Evaluation of the roles played by Hcp and VgrG type 6 secretion system effectors in *Aeromonas hydrophila* SSU pathogenesis

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Aeromonas hydrophila, a Gram-negative bacterium, is an emerging human pathogen equipped with both a type 3 and a type 6 secretion system (T6SS). In this study, we evaluated the roles played by paralogous T6SS effector proteins, hemolysin co-regulated proteins (Hcp-1 and -2) and valine glycine repeat G (VgrG-1, -2 and -3) protein family members in *A. hydrophila* SSU pathogenesis by generating various combinations of deletion mutants of their genes. In addition to their predicted roles as structural components and effector proteins of the T6SS, our data clearly demonstrated that paralogues of Hcp and VgrG also influenced bacterial motility, protease production and biofilm formation. Surprisingly, there was limited to no observed functional redundancy among and/or between the aforementioned T6SS effector paralogues in multiple assays. Our data indicated that Hcp and VgrG paralogues located within the T6SS cluster were more involved in forming T6SS structures, while the primary roles of Hcp-1 and VgrG-1, located outside of the T6SS cluster, were as T6SS effectors. In terms of influence on bacterial physiology, Hcp-1, but not Hcp-2, influenced bacterial motility and protease production, and in its absence, increases in both of the aforementioned activities were observed. Likewise, VgrG-1 played a major role in regulating bacterial protease production, while VgrG-2 and VgrG-3 were critical in regulating bacterial motility and biofilm formation. In an intraperitoneal murine model of infection, all Hcp and VgrG paralogues were required for optimal bacterial virulence and dissemination to mouse peripheral organs. Importantly, the observed phenotypic alterations of the T6SS mutants could be fully complemented. Taking these results together, we have further established the roles played by the two known T6SS effectors of *A. hydrophila* by defining their contributions to T6SS function and virulence in both *in vitro* and *in vivo* models of infection.

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Abbreviations: CI, competitive index; CV, crystal violet; d.p.i., days post-infection; i.p., intraperitoneal; T3SS, type 3 secretion system; T6SS, type 6 secretion system.

The GenBank/EMBL/DDBJ accession number for the *hcp-1* and *vgrG-1* genes as well as their flanking DNA sequence of *Aeromonas hydrophila* SSU is JX646703.

One supplementary table is available with the online version of this paper.

INTRODUCTION

Of the 27 aeromonads characterized to date, *Aeromonas hydrophila* is the most commonly isolated human pathogen, and the organism is usually contracted via the faecal–oral route by consuming contaminated water or food (Altwegg *et al.*, 1991; Edberg *et al.*, 2007; Kirov, 1993; Palú *et al.*, 2006). In fact, formation of biofilm allows this pathogen to resist chlorination of treated waters as well as numerous antimicrobial agents (Palú *et al.*, 2006). The

majority of *Aeromonas* infections manifest themselves as self-limiting gastroenteritis; however, in some instances (particularly in the young, elderly and other immunocompromised individuals), disease can range from superficial skin sequelae to more systemic infections including: cellulitis, bacteraemia, peritonitis and haemolytic-uraemic syndrome (Brouqui & Raoult, 2001; Chopra *et al.*, 1993; Chopra *et al.*, 1996; Chopra & Houston, 1999; Galindo *et al.*, 2006; Janda *et al.*, 1994; Kühn *et al.*, 1997; Merino *et al.*, 1995). However, not all infections occur in immunocompromised individuals. In May 2012, a University of Georgia student suffered a zip-lining accident and contracted *A. hydrophila* from the warm brackish waters of the Tallapoosa River, resulting in horrific necrotizing fasciitis (Castillo, 2012). Therefore, it becomes imperative to develop effective counter-measures against this emerging pathogen lurking in our waters, soil and processed foods. Since *A. hydrophila* is primarily a water-borne pathogen that also infects fish populations, several vaccines have been tested to reduce losses suffered by the fishing industry; however, currently no human vaccine is available (Sierra *et al.*, 2011).

In addition to three enterotoxins, including the cytotoxic enterotoxin (Act), which is a substrate for the type 2 secretion system (T2SS) (Chopra & Houston, 1999), *A. hydrophila* SSU, a diarrhoeal isolate, possesses two additional weapons which it can readily deploy to fend off an immune cell attack during an infection. A novel type 3 secretion system (T3SS) effector has been identified, AexU, which disrupts host macrophages and promotes their apoptosis by ADP-ribosyltransferase as well as GTPase-activating protein activity (Sierra *et al.*, 2007; Sierra *et al.*, 2010). Whereas the T3SS serves as an injectisome that translocates bacterial toxins directly into the host cell cytoplasm, *A. hydrophila*'s second major weapon serves as both an injectisome, like the T3SS, as well as a secretion apparatus that allows some microbial toxins to be secreted extracellularly, promoting bacterial dissemination.

This second weapon is the type 6 secretion system (T6SS), and two major T6SS effector proteins have been identified to date, namely hemolysin co-regulated protein (Hcp) and valine glycine repeat G (VgrG) protein family members (Suarez *et al.*, 2008, 2010a, b). Importantly, both the T3- and T6- secretion systems operate independently, and we have shown that the secretion of Hcp was not affected in the mutants of *A. hydrophila* SSU that did not have the functional T3SS or the flagellar system. Likewise, translocation of the T3SS effector AexU was not affected in the T6SS mutants (e.g. $\Delta vasH$ and $\Delta vasK$) (Suarez *et al.*, 2008).

The T6SS is derived from phage injection machinery, and, through horizontal gene transfer, has been acquired and identified in 25 % of sequenced Gram-negative genomes (Records, 2011). The identification of T6SS in human pathogens can be traced back to seminal discoveries made in both *Pseudomonas aeruginosa* and *Vibrio cholerae* in which a cluster of chromosomally encoded genes have been

found to be involved in bacterial virulence (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). Indeed, multiple copies of *hcp*s and *vgrG*s have commonly been seen across all the bacteria that possess the T6SS, and, interestingly, some bacteria even possess multiple copies of the T6SS cluster (Mougous *et al.*, 2006; Podladchikova *et al.*, 2011). In *A. hydrophila*, the two effector proteins (Hcps and VgrGs) that disarm host defences by targeting genes important for innate immune cells (among other genes) cluster to only one region of the chromosome (Seshadri *et al.*, 2006; Suarez *et al.*, 2008).

Previously, we have demonstrated that Hcp is translocated into the host cytoplasm and induced apoptosis via caspase 3 activation (Suarez *et al.*, 2008) and that Hcp paralysed macrophages and prevented phagocytosis (Suarez *et al.*, 2010b). The T6SS needle conduit comprises VgrG proteins, and one current model has indicated that the carboxyl terminus of VgrG punctures the host cell membrane, allowing the active domain of VgrG and Hcp to be translocated into the host cytoplasm (Cascales, 2008). We previously showed that VgrG-1 in *A. hydrophila* ATCC 7966^T carries a C-terminal extension domain with ADP-ribosylation activity, and, through its T6SS, it targets host actin and promotes host cell apoptosis (Suarez *et al.*, 2010a). VgrGs with C-terminal extension domains have been termed 'evolved VgrGs', and another such VgrG-1 with an actin cross-linking activity domain has been reported in *Vibrio cholerae* (Pukatzki *et al.*, 2007). Interestingly, each effector has multiple copies, presumably paralogues of each other.

