bacteria harbor the T6SS gene cluster [33]. Some of these bacteria that carry the T6SS gene cluster, which has been variously named in different pathogens, include *Yersinia pestis*, *Legionella pneumophila* (the T6SS gene cluster named as IAHP [**I**cmF-**a**ssociated **h**omologous **p**rotein]), *Pseudomonas aeruginosa* (the gene cluster known as HSI [Hcp secretion Island]), **E**ntero**a**ggregative ***E**scherichia **c**oli* (EAEC) O42 (the gene cluster known as aai [AggR-Activaded Island]), *Salmonella enterica* (the gene cluster known as Sci), *Agrobacterium tumefaciens*, *Edwardsiella ictaluri*, *Edwardsiella tarda*, *Rhizobium leguminosarum*, *Burkholderia mallei* [33,37,38,40,47–49], and recently in *A. hydrophila* SSU (GenBank accession number DQ667172) and *A. hydrophila* ATCC 7966 [39]. The latter is an environmental isolate (type strain ATCC 7966 of *A. hydrophila* subspecies *hydrophila*), and we recently annotated its genome sequence in collaboration with The Institute for Genome Research (TIGR). However, the role of this secretion system in the virulence of bacterial pathogens, in general, is largely unknown.

Hcp and VgrG family of proteins are known effectors of T6SS [32,35,38,40]. It has been reported that a member (VgrG1) of the Vgr family from *V. cholerae* shares with RtxA toxin a subdomain that mediates actin crosslinking and cytotoxicity when it is expressed in the cytoplasm of eukaryotic cells. However, this activity has been associated with a second domain of this protein which is not present in VgrG2 and VgrG3 found in *A. hydrophila* (Table 1) [50]. Further, studies have shown that mutations affecting the ATPase domain of *clpV1* (a member of the *clpB* family) in *P. aeruginosa* abrogates Hcp secretion [35]. These results suggest that the *clpB* gene in *A. hydrophila* T6SS gene cluster (Table 1) could play a similar role in unfolding proteins to be secreted, as well as in providing energy for this process.

The T6SS gene cluster in *A. hydrophila* SSU contains homologs of *vasA*, *vasF*, *vasK*, and *vasH* genes found in *V. cholerae* (ORF 7, 12, 17, and 14 respectively (Table 1). For the first three genes, their functions remain poorly characterized (COG3519, COG3455, and COG3523, respectively). The *vasH* gene encodes a sigma-54 factor-dependent transcriptional regulator carrying a factor for inversion stimulation (Fis)-type helix-turn-helix and homeodomain-like motifs, which are involved in DNA-binding and ATPase activity (AAA+ core). Approximately one-half of the proteins that interact with RNA polymerase sigma factor-54 domain can be phosphorylated by a sensor kinase. The ATPase activity in most of these proteins has been associated with conformational changes that promote interaction with Sigma-54 factor, and the Fis-type domain and the homeodomain are directly associated with DNA-binding [51–53]. Thus, *vasH* in *A. hydrophila* SSU could be a key component necessary for the expression of components of T6SS which mediates the association between RNA polymerase and DNA.

Hcp and VgrG proteins are proposed effectors associated with the T6SS gene cluster as they are associated with cytotoxicity in some *in vitro* models, such as the rounded phenotype in the J774.1 murine macrophages, and killing of *Dictyostelium* amoeba in *V. cholerae* [32]. However, it has also been reported that expression of the *hcp* gene is required for VgrG secretion [32]. This, together with the ability of Hcp to form hexameric rings after secretion, suggest that Hcp could be part of the translocon and that VgrG then passes through the Hcp channel [32,35,41].

In Fig. 2A, we showed importance of *vasH* and *vasK* genes in the T6SS gene cluster of *A. hydrophila* SSU. Likewise, studies conducted by Mougous *et al*. (2006) demonstrated by fluorescence microscopy that IcmF (homolog to VasK) in *P. aeruginosa* could be required (but not totally essential) for the efficient assembly of the T6- secretion apparatus. Therefore, we consider that VasK may constitute a structural protein of the T6SS in *A. hydrophila* SSU that provides the stability necessary to secrete Hcp into the extracellular milieu in absence of cell-to- cell contact. However, when the T6SS apparatus makes a cell- to-cell contact with eukaryotic membranes, its (VasK) function could be redundant due to the stability that the translocon (e.g., Hcp) could render, thus still allowing Hcp to be translocated into the host cell (Fig. 2A). Finally, the ability of *A. hydrophila* SSU Hcp to bind to murine macrophages (Fig. 2C) supported the contention proposed for Hcp from *P. aeruginosa* that Hcp alone could be able to attach to the cellular membrane [35].

Previous reports indicated that a homolog of *vasK*, called *icmF* and *sciS* in *L. pneumophila* and *S. enterica*, respectively, is involved in the intracellular survival and replication of these bacteria [37,54]. Similarly, our findings indicated that mutations in components of the T6SS gene cluster (e.g., *vasH* and *vasK*) could alter the virulence of *A. hydrophila* by altering the ability of bacteria to be phagocytosed. Our future studies will delineate how deletion of *vasH* and *vasK* genes leads to increased phagocytosis of the bacteria.

Finally, we provided evidence that expression of the *hcp* gene in HeLa cells led to host cell apoptosis (Fig. 4). It has been proposed that Hcp from *P. aeruginosa* could be able to form a channel allowing the release of ions as well as the transport of macromolecules [35]. Bacterial pore-forming proteins like alpha toxin from *Staphylococcus aureus*, listeriolysin O from *Listeria monocytogenes* and alpha-hemolysin from *E. coli* are able to induce apoptosis by the selective release of ions leading to DNA fragmentation [55]. However, alpha toxin can induce apoptosis or necrosis depending upon its concentration [55]. The induction of apoptosis by Hcp in *A. hydrophila* might be related to the formation of pores in the cell membrane, and, since the amount of Hcp produced by the HeLa cells is not high because the pBI-EGFP vector possesses a minimal cytomegalovirus promoter which lacks the enhancer, the effects that we are seeing are consistent with the induction of apoptosis rather than necrosis. Our future studies will be targeted at studying Hcp-induced apoptosis in details.

In Fig. 5A, we demonstrated circulating antibodies to Hcp in mice after infection with the WT *A. hydrophila* SSU. Following infections with *P. aeruginosa*, similar results were reported in patients with chronic respiratory infections in that they showed a high titer of antibodies against Hcp [35]. In addition, animals of different species showed, following infection with glanders disease, the presence of anti-Hcp antibodies [38]. Since anti-Hcp antibodies were present in the sera of mice infected with the sub-lethal dose of *A. hydrophila* and these antibodies were protective against subsequent challenge of animals with the WT bacterium (Table 2), we speculate that Hcp could be a potential target for vaccine development.

Importantly, our data reported in Figs. 2A and 5B indicated that although Δ*vasK* mutant could translocate Hcp, it was attenuated in a mouse model of infection. These results raise the question whether the secreted Hcp plays a more important role in bacterial virulence than the

translocated one and will be studied in our future studies. The susceptibility of the Δ*vasH* and Δ*vasK* mutants to be phagocytosed, compared to that of the WT *A. hydrophila*, could explain the increased survival of the mice infected with these mutants. Thus, Δ*vasH* and Δ*vasK* mutants could be cleared from the intraperitoneal space before infection becomes systemic. Further, since these mutants killed mice at higher doses (5×10^7 cfu compared to 8×10^6 cfu), these data implied that the presence of T3SS effectors, as well as Act could be contributing to animal lethality, which confirms our previous studies [21,30].

It is also interesting to note that both Δ*vasH* and Δ*vasK* mutants showed increased phagocytosis (Fig. 3A) in spite of the fact that the Δ*vasK* mutant could translocate Hcp efficiently (Fig. 2A). These data could indicate: i) secreted Hcp might play an important role in phagocytosis, and ii) the translocated Hcp might have been altered in its function in the Δ*vasK* mutant. Our future studies will be focused on these aspects as it relates to Hcp.

In summary, we reported the presence of a functional T6SS gene cluster in *A. hydrophila* SSU and showed, for the first time, the ability of this secretion system to translocate Hcp into the eukaryotic cells and to induce apoptosis that was mediated by caspase 3 activation. Additionally, we showed that immunization of mice with Hcp protected animals from subsequent challenge with the lethal dose of the WT bacterium.

4. Materials and Methods

4.1. Cell Lines and transfections

HT-29, a human colonic intestinal epithelial cell line, and RAW 264.7, a murine macrophage cell line, were obtained from American Type Culture Collection (Manassas, VA). These cells were grown as previously described [29]. HeLa Tet-Off™, a human cervical epithelial cell line, was obtained from Clontech (Mountain View, CA). Cells were cultured in Dulbecco's modified eagle medium (DMEM) with high glucose (Invitrogen-Gibco, Carlsbad, CA), supplemented with 10% tetracycline free fetal bovine serum (FBS) (Clontech) and 100 μg/ml of G-418 (Cellgro, Herndon, VA) as described previously [29].

HeLa Tet-off™ cells were transfected by electroporation with a pBI-EGFP plasmid (Clontech) that separately controls expression of the gene encoding EGFP and the gene of interest. The DNA fragment encoding Hcp (519 bp) was cloned at the NheI and MluI restriction enzyme sites of the vector pBI-EGFP. The efficiency of transfection was determined as the percentage of cells expressing EGFP by fluorescent microscopy (Axiovert 200M, Carl Zeiss, Thornwood, NY) and flow cytometry (FACScan, Becton Dickinson, San Diego, CA). HeLa Tet-Off cells transfected with the pBI-EGFP vector alone (without any insert) were used as a negative control in different assays. Western blot analysis and flow cytometry, after intracellular staining using specific antibodies, were also performed to examine the production of Hcp in HeLa Tet-Off cells.

4.2. Bacterial cultures and vectors

Escherichia coli HMS174-DE3 cells, obtained from Novagen (Madison, WI), were grown after transformation with appropriate recombinant plasmid DNA in Luria Bertani (LB) medium supplemented with appropriate antibiotics [29]. The plasmid DNA was isolated from *E. coli* DH5α clones transformed with pBI-EGFP recombinant plasmids, which were similarly cultivated [29]. The gene encoding Hcp was cloned into a pET-30a vector for hyper-expression and purification purposes. Briefly, the appropriate DNA fragment was cloned at the XhoI and BglII restriction enzyme sites of the vector, resulting in the fusion of Hcp with the 6x-histidine (His)-tag. The other cultures and plasmids used are listed in Table 3.

4.3. Generation of Δ*vasK* and Δ*act*/Δ*vasK* knockout mutants

Two pairs of primers (VasKup5/VasKup3 and VasKdn5/VasKdn3) (Table 4) were used to PCR amplify the upstream and downstream flanking DNA sequences to the *vasK* gene from the genomic DNA (gDNA) of *A. hydrophila* SSU. After the PCR reactions, the resulting up- and down- stream flanking DNA fragments (1472- and 1198-bp, respectively) were ligated together through the introduced common BglII enzyme site and cloned into the pBluescript vector at XbaII/KpnI restriction enzyme sites, generating a recombinant plasmid pBlue*vasK*UD. Subsequently, a streptomycin/spectinomycin (Sm^r/Sp^r) gene cassette flanked by the BamHI site was removed from plasmid pHP45Ω and inserted at the BglII site (compatible with the BamHI site) of pBlue*vasK*UD which generated the recombinant plasmid pBlue*vasK*UDSm^r/Sp^r. After digestion with XbaII/KpnI restriction enzymes, the DNA fragment containing the up- and down- *vasK* flanking DNA sequences, as well as the Sm^r/Sp^r gene cassette, was removed from the above plasmid and ligated into the pDMS197 suicide vector (harbors tetracycline resistance [Tc^r] and the *sacB* gene [encodes levansucrase, which is lethal to bacteria when the *sacB* gene is induced with sucrose]) at the compatible restriction enzyme sites, and the resulting plasmid (pDMS197*vasK*UDSm/Sp) was transformed into *E. coli* SM10 (λ*pir*). The recombinant *E. coli* [pDMS197*vasK*UDSm/Sp] clone was conjugated with either the WT *A. hydrophila* SSU-R (rifampin [Rif] resistant) or its Δ*act* mutant to generate Δ*vasK* single- and Δ*act*/Δ*vasK* double- knockout mutants of *A. hydrophila* SSU, respectively. The transconjugants were selected based on their resistance to appropriate antibiotics and sucrose and subjected to further analyses. Briefly, transconjugants were plated onto LB agar plates with Rif (200 μg/ml), Sm^r/Sp^r (50 μg/ml) and 15% sucrose. Single colonies that replicated on plates with Sm and Sp antibiotics, but were sensitive to Tc, were verified by Southern blot analysis [56] using the *vasK* gene probe.

4.4. Generation of Δ*vasH* and Δ*act*/Δ*vasH* knockout mutants

To generate the Δ*vasH* knockout mutant of *A. hydrophila* SSU in the WT background strain, first the recombinant plasmid pDMS197*vasH* was constructed. To clone the *vasH* gene in the pDMS197 vector, this gene was PCR amplified using gDNA of *A. hydrophila* SSU-R as a template and a pair of specific primers (*vasH*-N-XbaI, *vasH*-C-XmaI) (Table 4). The PCR product and vector were digested with XbaI/XmaI enzymes, ligated and electroporated into the *E. coli* SM10 (λ*pir*) strain. To clone the Sm/Sp^r gene cassette into the *vasH* gene of pDMS197*vasH* plasmid, the cassette was PCR amplified using the pHP45Ω plasmid and N-KpnI and C-KpnI primers (Table 4). Then *E. coli* SM10 strain containing the final recombinant plasmid pDMS197*vasH*Sm/Sp was conjugated with *A. hydrophila* WT and its Δ*act* mutant strain (SSU-R). Transconjugants were plated as described above, and the correct identity of the clone verified by Southern blot analysis using the *vasH* gene probe.

4.5. Complementation of *A. hydrophila* SSU Δ*vasH and* Δ*vasK* knockout mutants

To complement the *vasH* gene, the latter was PCR amplified using gDNA of *A. hydrophila* as a template and proper primers (*vasH*-N/BamHI and *vasH*-C/EcoRI) (Table 4). This DNA fragment (1.5 kb) was cloned in the pBR322 vector (Tc^r and Ap^r) at BamHI-EcoRI sites and transformed into the *E. coli* TOP10 strain. The pBR322-*vasH* (Tc^s and Ap^r) recombinant plasmid was isolated from the *E. coli* strain and electroporated in the *A. hydrophila* Δ*vasH* mutant. To complement the *vasK* gene in the *A. hydrophila* mutant, pCR2.1-*vasK* plasmid was digested with EcoRI restriction enzyme and the gene (3.5-kb fragment) was excised from gel, ligated with pBR322 vector and transformed into *E. coli* TOP10 cells. The pBR322-*vasK* recombinant plasmid was isolated from the *E. coli* strain and electroporated into the *A. hydrophila* mutant strain. We then generated as a control the *A. hydrophila* WT strain containing the pBR322 vector alone. To test the presence of the pBR322-*vasH* and pBR322-*vasK* recombinant plasmids and of pBR322 vector in *A. hydrophila*, the plasmid DNA was

isolated from all of the above-mentioned strains and digested with appropriate restriction endonucleases.