In this study, we have identified additional *hcp* and *vgrG* paralogues in the genome of *A. hydrophila* SSU and delineated the contributions of Hcp-1 and -2, as well as VgrG-1, -2 and -3 to T6SS function, bacterial motility, protease production and biofilm formation. Furthermore, we evaluated the contribution of each Hcp and VgrG paralog to bacterial virulence using a septicemic murine model of infection.

METHODS

Bacterial strains, plasmids and reagents. The sources of *A. hydrophila* SSU and *Escherichia coli* strains, as well as the plasmids used in this study, are listed in Table 1. These bacteria were grown in Luria-Bertani (LB) medium at 37 °C with continuous shaking (180 r.p.m.). The suicide vector used, pDMS197, has a conditional R6K origin of replication (*ori*) and a levansucrase gene (*sacB*) from *Bacillus subtilis* (Edwards *et al.*, 1998). The antibiotics tetracycline (Tc), streptomycin (Sm) and rifampicin (Rif) were obtained from Sigma and used at concentrations of 15 µg ml⁻¹, 50 µg ml⁻¹ and 100 µg ml⁻¹, respectively. Restriction endonucleases and T4 DNA ligase were obtained from Promega and New England BioLabs. The Advantage cDNA PCR kit was purchased from BD Bioscience Clontech. Chromosomal DNA from various *A. hydrophila* mutants was isolated using a QIAamp DNA Mini kit (Qiagen). The digested plasmid DNA and the DNA fragments from the agarose gel were prepared and purified using a QIAprep Miniprep kit (Qiagen).

Identification of *hcp-1* and *vgrG-1* in the genome of *A. hydrophila* SSU. Based on the genome sequences of *V. cholerae*

Table 1. Strains and plasmids used in this study

Strain or plasmid*	Relevant characteristic(s)	Source or reference
<i>A. hydrophila</i> SSU	<i>A. hydrophila</i> human diarrhoeal isolate SSU	†CDC, Atlanta, GA
SSU-R	Rifampicin resistance (Rif ^r) strain of SSU	Laboratory stock
<i>ΔvasH</i>	<i>vasH</i> deletion mutant of <i>A. hydrophila</i> SSU-R	Suarez <i>et al.</i> (2008)
<i>ΔvasK</i>	<i>vasK</i> deletion mutant of <i>A. hydrophila</i> SSU-R	Suarez <i>et al.</i> (2008)
<i>Δhcp1</i>	<i>hcp1</i> in-frame deletion mutant of <i>A. hydrophila</i> SSU-R	This study
<i>Δhcp2</i>	<i>hcp2</i> in-frame deletion mutant of <i>A. hydrophila</i> SSU-R	This study
<i>ΔvgrG1</i>	<i>vgrG1</i> in-frame deletion mutant of <i>A. hydrophila</i> SSU-R	This study
<i>ΔvgrG2</i>	<i>vgrG2</i> in-frame deletion mutant of <i>A. hydrophila</i> SSU-R	This study
<i>ΔvgrG3</i>	<i>vgrG3</i> in-frame deletion mutant of <i>A. hydrophila</i> SSU-R	This study
<i>ΔvgrG1/2</i>	<i>vgrG1</i> and <i>vgrG2</i> double in-frame deletion mutant of <i>A. hydrophila</i> SSU-R	This study
<i>ΔvgrG1/3</i>	<i>vgrG1</i> and <i>vgrG3</i> double in-frame deletion mutant of <i>A. hydrophila</i> SSU-R	This study
<i>ΔvgrG2/3</i>	<i>vgrG2</i> and <i>vgrG3</i> double in-frame deletion mutant of <i>A. hydrophila</i> SSU-R	This study
<i>ΔvgrG1/2/3</i>	<i>vgrG1</i> , <i>vgrG2</i> and <i>vgrG3</i> triple in-frame deletion mutant of <i>A. hydrophila</i> SSU-R	This study
WT/pBR322	SSU-R transformed with pBR322, Rif ^r , Tc ^r	Laboratory stock
<i>ΔvasH</i> /pBR322	<i>ΔvasH</i> transformed with pBR322, Rif ^r , Tc ^r	This study
<i>ΔvasK</i> /pBR322	<i>ΔvasK</i> transformed with pBR322, Rif ^r , Tc ^r	This study
<i>Δhcp1</i> /pBR322	<i>Δhcp1</i> transformed with pBR322, Rif ^r , Tc ^r	This study
<i>ΔvgrG1</i> /pBR322	<i>ΔvgrG1</i> transformed with pBR322, Rif ^r , Tc ^r	This study
<i>ΔvgrG2</i> /pBR322	<i>ΔvgrG2</i> transformed with pBR322, Rif ^r , Tc ^r	This study
<i>ΔvgrG3</i> /pBR322	<i>ΔvgrG3</i> transformed with pBR322, Rif ^r , Tc ^r	This study
<i>ΔvgrG2/3</i> /pBR322	<i>ΔvgrG2/3</i> transformed with pBR322, Rif ^r , Tc ^r	This study
<i>ΔvasK</i> /comp	<i>ΔvasK</i> transformed with pBR322- <i>vasK</i> , Rif ^r , Tc ^r	Suarez <i>et al.</i> (2008)
<i>Δhcp1</i> /comp	<i>Δhcp1</i> transformed with pBR322- <i>hcp1</i> , Rif ^r , Tc ^r	This study
<i>ΔvgrG1</i> /comp	<i>ΔvgrG1</i> transformed with pBR322- <i>vgrG1</i> , Rif ^r , Tc ^r	This study
<i>ΔvgrG2</i> /comp	<i>ΔvgrG2</i> transformed with pBR322- <i>vgrG2</i> , Rif ^r , Tc ^r	This study
<i>ΔvgrG3</i> /comp	<i>ΔvgrG3</i> transformed with pBR322- <i>vgrG3</i> , Rif ^r , Tc ^r	This study
<i>ΔvgrG2/3</i> /vgrG2comp	<i>ΔvgrG2/3</i> transformed with pBR322- <i>vgrG2</i> , Rif ^r , Tc ^r	This study
<i>E. coli</i> strains		
SM10(<i>λpir</i>)	Km ^r , <i>thi-1 thr leu tonA lacY supE recA::RP4-2-Tc::Mu pir</i>	Edwards <i>et al.</i> (1998)
DH5α	<i>recA</i> , <i>gyrA</i>	Laboratory stock
Plasmids		
pDMS197	A suicide vector, <i>oriT oriV sacB</i> , Tc ^r	Laboratory stock
pDMShcp1	Suicide vector pDMS197 containing upstream and downstream flanking DNA fragments to the <i>hcp-1</i> gene of <i>A. hydrophila</i> SSU; Tc ^r	This study
pDMShcp2	Suicide vector pDMS197 containing upstream and downstream flanking DNA fragments to the <i>hcp-2</i> gene of <i>A. hydrophila</i> SSU; Tc ^r	This study
pDMSvgrG1	Suicide vector pDMS197 containing upstream and downstream flanking DNA fragments to the <i>vgrG-1</i> gene of <i>A. hydrophila</i> SSU; Tc ^r	This study
pDMSvgrG2	Suicide vector pDMS197 containing upstream and downstream flanking DNA fragments to the <i>vgrG-2</i> gene of <i>A. hydrophila</i> SSU; Tc ^r	This study
pDMSvgrG3	Suicide vector pDMS197 containing upstream and downstream flanking DNA fragments to the <i>vgrG-3</i> gene of <i>A. hydrophila</i> SSU; Tc ^r	This study
pBR322	Ampicillin resistance (Ap ^r), Tc ^r	Stratagene
pBR322- <i>hcp1</i>	<i>A. hydrophila hcp1</i> gene with its putative promoter region cloned in pBR322 at the <i>EcoRI/PstI</i> sites, Tc ^r	This study
pBR322- <i>hcp2</i>	<i>A. hydrophila hcp2</i> gene with its putative promoter region cloned in pBR322 at the <i>EcoRI/PstI</i> sites, Tc ^r	This study
pBR322- <i>vgrG1</i>	<i>A. hydrophila vgrG1</i> gene with its putative promoter region cloned in pBR322 at the <i>PstI</i> sites, Tc ^r	This study
pBR322- <i>vgrG2</i>	<i>A. hydrophila vgrG2</i> gene with its putative promoter region cloned in pBR322 at the <i>PstI</i> sites, Tc ^r	This study
pBR322- <i>vgrG3</i>	<i>A. hydrophila vgrG3</i> gene with its putative promoter region cloned in pBR322 at the <i>PstI</i> sites, Tc ^r	This study