4.6. Electroporation

Electroporation of *E. coli* as well as of the HeLa Tet-Off cells with various plasmids was performed as we recently described [29]. Well-isolated bacterial colonies were selected and grown in LB medium for the plasmid DNA isolation using a QIAprep® Miniprep Kit (Qiagen, Inc., Valencia, CA). The DNA was subjected to restriction enzyme analysis and DNA sequencing at the Biomolecular Resource Facility at UTMB to confirm target gene sequences. HeLa cells after electroporation were recovered in complete medium (DMEM/10% FBS/ G-418), plated, and grown under standard tissue culture conditions [29].

4.7. Two-dimensional gel electrophoresis

Supernatants from overnight cultures (10 ml grown in LB medium at 37°C/8 hr with shaking [150 rpm]) of *A. hydrophila* WT were prepared after centrifugation and filtering of the culture filtrates through 0.22-μm filters. These supernatants were concentrated by TCA precipitation (final concentration 10%). Two-dimensional gel electrophoresis was performed using an Ettan IPGphor isoelectric focusing system (GE Healthcare, Piscataway, NJ), per the manufacturer's instructions and as previously described in detail [57,58]. After electrophoresis, the gels were washed (10% methanol and 7% acetic acid) for 30 min and stained with SYPRO Ruby protein stain (BioRad, Hercules, CA). The images were taken with a Fluochem 8800 (Alpha Innotech, San Leandro, CA), and the data were analyzed using a Progenesis Work station (Nonlinear Dynamics, Durham, NC) at the Protein Chemistry Core Laboratory, UTMB. Based on the molecular mass and isoelectric point (pI) of the proteins, several spots in the vinicity of 15–25 kDa and a pI of 4.9–5.4, which corresponded to the size (19 kDa) and pI (5.24) of Hcp, were robotically excised from the stained gels, subjected to trypsin digestion, and the tryptic fragments were then analyzed by matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry using 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA). Importantly, Hcp was produced in relatively higher amounts than the other spots that were excised from the gel.

4.8. Recombinant Hcp (rHcp) purification

Purification of the Hcp was achieved using the Probond™ purification system (Invitrogen-Gibco) as previously described [29]. The proteins from the nickel column were eluted with 250 mM imidazole and subjected to sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis (SDS-PAGE) to verify the identity of hyper-produced Hcp protein by performing Coomassie blue staining of the gels. Fractions containing the protein were mixed and dialyzed against phosphate-buffered saline (PBS) overnight at 4ºC. The protein concentration was measured by a Bradford Assay (BioRad) and the samples stored at −20ºC.

4.9. Antibody production

Swiss Webster mice (Taconic Farms, Germantown, NY) were immunized i.p. with 10 μg of rHcp protein mixed with a synthetic adjuvant as previously described [21]. Sera were obtained from mice and the antibody titers determined by an enzyme-linked immunosorbent assay (ELISA) using rHcp protein as the source of antigen [59]. Dilutions of the sera that were in the middle of the logarithmic portion (~1:1000) of the ELISA titration curves were tested by Western blot analysis for their reactivity to the antigen.

4.10. Western blot analysis

For assessment of antibody reactivity in sera, rHcp protein (2 μg/lane) was subjected to SDS-PAGE and transferred to Hybond™-ECL™ nitrocellulose membranes (GE Healthcare)

following the standard Western blot procedure [59]. Membranes were cut into strips corresponding to the lanes of the gel, blocked with 1% bovine serum albumin [BSA]/5% skim milk, and were subsequently incubated with anti-Hcp sera, taken from individual mice (1:1000) and diluted in Tris-buffered saline (TBS), pH 7.6, and 0.5% skim milk for 1 hr with constant shaking at room temperature. The strips were then incubated for 1 hr with secondary antibody (Goat α-mouse IgG [diluted 1:10000] conjugated with horse-radish peroxidase ([HRP] [Southern Biotechnology Associates, Inc., Birmingham, AL]). Five washes of strips were performed between various steps using TBS/0.05% Tween 20 for 10 min each. The blots were developed with Super Signal® West Pico Chemiluminescent substrate (Pierce, Rockford, IL) followed by X-ray film exposure.

Likewise, HeLa cells were lysed in SDS-Tris-Glycine buffer [59] after 24 hr of transfection and subjected to electrophoresis and Western blot analysis as described above.

4.11. Intracellular staining

HeLa Tet-Off cells after 24 hr of transfection were permeabilized using CytoFix™/ CytoPerm™ (Becton Dickinson, San Diego, CA). The α-Hcp antibody containing the hyper-immune serum (diluted 1:100), as well as the pre-immune serum (diluted 1:100), was used as the source of primary antibodies. Hcp in HeLa cells was then visualized using phycoerythrin (PE)-conjugated α-mouse IgG antibody (Santa Cruz, Santa Cruz, CA) as previously described [29]. The samples were acquired in a FACScan™ (Becton Dickinson) and analyzed using CellQuest™ (Becton Dickinson) software and WinMDI©.

4.12. Translocation assays

For these assays, we followed the methodology reported by Sha *et al* (2007) with minor modifications [21]. Briefly, bacterial strains grown to log phase were washed and re-suspended in PBS, and their turbidity measured at OD_{600} nm. Human colonic epithelial cells, HT-29, were grown in 6 well plates to ~80% confluence in DMEM medium supplemented with 10% FBS before infection with bacteria, and infection was performed at an MOI of 5 in DMEM/ 0.5% FBS medium. The bacteria and host cells were co-cultured for 2 hr at 37ºC in 5% of CO_2. Subsequently, four fractions were collected: i) supernatant fraction, ii) cytoplasmic fraction, iii) eukaryotic host membrane fraction, and iv) whole bacterial lysates. The supernatant fraction was collected by removing the medium and centrifuging it at 1000 *x g* for 10 min. The supernatants were separated from the pellet and filtered through a 0.22-μm membrane filter. Proteins present in the supernatant fraction were precipitated with trichloroacetic acid (TCA) (10% final concentration) and pelleted by high- speed centrifugation at 14000 *x g* for 15 min at 4ºC. The pellet was re-suspended in 1X Lammeli loading buffer [59].

The cytoplasmic fraction was collected following lysis of the host cells with 500 μl of sterile water. The cells were disrupted by gentle pipetting and then centrifuged at 6000 *x g* for 10 min at 4ºC. The eukaryotic membrane fraction was obtained by extraction of the proteins from the above pellet with 500 μl of the cell lysis buffer (200 mM NaCl, 5 mM EDTA, 10% glycerol, 1 mM PMSF, 0.1% Triton X-100, 10 mM tris-HCl pH 7.0). Only eukaryotic cell membranes were affected by this method, while the bacterial membrane integrity was maintained. After centrifugation, the remaining pellet was re-suspended directly in 1X loading buffer. This fraction was considered as the whole bacterial lysate fraction. Samples as obtained above were separated by 4–20% gradient SDS-PAGE, and then proteins were electro-transferred to nitrocellulose membranes for performing Western blot analysis as described earlier. As controls of contamination between fractions, antibodies to actin (eukaryotic cytoplasmic protein), calnexin (eukaryotic membrane protein), and DnaK (bacterial cytosolic protein) were

run in parallel for Western blot analysis. Although less likely, the cytoplasmic fraction could contain bacterial secreted proteins that are present in endocytic vesicles.

4.13. Hcp binding to RAW 264.7 macrophages

To perform this experiment, supernatants of *A. hydrophila* SSU Δ*act* and double knockout mutant Δ*act*Δ*vasH* were used to avoid the cytotoxic effects mediated by Act. Briefly, bacteria were grown overnight in the LB medium containing appropriate antibiotics. The next day, the cells were washed 3X with PBS, quantified by optical density measurements at 600nm and grown in DMEM supplemented with 1% FBS at a concentration of 5×10^6 cfu/ml. After 2 hr, the medium was centrifuged and filtered through a 0.22-μm filter. Subsequently, 2×10^6 RAW 264.7 cells/well were grown in 6-well plates. After the cells were attached, the medium was exchanged with 2 ml of conditioned medium used to grow bacteria (see above) and incubated for 2 hr at 37ºC. Then, the host cells were washed 3X with PBS and lysed with 500 μl of water. The supernatant was collected as a cytoplasmic fraction, and the pellet as a membrane fraction. These samples were subjected to SDS-4–20%-PAGE, transferred to nitrocellulose membranes and tested by Western blot analysis for the presence of Hcp using specific antibodies. Similar assays using rHcp were preformed. For these assays, DMEM supplemented with 1% FBS containing rHcp (5 μg/ml) was used instead of bacteria-grown conditioned DMEM.

4.14. Phagocytosis

RAW 264.7 murine macrophages were plated and grown at a density of 1×10^5 cell/well in a 96-well plate in DMEM with 10% FBS under normal tissue culture conditions for 2 hr. Subsequently, 5×10^5 bacteria that were previously washed and re-suspended in PBS were added. The plate was centrifuged at 300 *x g* for 5 min to facilitate contact between macrophages and bacteria and then incubated for 30 min at 37ºC. Next, gentamicin was added at a final concentration of 100 μg/ml for 1 hr to kill extracellular bacteria. Subsequently, the cells were washed twice with PBS, and lysated in 200 μl of water. Different dilutions were plated on LB agar plates and incubated overnight at 37ºC. The colony formation units (cfu) were calculated based on the number of bacteria used for infection by determining the number of colonies inside the macrophages multiplied by the dilution factor.

4.15. Host cell apoptosis

Apoptosis of host cells expressing the *hcp* gene was assessed after 24 and 48 hr post-transfection by detection of cytoplasmic nucleosomes and measurement of caspase 3 activities as previously described [29]. The color reaction was measured in a microplate reader at 405 nm.

4.16. Host cell viability

To determine host cell viability, we performed the incorporation of 7-amino actinomycin D (7-AAD) and the colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide] assay for cell survival as we previously described [29]. For 7-AAD assays, the percentage of positive cells was determined by flow cytometry using CellQuest™ software. For the MTT assay, the color reaction was measured with a microplate reader at 570 nm [29].

4.17. LDH release assay

RAW 264.7 murine macrophages and HeLa cells were infected at an MOI of 0.5 with WT *A. hydrophila*, and Δ*act*, Δ*act*/Δ*vasH* and Δ*act*/Δ*vasK* mutants. During infection, cell morphology was monitored, and at various time points after infection, host cell cytotoxicity was measured by the release of lactate dehydrogenase (LDH) enzyme using CytoTox 96® kit (Promega, Madison, WI) in the tissue culture supernatant [30]. The percentage of cytotoxicity was calculated as recommended by the manufacturer using the following formula: [(OD_{490} Sample

− OD_{490} Spontaneous)/(OD_{490} Maximum Release − OD_{490} Spontaneous)] × 100. OD_{490} spontaneous indicated LDH release from uninfected cells into the culture supernatant and maximum release denoted LDH release obtained by lysis of the uninfected cells. Three independent experiments were performed in duplicate wells.

4.18. Detection of specific Hcp antibodies from sera of mice infected with WT *A. hydrophila* SSU

A group of 10 Swiss Webster mice were infected i.p. with WT *A. hydrophila* at a dose of approximately 1 LD_{50}. After 2 weeks of infection, sera from the surviving mice were pooled and used as the source of primary antibodies in the Western blot analysis with purified rHcp as an antigen [21]. As a negative control, pre-immune sera were used.

4.19. Animal experiments

Groups of 10 Swiss Webster mice were infected i.p. with WT *A. hydrophila*, Δ*vasH*, and Δ*vasK* mutants as well as their complemented strains in accordance with an approved IACUC protocol. We also used WT *A. hydrophila* with pBR322 vector alone as a control in these experiments. Deaths were recorded for 16 days post-infection. The bacterial doses used represented approximately 2 LD_{50s} of WT *A. hydrophila* [60]. In another experiment, animals were immunized with purified rHcp (as described for the antibody production experiment) and then challenged with the WT *A. hydrophila* at a dose of 3 LD_{50s} by the i.p. route after 1 month of immunization. Control animals included those that were given the adjuvant alone (without the antigen) and then infected with the WT bacterium. Deaths were recorded for 16 days post-infection. Fisher's exact test was used for statistical analysis, and two independent experiments were performed.

4.20. Statistical analysis

Two-way ANOVA and Bonferroni posttests were used for statistical analysis of the data using GraphPad Prism® version 4.02 for windows™ (Software MacKiev, San Diego, CA). The animal mortality data were analyzed by the Fisher exact test.

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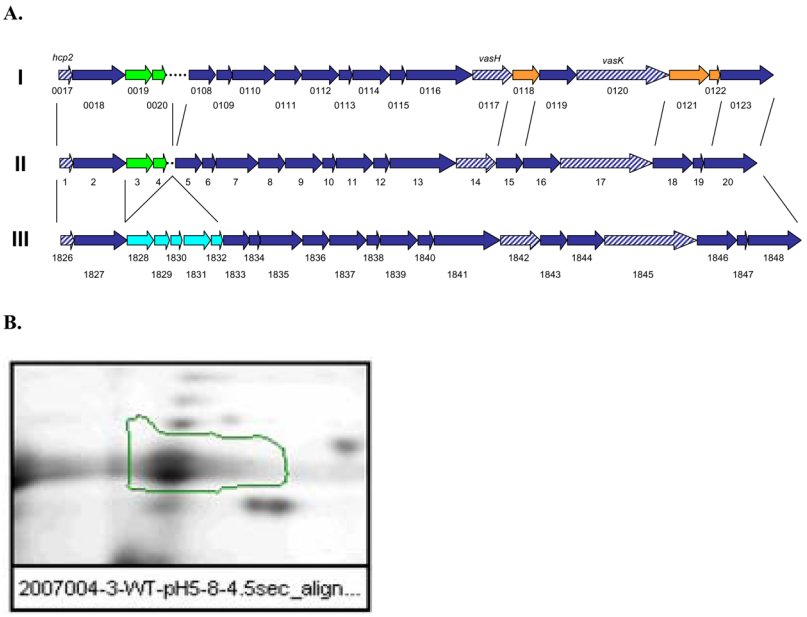
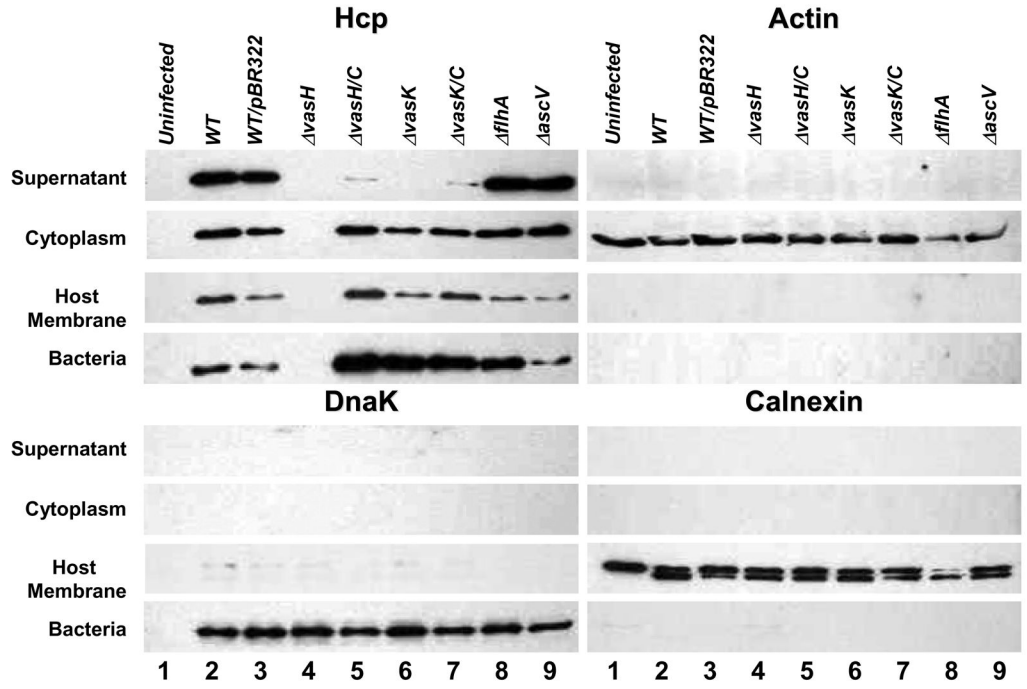