*comp, Complemented.

†CDC, Center for Disease Control and Prevention.

and *A. hydrophila* ATCC 7966 (Pukatzki *et al.*, 2006; Seshadri *et al.*, 2006), additional copies of *hcp* and *vgrG* genes, designated *hcp-1* and *vgrG-1*, were identified that mapped to chromosomal regions outside of the T6SS gene cluster. During our T6SS characterization, we noticed that two PCR amplicons/products of lengths 450 and 650 bp were amplified using the primer pair Pv1 and Pv2, which corresponded to the 3' end of *hcp-2* and the 5' end of *vgrG-2*, respectively (Table S1, available in *Microbiology Online*). For PCR, the program used was 94°C for 3 min (denaturation) followed by 30 cycles of 94°C for 1 min, 65°C for 1 min, and then 72°C for 1 min. The final extension was performed at 72°C for 7 min. DNA sequencing revealed that although both of the PCR products contained the expected portions of *hcp-2* and *vgrG-2*, the intervening DNA sequences between these two genes were not well conserved. The intervening sequence of the larger DNA fragment exactly matched the corresponding region of the T6SS cluster, while the smaller DNA fragment did not, so we designated them as *hcp-1* and *vgrG-1*, respectively. Primers Rev1 and Rev2 (Table S1) corresponding to the intervening region of the small DNA fragment were designed and synthesized. Genomic DNA of *A. hydrophila* SSU was digested with various restriction enzymes including: *Pst*I, *Sall*, *Hind*III and *Bam*HI. Following ligation, reverse PCRs were performed with the primer pair Rev1/Rev2. Two DNA fragments (2.0 kb and 4.3 kb) were PCR amplified from the *Sall*- and *Pst*I-digested genomic samples and were subsequently subjected to DNA sequencing at the Protein Chemistry Core laboratory of the University of Texas Medical Branch.

Generation of T6SS mutant strains. Based on the DNA sequences of *hcps* (1 and 2) and *vgrGs* (1, 2 and 3) of *A. hydrophila* SSU, the upstream and downstream flanking regions corresponding to the target genes were PCR amplified by using the UP and DN series of primer sets (Table S1). The flanking sequences were then in-frame ligated, through a common restriction enzyme site, and cloned into the pDMS197 suicide vector resulting in recombinant plasmids: pDMShcp1, pDMShcp2, pDMSvgrG1, pDMSvgrG2 and pDMSvgrG3, respectively. For generating single, double, or triple-knockout mutants, the recombinant plasmids were transformed (via electroporation, using a Genepulser Xcell; Bio-Rad) into the wild-type (WT) or the existing mutant strains of *A. hydrophila* SSU. The single-crossover mutants were first selected via tetracycline resistance and were then grown in LB medium without antibiotic pressure overnight at 37 °C with agitation (180 r.p.m.). The double-crossover mutants were then selected based on their losing the antibiotic resistance marker. The deletion of the corresponding target genes was verified via PCR by using the VR series of primer sets (Table S1). The in-frame deletion of the corresponding target genes from the mutants was further confirmed by genome sequencing with the CON series of primers (Table S1).

Generation of complemented strains of T6SS mutants. By using specific primer sets (Table S1), the DNA fragments containing specific genes (i.e. *hcp1*, *hcp2*, *vgrG1*, *vgrG2* and *vgrG3*) and their putative promoter regions were PCR amplified from the chromosomal DNA of *A. hydrophila* SSU. Subsequently, these fragments were ligated to the pBR322 vector at compatible restriction sites to generate recombinant plasmids (Table 1). By electroporation, these recombinant plasmids were transformed into their corresponding mutants to generate complemented strains (Table 1). As controls, empty vector plasmid pBR322 was also transformed into these mutant strains as well as the WT bacterium (Table 1).

Western blot analysis. Supernatants and cell pellets from WT and various isogenic mutants of *A. hydrophila* saturated cultures were separated by centrifugation. Cell pellets were directly lysed in SDS-PAGE loading buffer, while supernatant proteins were first precipitated with 10% TCA and then dissolved in the loading buffer. Supernatant and pellet samples were then subjected to SDS-PAGE followed by immunoblotting with mouse polyclonal antibodies raised against Hcp,

according to the procedure described by us previously (Suarez *et al.*, 2010b). In short, following SDS-PAGE, proteins were transferred to hybond-ECL nitrocellulose membranes (GE Healthcare). Membranes were blocked with 5 % non-fat milk and subsequently incubated with a 1:1000 dilution of anti-Hcp antibody for 1 h with shaking at room temperature. After 1 h of secondary antibody incubation, the blots were developed with Super Signals West Pico Chemiluminescent substrate (Pierce) followed by X-ray film exposure.

Motility assay. LB medium with 0.3 % Difco Bacto-agar (Difco Laboratories) was used to characterize the swimming motility (Kozlova *et al.*, 2008) of WT *A. hydrophila* SSU, its various isogenic mutants and their complemented strains. Saturated cultures of each *Aeromonas* strain were adjusted to the same optical density, and equal numbers of bacteria (10^6 c.f.u.) were stabbed into 0.3 % LB agar. Plates were incubated at 37 °C overnight, and motility was assayed by examining the radial migration of bacteria through the agar away from the centre stab towards the periphery of the plate.