Figure 1.

A. Diagram showing the genetic organization of the T6SS gene cluster of *A. hydrophila* SSU (II) in comparison with the similar cluster present in *Vibrio cholerae* N16691 (I) and *A. hydrophila* ATCC 7966 (III). Shown in green are the genes present only in *V. cholerae* and *A. hydrophila* SSU T6SS gene clusters. Dashed in blue are genes that were pursued during this study. Genes in cyan color are those that are present only in *A. hydrophila* ATCC 7966 strain (III). Genes in orange exhibit low identity (<25%) between *V. cholerae* and *A. hydrophila* SSU T6SS gene cluster. **B.** Two-dimensional gel electrophoresis of supernatants from *A. hydrophila* SSU after staining with Sypro Ruby. The spot for Hcp is highlighted, and its identity was confirmed by mass-spec analysis.



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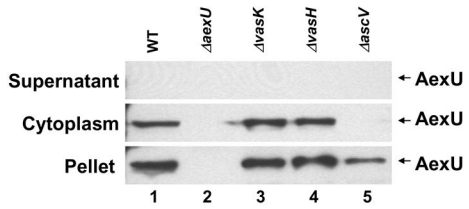


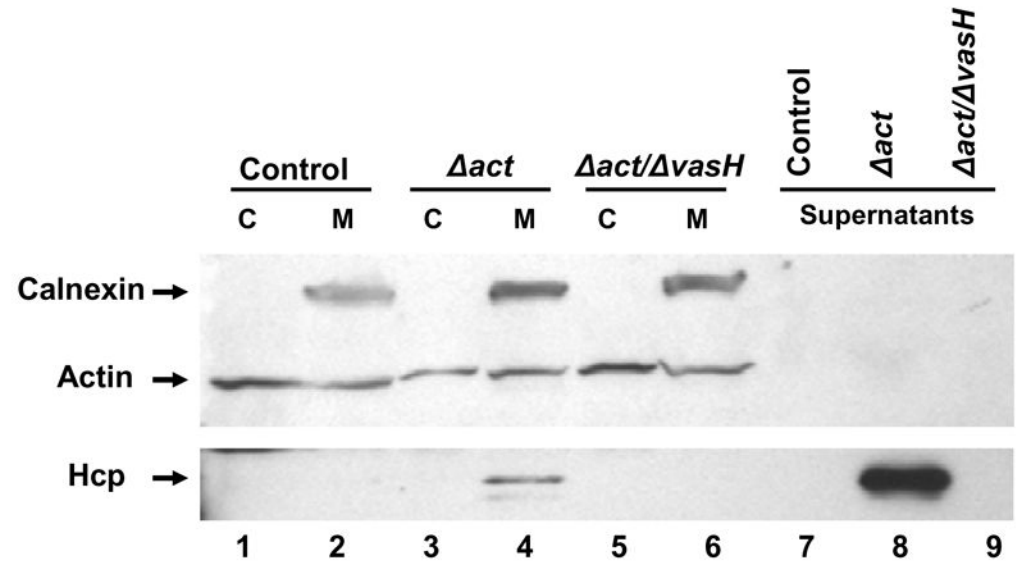
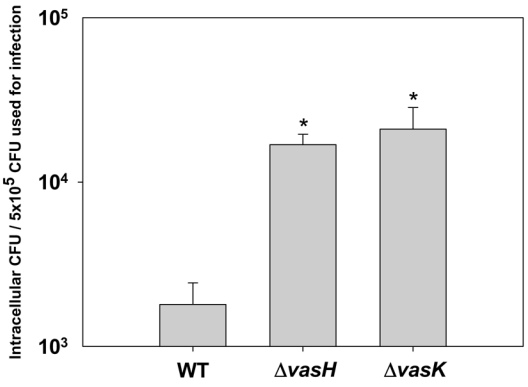


Figure 2.

A. Production of Hcp from *A. hydrophila* SSU and the translocation of Hcp into host cells. HT-29 human colonic epithelial cells were infected with different *A. hydrophila* strains (MOI of 5) for 2 hr at 37°C in DMEM/0.5% FBS medium. The culture supernatants were TCA precipitated (supernatant fraction). The infected host cells were osmotically lysed and centrifuged to obtain soluble (cytoplasmic fraction representing translocated effectors) and insoluble fractions. The insoluble fraction was resuspended in cell lysis buffer containing 0.1% Triton X-100 and centrifuged to obtain soluble (host cell membrane fraction) and insoluble (intact bacterial pellet) fractions. The samples were run on 4–20% gradient SDS-PAGE and subjected to Western blot analyses using anti-Hcp, anti-actin, anti-DnaK and anti-calnexin antibodies. **Lane 1:** Untreated HT-29 cells; **Lane 2:** *A. hydrophila* WT; **Lane 3:** WT bacteria containing pBR322; **Lane 4:** Δ*vasH* mutant; **Lane 5:** Δ*vasH* complemented strain; **Lane 6:** Δ*vasK* mutant; **Lane 7:** Δ*vasK* complemented strain; **Lane 8:** Δ*flhA* mutant, and **lane 9:** Δ*ascV* mutant **B.** Production and translocation of AexU are not affected by mutations in the T6SS components. HT-29 cells were infected with the WT *A. hydrophila* SSU **(lane 1)**, Δ*aexU* mutant **(lane 2)**, Δ*vasK* mutant **(lane 3)**, Δ*vasH* mutant **(lane 4),** and Δ*ascV* mutant **(lane 5)**. The culture supernatants were TCA precipitated (supernatant fraction). The infected host cells were osmotically lysed and centrifuged to obtain soluble (cytoplasmic fraction) and insoluble fractions (pellet). The samples were run on 4–20% gradient SDS-PAGE and subjected to Western blot analyses using anti-AexU antibodies. **C.** Hcp binds to the cell membrane of RAW 264.7 murine macrophages. Supernatants from *A. hydrophila* Δ*act* and *Δact*/*ΔvasH* mutants were added to RAW 264.7 cells and incubated for 2 hr at 37°C. The host cells were washed, osmotically lysed, and centrifuged to obtain soluble (cytoplasmic) and insoluble (membrane) fractions. Samples were run on a 4–20% gradient SDS-PAGE and subjected to Western blot analyses using the following antibodies: anti-calnexin, anti-actin and anti-Hcp. Control: Macrophages incubated with 1% FBS-DMEM **(lanes 1 and 2)**; Δ*act*: Macrophages incubated with supernatants from *A. hydrophila act* mutant **(lanes 3 and 4)**; Δ*act*/Δ*vasH*: Macrophages incubated with supernatants from *A. hydrophila* Δ*act*/Δ*vasH* mutant **(lanes 5 and 6)**. The supernatants from the Δ*act* **(lane 8)** and Δ*act*/Δ*vasH* **(lane 9)**

mutants of *A. hydrophila* SSU were used as a control for the presence of Hcp. C=Cytoplasmic fraction from RAW 264.7 macrophages. M=Membrane fraction from RAW 264.7 macrophages.

A.



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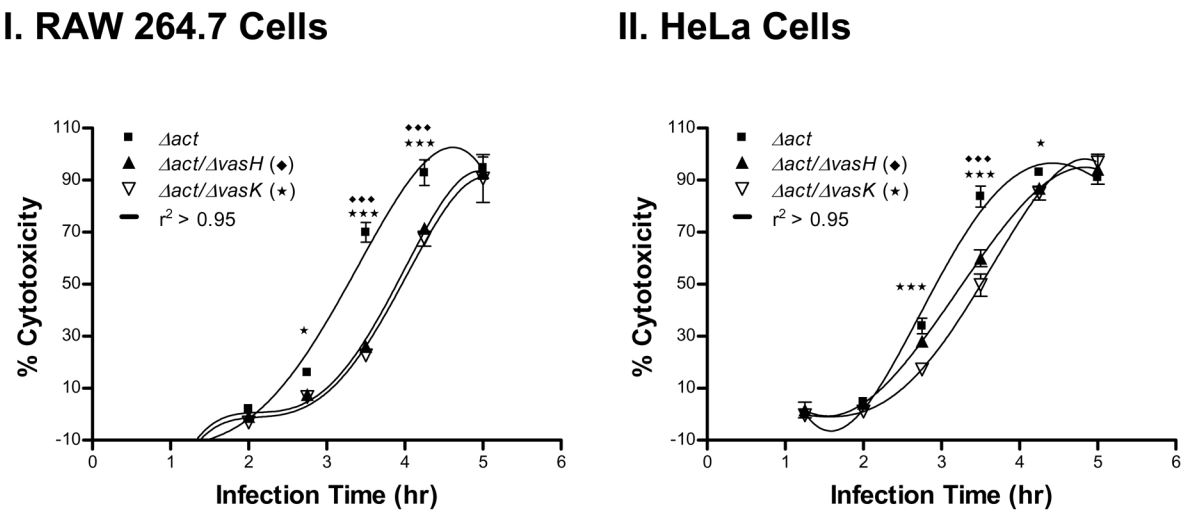

Figure 3.

A. Phagocytosis is enhanced in *A. hydrophila* Δ*vasH* and Δ*vasK* mutants. RAW 264.7 murine macrophages were infected at an MOI of 5 with *A. hydrophila* strains, namely, WT, Δ*vasH* and Δ*vasK* mutants. Thirty minutes after infection, the cells were washed and treated with 100 μg/ml of gentamicin for 1 hr. Then, RAW 264.7 cells were washed and lysed with water. The bacteria were plated at different dilutions and the colony forming units were determined.* denotes statistically significant values ($p<0.01$) compared to the parent strain (WT). **B**. Cytotoxicity associated with the T6SS. RAW 264.7 cells **(Panel I)** and HeLa cells **(Panel II)** were infected with Δ*act*/Δ*vasH* mutant (solid triangle) and Δ*act*/Δ*vasK* mutant (open triangle) double-knockout mutants and their parental strain Δ*act* mutant (solid square) at an MOI 0.5. At different time points, cytotoxicity was measured by the lactate dehydrogenase (LDH) enzyme release assay. *** or ♦♦♦ denotes statistically significant values ($p<0.001$) compared to the parent strain Δ*act*. * denotes statistically significant values ($p<0.05$) compared to the parent strain Δ*act*. Three independent experiments in duplicate wells were performed.

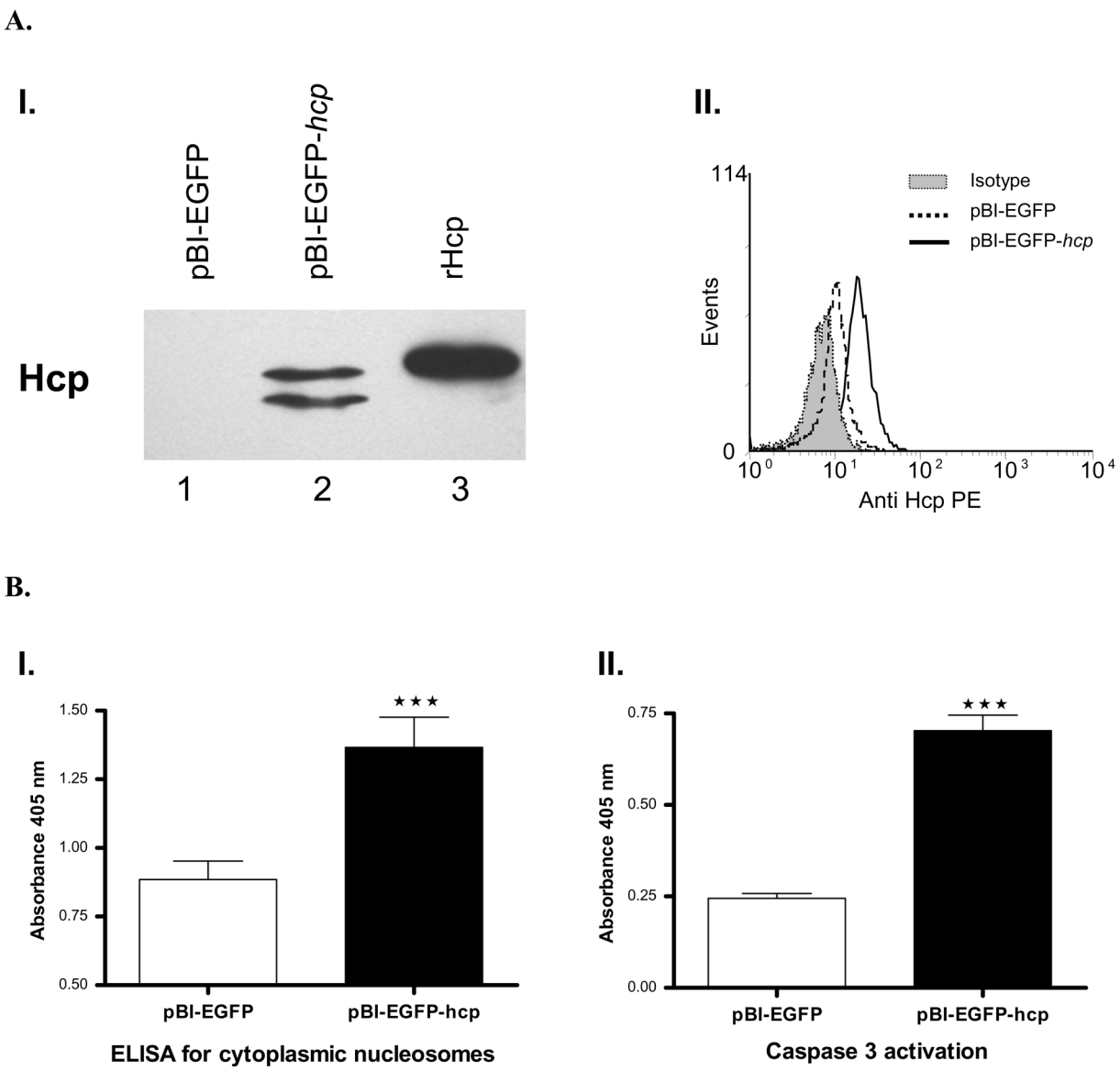

Figure 4.