Protease activity. Protease activity in culture filtrates of various *Aeromonas* strains was measured using our previously described procedure (Erova *et al.*, 2006). In short, protease activity was calculated per ml culture filtrate and was divided by the optical density of the culture to obtain the protease activity per unit of growth. Hide powder azure substrate (Calbiochem) was used for measuring protease activity because of the sensitivity and rapidity of the assay. Furthermore, this substrate could detect both metalloproteases and serine proteases, which are the two major classes of this enzyme produced by *Aeromonas* species.

Biofilm assay. We employed a modified biofilm ring assay, as we described previously (Khajanchi *et al.*, 2009). The *A. hydrophila* SSU WT strain, its various isogenic mutants and their corresponding complemented strains were grown directly in 3 ml LB medium contained in polystyrene tubes at 37 °C for 24 h with shaking. Biofilm formation was quantified according to a procedure described elsewhere (Morohoshi *et al.*, 2007). Finally, biofilm formation results were normalized to 1×10^9 c.f.u. to account for any minor differences in the growth rates of various bacterial strains used. The experiment was repeated independently three times.

Murine infections, bacterial dissemination and competition assay. Female, Swiss Webster mice (20 per group) from Charles River Laboratories (Wilmington, MA) were challenged via the intraperitoneal (i.p.) route with either 4×10^7 or 8×10^6 c.f.u. doses of WT or of various Hcp and VgrG mutant strains of *A. hydrophila* SSU. For complementation studies, mice (10 per group) were i.p. infected with WT/pBR322, Δ vgrG1/pBR322 and the Δ vgrG1/complemented strain (Table 1) at a dose of 4×10^7 c.f.u. Animals were assessed for morbidity and/or mortality over 10 days post-infection (d.p.i.).

For bacterial dissemination studies, mice (10 per group) were infected with WT and the Δ vgrG1/2/3 mutant of *A. hydrophila* SSU at the dose of 2×10^7 c.f.u. At 24 and 48 h p.i., five mice from each group (at each time point) were killed using carbon dioxide followed by cervical dislocation. Livers and spleens were removed immediately. Organs were mixed with 1 ml DPBS (Cellgro) and homogenized with a Kendall Disposable Tissue Grinder (Fisher HealthCare). Bacterial loads in different organs were determined by serial dilution and plating of the organ homogenates (Sha *et al.*, 2008). In a separate experiment, mice (10 per group) were infected with the WT strain or the Δ hcp1 mutant of *A. hydrophila* SSU at the dose of 8.0×10^6 c.f.u. The bacterial loads in the survivors 2 d.p.i. were evaluated.

For the competition assay, mice were i.p. challenged with a 1:1 ratio of WT *A. hydrophila* SSU (streptomycin resistance, Sm^r) and the Δ vgrG1/2/3 mutant (rifampicin resistance, Rif^r) (Table 1) at a dose of 2×10^7 or 4×10^7 c.f.u. each. We used a total of 10–15 mice in each

challenge dose group. At 48 h p.i., five mice from each challenge group were killed, and the mouse organs (spleen and liver) were processed to enumerate the bacterial load, as described above. To differentiate the WT bacterium from the $\Delta vgrG1/2/3$ mutant recovered from mouse organs, the homogenates were spread separately onto different antibiotic-containing agar plates (i.e. streptomycin versus rifampicin), and the corresponding bacterial loads were then determined. The competitive index (CI) was calculated as follows: $CI = (\text{mutant output}/\text{WT output})/(\text{mutant input}/\text{WT input})$ (Silver *et al.*, 2007).

Statistical analysis. Whenever appropriate, one-way ANOVA, Student's *t*-test, or Kaplan–Meier survival estimates were employed for statistical analysis of the data with GraphPad Prism V 4.02 for Windows (Software MacKiev). Experiments were repeated at least three times, and *P*-values of ≤ 0.05 were considered significant.

Nucleotide sequence accession number. The DNA sequences of *hcp-1* and *vgrG-1* genes as well as their flanking DNA were submitted to GenBank under accession number JX646703. The NCBI reference sequence number for *A. hydrophila* SSU genome is NZ_AGWR00000000.1.

RESULTS

T6SS gene cluster and function

We previously reported a T6SS gene cluster in *A. hydrophila* SSU that contained 20 ORFs, including one copy of *hcp* (designated *hcp-2*) and two copies of *vgrG* genes (designated *vgrG-2* and *vgrG-3*) (Suarez *et al.*, 2008). In this study, we identified additional *hcp* and *vgrG* genes (designated *hcp-1* and *vgrG-1*) located at distal genomic sites (outside of the T6SS cluster) of *A. hydrophila* SSU. Interestingly, *hcp-1* and *vgrG-1* genes were genetically linked through a 263 bp non-coding intergenic region, as was also seen in *V. cholerae* and *A. hydrophila* ATCC 7966 genomes (Pukatzki *et al.*, 2006; Seshadri *et al.*, 2006). The *hcp-1* and *hcp-2* genes of *A. hydrophila* SSU encoded almost identical proteins with 172 amino acid residues; the only differences observed were at positions 84 and 146, in which alanine and methionine in Hcp-1 were replaced with serine and leucine in Hcp-2, respectively (Fig. 1a). The three VgrGs were 681–682 aa residues in length and shared approximately 87% homology, with sequences becoming less conserved at the carboxyl termini (Fig. 1b).

To evaluate the roles of Hcps and VgrGs in the pathogenesis of an *A. hydrophila* SSU infection, their corresponding encoding genes were in-frame deleted either individually or in combinations, generating single ($\Delta hcp1$, $\Delta hcp2$, $\Delta vgrG1$, $\Delta vgrG2$, $\Delta vgrG3$), double ($\Delta vgrG1/2$, $\Delta vgrG1/3$, $\Delta vgrG2/3$) and triple ($\Delta vgrG1/2/3$) deletion mutants. Despite the successful generation of single $\Delta hcp1$ and $\Delta hcp2$ mutants, we were unable to delete both copies of *hcp*, suggesting that having at least one paralogue of *hcp* is essential for *A. hydrophila* SSU viability, but this needs to be proven. All mutants demonstrated no aberrant growth rates relative to the WT bacterium (data not shown).

The secretion of Hcp has become a reliable indicator of a functional T6SS in numerous bacteria, despite the

Hcp-encoding gene not always being found in T6SS clusters (Pukatzki *et al.*, 2009). Therefore, we evaluated the ability of each of the aforementioned nine mutant strains to secrete Hcp by using a polyclonal anti-Hcp antibody that does not distinguish between Hcp-1 and Hcp-2 (Suarez *et al.*, 2010b). As shown in Fig. 2, although all tested strains had similar levels of Hcp in the cell pellet fraction (Fig. 2b), the secretion of Hcp was abrogated in the $\Delta vgrG2$, $\Delta vgrG3$, $\Delta vgrG1/2$, $\Delta vgrG1/3$ and $\Delta vgrG1/2/3$ mutants while it was significantly increased in the $\Delta hcp2$ and $\Delta vgrG2/3$ mutant strains (Fig. 2a).