A. Expression and production of Hcp in transfected HeLa Tet-Off cells. **Panel I**. Western blot analysis showing production of Hcp in whole cell lysates of HeLa Tet-Off cells after 24 hr of transfection with pBI-EGFP-*hcp* **(lane 2)** or pBI-EGFP (empty vector) **(lane 1)** plasmid. Recombinant Hcp was used as a positive control **(lane 3). Panel II**. Expression of the *hcp* gene in HeLa Tet-Off cells after permeabilization and intracellular staining using anti-Hcp antibodies. Mouse pre-immune serum was used as an isotype control. The cells were acquired using a FACScan flow cytometer and analyzed using WinMDI software, gated on EGFP-positive cells. **B.** Induction of apoptosis in HeLa Tet-Off cells transfected with the *hcp* gene. **Panel I**. Detection by ELISA of cytoplasmic nucleosomes in HeLa Tet-Off cells transfected with hcp after 24 hr. *** denotes statistically significant values ($p<0.001$) compared to those in cells transfected with the pBI-EGFP (empty vector) plasmid. Standard deviations were calculated from duplicate samples from one representative experiment. A minimum of three experiments were performed with similar results. **Panel II**. Colorimetric caspase 3 detection in total lysates of HeLa Tet-Off cells transfected with the *hcp* gene after 24 hr. Figures are

representative of three independent experiments. *** denotes statistically significant values ($p<0.001$) compared to those of cells transfected with the pBI-EGFP (empty vector) plasmid. Standard deviations were calculated from duplicate assays from one experiment. A minimum of three experiments were performed with similar results.

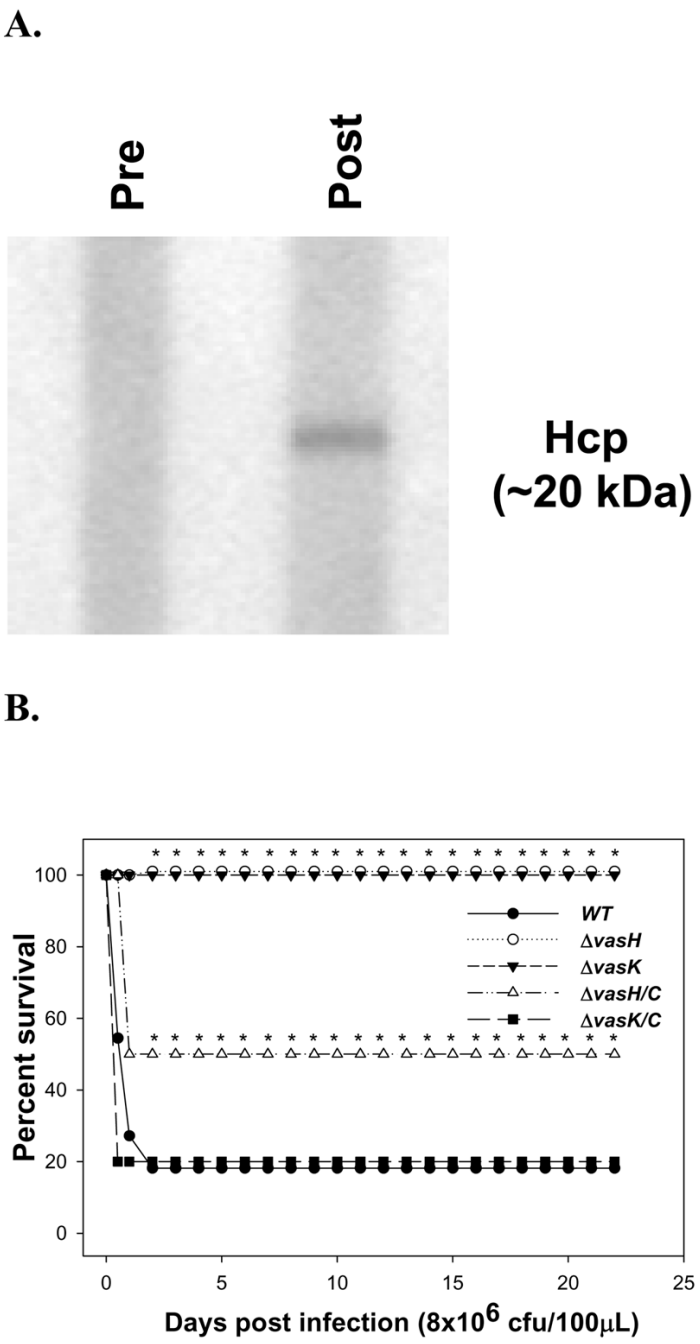

Figure 5.

Role of Hcp during *A. hydrophila* infection in a mouse model. **A**. Swiss Webster mice were infected i.p., with WT *A. hydrophila* at a dose of 1 LD_{50} (1×10^5 cfu/100μl). After 2 weeks of infection, sera from the surviving mice were collected and pooled. The sera was diluted 1:100 and used as a source of primary antibodies in Western blot analysis against rHcp. Pre-immune serum was used as a negative control. **B.** Groups of 10 Swiss Webster mice were infected i.p. (8×10^6 cfu) with *A. hydrophila*: WT (solid circles), Δ*vasH* mutant (open circle) and Δ*vasK* mutant (solid triangle). Same doses of the complemented strains were also used, Δ*vasH/C* (open triangle) and Δ*vasK/C* (solid square). Deaths were recorded for 16 days post-infection. The bacterial doses represented approximately 2 LD_{50} of WT *A. hydrophila*. * denotes

statistically significant values ($p<0.001$) of the mutants (*vasH* and Δ*vasK*) compared to the WT bacterium and of Δv*asH* mutant compared to the Δ*vasH*/C strain ($p<0.05$) using the Fisher exact test. The death curve for WT *A. hydrophila* with pBR322 vector alone was similar to that of the WT bacterium (without the vector) (not shown).

Table 1

Comparison of T6SS gene cluster of *A. hydrophila* SSU with that of *V. cholerae* N16961 and *A. hydrophila* ATCC 7966 sequenced strain

A. hydrophila SSU[1]	*V. cholerae*[2]	Identity/ homology (%)[1] vs. [2]	*A. hydrophila* ATCC 7966[3]	Identity/ homology (%)[1] vs. [3]	Gene product name	Conserved Domains
1	VCA0017	71/79	AHA_1826	95/98	Hemolysin coregulated protein Hcp	COG3157
2	VCA0018	51/52	AHA_1827	80/85	VgrG protein	COG3501, DUF586
3	VCA0019	52/38			hypothetical protein	
4	VCA0020	25/37			hypothetical protein	
			AHA_1828		hypothetical protein	
			AHA_1829		hypothetical protein	
			AHA_1830		hypothetical protein	
			AHA_1831		hypothetical protein	TRP repeat, Sel1
			AHA_1832		hypothetical protein	ImpG homolog
5	VCA0108	74/82	AHA_1833	92/97	hypothetical protein	COG3517
6	VCA0109	24/31	AHA_1834	94/95	hypothetical protein	COG3518, GPW/gp25
7	VCA0110	58/52	AHA_1835	96/98	VasA	COG3519, DUF879
8	VCA0111	51/43	AHA_1836	93/97	hypothetical protein	COG3520, Acyl-CoA dehydrogenase/oxidase C-term
9	VCA0112	18/34	AHA_1837	92/94	hypothetical protein	COG3456, SMAD/FHA
10	VCA0113	40/39	AHA_1838	95/98	Putative Lipoprotein	COG3521
11	VCA0114	61/60	AHA_1839	94/98	hypothetical protein	COG3522
12	VCA0115	41/45	AHA_1840	9497	VasF/IcmH	COG3455
13	VCA0116	63/60	AHA_1841	94/98	ClpA/ClpB family protein	COG0542, AAA, AAA_2, AAA+, chaperone
14	VCA0117	27/40	AHA_1842	94/96	Sigma-54 dependent transcriptional regulator/VasH	COG3829, AAA, Interaction RNA pol sigma factor 54, Fis-type Helix-turn-helix, core AAA + ATPase, Homeodomain-like.
15	VCA0118	7/27	AHA_1843	90/91	hypothetical protein	
16	VCA0119	26/32	AHA_1844	95/97	ImpA related domain protein	COG3515, ImpA-related N-ter
17	VCA0120	53/41	AHA_1845	96/99	ImcF family protein/VasK	COG3523, DUF1215, IcmF-related, conserved hypothetical ATP binding protein
18	VCA0121	1/17	AHA_1846	96/98	ImpA related domain protein	COG3515, ImpA-related N-ter
19			AHA_1847	96/97	paar motif protein	COG4104, PAAR
20	VCA0123	48/52	AHA_1848	87/93	Rhs element Vgr family protein	COG3501, DUF586, Phage_GPD

COG: Cluster of Orthologous Groups of proteins; Most of these analysis data were obtained from the National Center of Biotechnology Information (http://www.ncbi.nlm.nih.gov/sites/entrez/) and the Integrated Microbial Genomes web page (http://img.jgi.doe.gov). The alignments were performed using the Clustalw software program (http://clustalw.genome.jp).

Table 2

Protection of mice immunized with rHcp against lethal challenge of WT *A. hydrophila* SSU

	Survivals
Immunized	5/5
Non Immunized	0/5

Animals were challenged with $3LD_{50}$ (5×10^{7} cfu/100μl)

Table 3

Various bacterial strains and plasmids used in this study.

Strain or Plasmid	Relevant Characteristic(s)	Component of	Source or Reference
***A. hydrophila* SSU**	Diarrheal isolate of *A. hydrophila*		CDC*
SSU-R	Rifampin resistant (Rif^r) strain of *A. hydrophila* SSU		Laboratory Stock
ΔascV	*ascV* mutant of *A. hydrophila* SSU-R; Rif^r, Sp^r/Sm^r	T3SS	[61]
Δact	*act* mutant of *A. hydrophila* SSU-R; Rif^r, Km^r	T2SS toxin	[60]
ΔaexU	*aexU* toxin mutant of *A. hydrophila* SSU-R; Rif^r, Km^r	T3SS effector	[21]
ΔflhA	*flhA* mutant of *A. hydrophila* SSU-R; Rif^r, Sp^r/Sm^r	Flagellar system	This study
ΔvasH	*vasH* mutant of *A. hydrophila* SSU-R; Rif^r, Sp^r/Sm^r	T6SS	This study
ΔvasK	*vasK* mutant of *A. hydrophila* SSU-R; Rif^r, Sp^r/Sm^r	T6SS	This study
Δact/ΔvasH	*act* and *vasH* double knockout mutant of *A. hydrophila* SSU-R; Rif^r, Km^r, Sp^r/Sm^r	T2SS/T6SS	This study
Δact/ΔvasK	*act* and *vasK* double knockout mutant of *A. hydrophila* SSU-R; Rif^r, Km^r,Sp^r/Sm^r	T2SS/T6SS	This study
ΔvasH/C	*vasH* mutant of *A. hydrophila* SSU-R complemented with pBR322-*vasH*; Rif^r, Sp^r/Sm^r, Tc^r	T6SS	This study
ΔvasK/C	*vasK* mutant of *A. hydrophila* SSU-R complemented with pBR322-*vasK*; Rif^r,Sp^r/Sm^r, Tc^r	T6SS	This study
WT/pBR322	*A. hydrophila* SSU-R transformed with pBR322 vector alone; Rif^r, Tc^r, Ap^r		Laboratory Stock
E.coli			
DH5α	*recA, gyrA*		Life Technologies
TOP10	*mcrA, hsdR, lacZ M15,endA1, recA1*		Invitrogen
SM10	λ *pir*		[60]
HMS174 (DE3)	*recA* mutated K-12 background, that carries a chromosomal copy of the T7RNA polymerase under control of *lac*UV5 promoter.		Novagen
Plasmids			
pBR322	Low copy plasmid used for complementation; Tc^r, Ap^r		Pierce
pET30a	pBR322-derived expression vector with T7 *lac* promotor, up- and down- stream His tags; Ap^r.		Novagen
pDMS197	Suicide vector; R6K ori, *sacB*, Tc^r.		[62]
pBI-EGFP	Eukaryotic expression vector regulated by a tetracycline element response (TER) and a minimal CMV promoter; Ap^r		Clontech
pBluescript	Vector used for cloning proposes; Ap^r.		Stratagen
pUC4K	Contains 1.2 Kb kanamycin resistance gene cassette; Km^r		Amersham
pHPΩ45	Contains a 2.0 Kb streptomycin/spectinomycin resistance gene cassette; Sm^r/Sp^r.		[63]
pCR2.1	Used for PCR cloning proposes; Ap^r, Km^r		Invitrogen

* Centers for Diseases Control and Prevention

Table 4

Sequences of the primers used in this study

Primer sequence	Restriction site	Description
5′-GCTCTAGACCGGTGAACCCATCAAGCGCGTCCACT-3′	XbaI	for cloning *vasH* gene into pDMS197
5′-TCCCCCCGGGCTGGTGGCCAGCAGCAGAGGCAATA-3′	XmaI	
5′-CGGGGTACCGAATTCCCGGGGATCCGGTGATTGAT-3′	KpnI	Amplification of Sm/Sp cassette
5′-CGGGGTACCGAATTCCCGGGGATCCGGTGATTGAT-3′	KpnI	
5′-CTGTCTAGACGCCTGCTCACCAGCCTGC-3′	XbaI	*vasK* upstream flanking sequence
5′-CCTAGATCTCATAAAATGTGATCCAACTCCGACTC-3′	BglII	
5′-GTCAGATCTGCCTGAATCCCTCTATTGATCGCG-3′	BglII	*vasK* downstream flanking sequence
5′-ATCGGTACCAAGCTGCGTCTGCCACTGC-3′.	XbaI	
5′-CGCGGATCCCCGGTGAACCCATCAAGCGCGTCCAC-3′	BamHI	for cloning *vasH* gene into pBR322
5′-CCGGAATTCCTGGTGGCCAGCAGCAGAGGCAATAG-3′	EcoRI	
5′-TCATAGATCTAATGCCAACTCCATGTTATTCAG-3′	BglII	for cloning *hcp* in pET30a
5′-GGTCCTCGAGTTAGGCCTCGATCGGCGCGCG-3′	XhoI	
5′-ACGTACGCGTATGCCAACTCCATGTTATATCAGC-3′	MluI	for cloning *hcp* in pBI-EGFP
5′-GTGAGCTAGCTTAGGCCTCGATCGGCGCGCGCCAGT-3′	NheI	