In sharp contrast, the deletion of either *hcp-1* or *vgrG-1* did not affect the secretion of Hcp (Fig. 2a) suggesting that Hcp-1 and VgrG-1 were redundant for assembly of a functional T6SS structure. These data also indicated that both VgrG-2 and VgrG-3 might play a crucial role in forming the T6SS structure, as deleting either of them reduced T6SS secretion of Hcp while deleting both resulted in dysregulated hypersecretion of Hcp (Fig. 2). Interestingly, increased secretion of Hcp was also observed in the $\Delta hcp2$ mutant similar to that of the $\Delta vgrG2/3$ double mutant (Fig. 2a), indicating that Hcp-1 and VgrG-1 also participated in forming a functional T6SS structure perhaps through a compensatory mechanism when their corresponding paralogues (i.e. *hcp-2* or *vgrG-2* and *vgrG-3*) were deleted from the genome. Thus, Hcps and VgrGs may have limited redundancy of function in terms of forming functional T6SS structures under certain circumstances.

The roles played by Hcp and VgrG proteins in bacterial motility

In addition to evaluating T6SS function, our engineered Δhcp and $\Delta vgrG$ mutant strains were evaluated for their motility. We chose to look at motility since it is required for bacterial virulence (Rabaan *et al.*, 2001). As shown in Fig. 3(a), the $\Delta hcp2$ strain's motility mirrored that of the WT strain while deleting *hcp-1* significantly increased bacterial motility, demonstrating no functional redundancy for Hcp-1 and Hcp-2 in influencing *A. hydrophila*'s motility. With regard to VgrG-encoding genes, deletion of *vgrG-3* negatively influenced motility while deletion of *vgrG-2*, in sharp contrast, positively influenced motility. The deletion of *vgrG-1* appeared to have no effect on bacterial motility (Fig. 3b). Interestingly, bacterial motility remained unchanged in all the *vgrG* double- and triple-knockout mutants (Fig. 3b), possibly due to the regulatory roles of VgrG-2 and VgrG-3 requiring the presence of VgrG-1 for reasons that remain unclear, despite VgrG-1 alone not having any direct influence on motility (Fig. 3b). In support of this statement, VgrGs have been reported to interact with one another, forming complex structures (Bröms *et al.*, 2012; Hachani *et al.*, 2011; Pukatzki *et al.*, 2007).

Production of protease

In addition to motility, the production of proteases, in particular metalloproteases, also promotes *A. hydrophila*

(a)	Hcp-1	MPTPCYISIEGKTQGNITAGAFSTSDSVGNIFVQGHEDMLVQEFQHVVTVPDTPQSGQPA	60
	Hcp-2	MPTPCYISIEGKTQGNITAGAFSTSDSVGNIFVQGHEDMLVQEFQHVVTVPDTPQSGQPA	60

	Hcp-1	GQRVHKPFKFTVALNKAVPLMYNALASGEMLPKVTCLKWYRTSVEGKQEHFFSTVLTDATI	120
	Hcp-2	GQRVHKPFKFTVALNKAVPLMYNSLASGEMLPKVTCLKWYRTSVEGKQEHFFSTVLTDATI	120
		*****;*****	
	Hcp-1	VDIDCQMPHCQDPAKSDFTQLIQVSMAYRKIDWEHTVAGTSGADDWRAPIEA	172
	Hcp-2	VDIDCQMPHCQDPAKSDFTQLIQVSLAYRKIDWEHTVAGTSGADDWRAPIEA	172
		*****;*****	
(b)	VgrG-1	MADSTGLQFTVKVGALPENTFVVAEFALDEALNRPFNLRLELASAQPDIDFGAVLDQPC	60
	VgrG-2	MADSTGLQFTVKVGALPENTFVVAEFALDEALNRPFNLRLELASAQPDIDFGAVLDQPC	60
	VgrG-3	MADSTGLQFTVKVGALPESTFVVAEFALDEALNRPFNLRLELASAQPDIDFGAVLDQPC	60

	VgrG-1	LLVWYNGELQRRVCGVVSDFAQGDSGFRRTYQLRVLPAWRLSLRQNSRIFQAQKPDEI	120
	VgrG-2	LLVWYNGELQRRVCGVVSDFAQGDSGFRRTYQLMVQPALWRLSLRQNSRIFQAQKPDEI	120
	VgrG-3	LLVWYNGELQRRVCGVVSDFAQGDSGFRRTYQLLVLPAWRLSLRQNSRIFQAQKPDEI	120
		*****;*****	
	VgrG-1	LSILLQEHGITDYAFALKNEHAKREYCVQYRESLDLDFVNRLAAEEGMFYFHEFEAGKHRI	180
	VgrG-2	LSILLQEHGITDYAFALKNEHAKREYCVQYRETGLDFVNRLAAEEGMFYFHEFEVGKHRI	180
	VgrG-3	LSILLQEHGITDYAFALKNEHAKREYCVQYRETDLDFVNRLAAEEGMFYFHEFEAGKHRI	180
		*****;*****	
	VgrG-1	VFADDAALTAQPELFFNLGNRSLEQGPYVRQFHYREAVRPSDVELKDYSFKTPAYGLSH	240
	VgrG-2	VFADDAALTAGPELFFNLGNRSLEQGPYVRQFHYREAVRPSDVELKDYSFKTPAYGLSH	240
	VgrG-3	VFADDAALTAQPELFFNLGNRSLEQGPYVRQFHYREAVRPSDVELKDYSFKTPAYGLSH	240
		*****;*****	
	VgrG-1	KKVGAELTHQRDTYQHFDPFGRYKEDPSGKAFQHRDLALRNDVAGQAKSNCAALLPGQ	300
	VgrG-2	KKVGAELTHQRDTYQHFDPFGRYKEDPSGKAFQHRDLALRNDVAGSGKSNSSALQPGQ	300
	VgrG-3	KKQGAELTHQRDTYQHFDPFGRYKQGGSGKAFQHRDLALRNDVAGSGKSNCAALLQPGQ	300
		* * * * *;*****;*****	
	VgrG-1	SFSLTEHPNGSLNTDQIVRIQHTGLQPQALEEEGGSGPTVYHNEFGVVKASTTWRARIG	360
	VgrG-2	TFSLTEHPNDLNTDQIVRIQHTGLQSQALEEEGGSGPTVYHNEFGVVKASTTWRARIG	360
	VgrG-3	TFSLTEHPNGSLNTDQIVRIQHTGLQPQALEEEGGSGPTVYHNEFGVVKASTTWRARIG	360
		*****;*****;*****	
	VgrG-1	SPEAPHKPMVDGPQIAIVVGPDEEIIYCDHGRVKLQFPWDYRGSSNDQSSCWVRVSQGW	420
	VgrG-2	SPEAPHKPMVDGPQIAIVVGPDEEIIYCDHGRVKLQFPWDYRGSSNDQSSCWVRVSQGW	420
	VgrG-3	SPEALHKPMVDGPQIAMVVGPEEIIYCDHGRVKLQFPWDYRGSSNDQSSCWVRVSQGW	420
		*****;*****;*****	
	VgrG-1	AGGQYGMMAIPRIGHEVIVSFLEGDPDQPIVTGRTYHATNRPPYELPANKTRTVLRTETH	480
	VgrG-2	AGGQYGMMAIPRIGHEVIVSFLEGDPDQPIVTGRTYHATNRPPYELPANKTRTVLRTETH	480
	VgrG-3	AGGQYGMMAIPRIGHEVIVSFLEGDPDQPIVTGRTYHATNRPPYELPANKARTVLRTETH	480
		*****;*****	
	VgrG-1	QGEFNFELRFEDQVQEEIYIHGQKDLNVLINDAAWHIKHDEHTDIDNERVTRIKANDH	540
	VgrG-2	QGEFNFELRFEDQAGKEEYIHGQKDLNVLINDAAWHIKHQHTDIDNERVTRIKANDH	540
	VgrG-3	QGEFNFELRFEDQAGQEEIYIHGQKDLNVLINNAVWHIKHDEHIDNERVTRIKANDH	540
		*****;*****;*****	
	VgrG-1	LTVEGEKRDQIKADYSLTVDTSMHQLGDSWLTQAGQEVHVKAGAKVLEAGSEITVKVG	600
	VgrG-2	LTVEGEKRDQIKADYSLTVDSLHQLGQSLLEAGSEVHHKAGMKIVMEAGAEITLKV	600
	VgrG-3	LTVEGEKRDQIKADYSLTVDSLHQLGQSLLEAGQEVHVKVGDKLVEAGSEITLKG	600
		*****;*****;*****	
	VgrG-1	GCFIKVDGGGVTLVGPTIKMNSGGSPSSGSGWGGKSPVDPLGVSVPPKPKVPLTPAQLAT	660
	VgrG-2	GSFVKIDPSGVTLSGGSIKMNSSGSGSGWAGQMPIQPGAVEVVAAPPMPDPAQIAT	660
	VgrG-3	GSFVKVDPSGIKLMGPAIKLNAGGSAGSGSGWGGKAPVVPKGVVEVKAPELVKAIPT	660
		*.:.:. * .: * .: * .: * .: * .: * .: * .: * .: * .: *	
	VgrG-1	MKSAAPFCEECEKKEGGCEI	681
	VgrG-2	LKSAEPVCEICEQLARQEGGG	681
	VgrG-3	EKAMEALLKEQGAQTFFVPSR	682
		*: .: .:	

Fig. 1. Amino acid sequence comparison between Hcp-1 and Hcp-2 (a) and among VgrG-1, VgrG-2 and VgrG-3 (b) of *A. hydrophila* SSU using online CLUSTAL 2.1 Multiple Sequence Alignments software. *Conserved amino acid residues; :, amino acid residues with strongly similar amino acid groups; ., amino acid residues with weakly similar amino acid groups.

virulence (Erova *et al.*, 2006). To determine contributions made by Hcp and/or VgrG proteins to protease activity, we measured the ability of *Aeromonas* mutant culture filtrates

to catalyse the degradation of Hide powder azure substrate. As shown in Fig. 4(a), deletion of *hcp-1* significantly increased protease activity, while deleting *hcp-2* did not

Fig. 2. Secretion of Hcp by the *A. hydrophila* SSU T6SS mutants. Immunoblot analysis was performed using polyclonal anti-Hcp antibodies to measure the production of Hcp in both the supernatants (a) and pellets (b) of WT *A. hydrophila* and its various Hcp and VgrG mutant strains.

alter this enzymic activity. With regard to VgrG-encoding genes, deletion of either *vgrG-2* or *vgrG-3* alone or in combination significantly decreased protease activity in the bacterial filtrates, indicating that VgrG-2 and VgrG-3 worked to positively influence protease activity. VgrG-1 seemed to not contribute to this virulence-associated function (Fig. 4b). However, in a seemingly contradictory manner, the $\Delta vgrG1/2$, $\Delta vgrG1/3$ and $\Delta vgrG1/2/3$ exhibited protease activity similar to that of the isogenic WT strain for reasons unknown at present (Fig. 4b).

Considering interactions of VgrGs in regulating bacterial motility (Fig. 3), it appeared most likely that VgrG-1 was playing a regulatory role rather than VgrG-2 and VgrG-3 in the production of protease. In other words, VgrG-1 negatively regulated protease production while VgrG-2 and VgrG-3 had the potential to negate this effect of VgrG-1. With this scenario in mind, it is then logical to speculate

that in the WT bacterium, the regulatory role of VgrG-1 is minimal at best in the presence of VgrG-2 and VgrG-3. However, deletion of either *vgrG-2* or *vgrG-3* or both significantly decreased the protease activity (Fig. 4b), as the possible regulatory role of VgrG-1 became apparent. In contrast, deletion of the *vgrG-1* gene such as in the $\Delta vgrG1$, $\Delta vgrG1/2$, $\Delta vgrG1/3$ and $\Delta vgrG1/2/3$ mutants did not affect the protease activity, as the remaining VgrG-2 and VgrG-3 seemed not to influence protease production (Fig. 4b).

Biofilm formation

We then measured solid-surface-associated biofilm formation of our T6SS mutant strains. We chose to evaluate biofilm formation since, like motility and protease production, it is also a virulence-associated function, enabling the bacteria to adhere to surfaces, resist phagocytosis and resist antibiotic-mediated toxicity. Following 24 h of growth and

Fig. 3. Swimming motility assay of the *A. hydrophila* SSU T6SS mutants. Semi-solid media plates were inoculated with WT and various Hcp (a) and VgrG (b) mutant strains of *A. hydrophila*. The plates were incubated at 37 °C overnight, and the distances of bacterial migration (cm) through the agar from the centre towards the periphery of the plate were measured. Student's *t*-test was used for data analysis, and a single asterisk represents a statistically significant difference relative to the WT strain with a *P*-value of <0.05, while a double asterisk represents a statistically significant difference relative to the WT strain with a *P*-value of <0.001. Both images of the motility on plates and quantification of the migration are shown.

Fig. 4. Protease activity of the *A. hydrophila* SSU T6SS mutants. The overnight culture filtrates from WT and various Hcp (a) and VgrG (b) mutant strains of *A. hydrophila* were mixed with 5 mg Hide powder azure substrate and incubated in a shaker incubator at 37 °C for 1–3 h. As the protease in the culture filtrates degraded the substrate, blue colour was released and quantified at OD₅₉₅. The protease activity was calculated per ml of culture filtrate per 10⁸ c.f.u. Student's *t*-test was used for the data analysis, and a single asterisk represents a statistically significant difference relative to the WT strain with a *P*-value of <0.05, while a double asterisk represents a statistically significant difference relative to the WT strain with a *P*-value of <0.001.

crystal violet staining, neither Hcp-1 nor Hcp-2 appeared to influence biofilm formation (Fig. 5a). Unlike Hcp-1 and -2, deletion of the VgrG-encoding genes generally resulted in decreased biofilm formation, and the significantly reduced biofilm formation observed in the $\Delta vgrG1/2/3$ mutant strain indicated that all three VgrGs participated in promoting biofilm formation of *A. hydrophila* SSU (Fig. 5b). Interestingly, the greatest reduction in biofilm formation was observed in $\Delta vgrG2$ and $\Delta vgrG2/3$ followed by $\Delta vgrG1/2/3$, $\Delta vgrG3$ and $\Delta vgrG1/2$ mutants, while the decrease was not apparent in the $\Delta vgrG1$ and $\Delta vgrG1/3$ mutants. These data indicated that different VgrG complexes formed in the

various mutants might contribute to these variations and those observed in the motility and protease production experiments (Figs 3 and 4).

Mouse survival

To evaluate the virulence potential of the various T6SS mutants, we employed our i.p. murine infection model (Erova *et al.*, 2006; Khajanchi *et al.*, 2009; Sierra *et al.*, 2010, 2011). Following a challenge dose of 4.0×10^7 c.f.u. with all of the $\Delta vgrG$ mutant strains, we noticed that the most significant attenuations were seen in the $\Delta vgrG1$, $\Delta vgrG2$,

Fig. 5. Biofilm formation by *A. hydrophila* SSU T6SS mutants. A crystal violet tube-based assay was used to detect biofilm production of WT and various Hcp (a) and VgrG (b) mutant strains of *A. hydrophila* SSU. The bar graph of optical quantification (OD₅₇₀) of biofilm formation and an image of the actual crystal violet (CV) staining tubes of WT and various VgrG mutants are shown. Student's *t*-test was used to analyse the data, and an asterisk represents a statistically significant difference relative to the WT strain with a *P*-value of ≤ 0.05 .

$\Delta vgrG1/2$, $\Delta vgrG1/3$ and $\Delta vgrG1/2/3$ mutants, resulting in mice survival rates of 90–100 %. The $\Delta vgrG2/3$ and $\Delta vgrG3$ infections resulted in mouse survival rates of 60–70 %. In sharp contrast to both these groups, only 10 % of WT-infected mice survived following the i.p. challenge (Fig. 6a). With the exception of the difference between $\Delta vgrG1$ and $\Delta vgrG3$, no statistically significant differences were observed among the 90–100 % survival rate group and the 60–70 % survival rate groups. Surprisingly, the $\Delta hcp1$ strain appeared to suffer no attenuation in virulence resulting in only a 20 % survival rate in infected mice, closely resembling the survival rates of WT-infected mice (data not shown).

To better delineate the role of Hcps *in vivo*, we challenged mice with either $\Delta hcp1$ or $\Delta hcp2$ at a slightly lower infectious dose of 8×10^6 c.f.u. Interestingly, at this bacterial challenge dose, we were able to detect contributions made by both Hcp paralogues to bacterial virulence. The $\Delta hcp1$ -infected mice demonstrated 60 % survival up to day 10 p.i., while $\Delta hcp2$ -infected mice were 100 % protected up to day 10 p.i., a statistically significant difference between the two groups ($P < 0.005$) (Fig. 6b). Mice infected with the WT strain at the same infectious dose had only 30 % survival rate at day 10 p.i. (Fig. 6b). These data indicated that both Hcps contributed to the overall bacterial virulence in mice.

In vivo bacterial dissemination

For dissemination studies, we evaluated spread of the WT, $\Delta vgrG1/2/3$ and $\Delta hcp1$ mutant strains into mouse livers and spleens following i.p. challenge. We chose the $\Delta vgrG1/2/3$

triple mutant since it is the best representative for evaluating the overall role of VgrGs. The $\Delta hcp1$ mutant was picked because it displayed an attenuated phenotype at a relatively low dose (Fig. 6b), and, thus, its further evaluation was warranted to confirm its attenuation. Following a 2.0×10^7 c.f.u. i.p. challenge with either the $\Delta vgrG1/2/3$ or the WT strain, at day 1 p.i., all 10 mice were dead in the WT-infected group with average bacterial counts of 1.5×10^7 and 1.6×10^8 c.f.u./organ in the liver and spleen, respectively (Fig. 7a). In the $\Delta vgrG1/2/3$ mutant-infected group, all 10 mice were alive 1 d.p.i., and of five examined mice, four of them had bacteria-free organs and only one mouse registered bacterial counts of 3.3×10^6 and 2.2×10^7 c.f.u./organ in its liver and spleen, respectively (Fig. 7a).

At 2 d.p.i., two dead mice challenged with the $\Delta vgrG1/2/3$ strain registered average bacterial counts of 1.0×10^7 and 1.8×10^6 c.f.u./organ in the liver and spleen, respectively, while no bacteria were detected in the organs of the three remaining surviving mice. No day 2 data were available for the WT-infected group since all of the mice died on the first day p.i. (Fig. 7b). We also evaluated bacterial dissemination to the livers and spleens of $\Delta hcp1$ -infected animals following an 8.0×10^6 c.f.u. i.p. challenge. By day 2 p.i., there were no observed $\Delta hcp1$ bacterial loads in liver or spleen. However, two animals in the WT-infected group had hepatic bacterial loads of 1.6×10^6 and 2.5×10^8 c.f.u./organ, respectively. Likewise, one animal exhibited a high splenic bacterial load of 1.2×10^7 c.f.u. (Fig. 7c). Taken together, we interpreted these data to indicate that the $\Delta vgrG1/2/3$ and $\Delta hcp1$ mutant were likely to be attenuated in virulence by virtue of their possible defect in disseminating into the periphery and their likely rapid clearance by the host immune system at 2 d.p.i.

Fig. 6. Survival curves of mice infected with *A. hydrophila* SSU T6SS mutants. Groups of 20 female, Swiss Webster mice were i.p. challenged with *A. hydrophila* SSU WT or various VgrG mutant strains at the dose of 4×10^7 c.f.u. (a) or *A. hydrophila* SSU WT and various Hcp mutant strains at the dose of 8×10^6 c.f.u. (b). Mouse survival was recorded during the course of the experiments, and the Kaplan–Meier survival estimate was used to analyse the survival of mice. The *P*-values represented statistically significant differences relative to the WT-infected group.

Fig. 7. Bacterial dissemination of *A. hydrophila* SSU T6SS mutants in infected mice. *In vivo* dissemination of the $\Delta vgrG1/2/3$ mutant relative to the WT strain following a 2×10^7 c.f.u. i.p. challenge was measured by viable c.f.u. plate counts of homogenized mice livers and spleens on day 1 (a) and day 2 (b) post-infection (p.i.). Similarly, *in vivo* dissemination of the $\Delta hcp1$ mutant relative to the WT strain following an 8×10^6 c.f.u. i.p. challenge was measured by viable c.f.u. plate counts of homogenized livers and spleens on day 2 p.i. (c). Average counts are represented as a horizontal bar with individual counts represented by circles. 'No survivors' denotes mice that had expired prior to sampling.

In vivo bacterial competition

To further confirm the dissemination data, we performed an *in vivo* bacterial competition assay. Mice were either 2×10^7 or 4×10^7 c.f.u. i.p. challenged with a 1:1 ratio of WT *A. hydrophila* SSU (Sm^r) and its isogenic $\Delta vgrG1/2/3$ (Rif^r) mutant (Table 1). At 48 h p.i., both hepatic and splenic bacterial loads of WT and $\Delta vgrG1/2/3$ strains in five mice were determined and differentiated according to their antibiotic resistance patterns. As shown in Fig. 8a, in the low challenge dose group, WT bacteria were recovered from livers and spleens of mice at 2.2×10^6 c.f.u./organ and

5.4×10^5 c.f.u./organ, respectively, while only a minimal number of mutant bacteria were isolated from these organs; the average CIs were determined to be between 10^{-5} and 10^{-4} in livers and spleens. As mentioned earlier, there was no *in vitro* growth defect noted for the $\Delta vgrG1/2/3$ mutant when compared to the WT bacterium.

A similar pattern was observed in the higher bacterial challenge dose group (Fig. 8b), in which WT bacterial counts were 5.2×10^6 c.f.u./organ (livers) and 8.6×10^6 c.f.u./organ (spleens), respectively. These bacterial numbers for the $\Delta vgrG1/2/3$ mutant were 2–3 orders of magnitude

Fig. 8. Competitive growth of WT *A. hydrophila* SSU and the T6SS mutant $\Delta vgrG1/2/3$ in mice. Mice were i.p. infected with a mixed culture of WT bacteria (streptomycin-resistant, Sm^r) and its $\Delta vgrG1/2/3$ mutant (rifampicin resistant, Rif^r) (Table 1) at the ratio of 1 : 1 with doses of 2×10^7 c.f.u. (a) and 4×10^7 c.f.u. (b). At 48 h p.i., the bacterial loads of WT and those of $\Delta vgrG1/2/3$ mutant in livers and spleens from five mice were determined according to their antibiotic resistance patterns. The CI in the liver and spleen [calculated as (mutant output/competitor output)/(mutant input/competitor input)] for each animal is shown. The horizontal lines represent mean CI values. Student's *t*-test was used to analyse the bacterial loads, and an asterisk represents a statistically significant difference relative to the WT strain (Sm^r) with $P < 0.05$.

lower, with 2.0×10^4 c.f.u./organ in livers and 3.6×10^3 c.f.u./organ in spleens. The average CIs were calculated to be 0.05 and 0.003 in the livers and spleens, respectively. These data clearly demonstrated that the WT bacteria out-competed the $\Delta vgrG1/2/3$ mutant in mice, and that the host immune system began to clear the $\Delta vgrG1/2/3$ mutant by 48 h p.i.

Complementation of the T6SS mutants

We complemented the mutants to possibly rule out any polar effects or secondary mutations contributing to the phenotypes of our in-frame deleted mutant strains. To complement the mutants, we used the pBR322 plasmid vector (Table 1), and a typical phenotypic alteration that was associated with each of the mutants was selected for evaluation. As shown in Fig. 9, biofilm formation was significantly decreased in the $\Delta vgrG2$ mutant harbouring the pBR322 vector alone; this phenotype was fully complemented in the $\Delta vgrG2$ complemented strain with the latter behaving similarly to that of the WT bacterium (Fig. 9a).

The single-deletion mutants, namely *hcp1*, *vgrG2* and *vgrG3*, demonstrated significantly altered bacterial motilities (Fig. 9b); however, *in-trans* complementation of these mutants resulted in restoration of bacterial motility to WT-like levels. Interestingly, suppressed motility was observed

for the double mutant $\Delta vgrG2/3$ when complemented with the ectopically expressed *vgrG2* gene despite the fact that the double mutant did not exhibit altered bacterial motility when compared with the WT bacterium. Furthermore, the complemented strain ($\Delta vgrG2/3/vgrG2$ comp) behaved similarly to the single mutant $\Delta vgrG3$, which displayed decreased/suppressed motility phenotype compared with the WT bacterium (Fig. 9b). These data further emphasized a possible role of at least *VgrG2* as a negative motility regulator in the presence of *VgrG1*.

In terms of protease activity, when the $\Delta hcp1$ mutant was complemented, the increased protease activity that was noted for the $\Delta hcp1$ mutant was decreased to WT-like levels (Fig. 9c). Surprisingly, no alterations of the aforementioned phenotypes were observed when the T6SS regulatory gene *vasH* was deleted ($\Delta vasH$) (Fig. 9a–c). On the other hand, when a T6SS structural component gene *vasK* ($\Delta vasK$) was deleted, a significant decrease in biofilm formation was noted. However, the protease activity and swimming motility associated with this mutant remained unchanged when compared with that of the WT bacterium (Fig. 9a–c). Considering that both $\Delta vasH$ and $\Delta vasK$ mutants displayed an attenuated phenotype in mice, an effect that was complemented (Suarez *et al.*, 2008), the alterations in the aforementioned *in vitro* phenotypes observed for the *hcp* and *vgrG* mutants do not seem to contribute to the overall

Fig. 9. Complementation of the phenotypic alterations associated with various *A. hydrophila* SSU T6SS mutants. The T6SS genes (i.e. *hcps* and *vgrGs*) with their putative promoter regions were cloned into plasmid pBR322 for complementing the corresponding T6SS mutants (Table 1). The complemented (comp) strains, along with the mutants, the WT bacterium and the *ΔvasH* as well as *ΔvasK* mutants (with inactive T6SS) were evaluated for biofilm formation (a), swimming motility (b), protease production (c), and virulence in mice (d). The Kaplan–Meier survival estimate was used to analyse the survival of mice. The *P*-values represented statistically significant differences relative to the WT-infected group. Student's *t*-test was used to analyse the data in other assays, and an asterisk represents a statistically significant difference relative to the WT bacterium with a *P*-value of <0.05.

bacterial virulence *in vivo*. However, these *hcp* and *vgrG* mutants might require a functional T6SS to demonstrate altered *in vitro* phenotypes. This was partly confirmed by the fact that deletion of *vasK* from the WT bacterium did attenuate biofilm formation, an effect that was successfully complemented.

Finally, to evaluate the complemented *ΔvgrG1* strain, we employed the mouse infection model as deletion of the *vgrG1* significantly attenuated the virulence of *A. hydrophila* SSU (Fig. 6a). Mice were i.p. challenged with 4×10^7 c.f.u. WT/pBR322, *ΔvgrG1*/pBR322 and its complemented strain, *ΔvgrG1*/comp. As shown in Fig. 9d, the WT bacterium killed 100% of infected mice by day 3 p.i., while all mice survived in the *ΔvgrG1*/pBR322-infected group. Importantly, 60% of mice infected with the *ΔvgrG1* complemented strain (*ΔvgrG1*/comp) died, a rate which was statistically compatible with that of the WT-infected group.

Unfortunately, the recombinant plasmid pBR322 harbouring the *hcp2* gene in the *Δhcp2* mutant was not successfully retained in spite of repeated attempts. In Table 2, we have provided summary of *in vitro* and *in vivo* phenotypic alterations observed in various T6SS mutants as well as their complementation profiles.

DISCUSSION

Studies have shown that Hcps and VgrGs are not only T6SS structural components but also effector proteins (Pukatzki *et al.*, 2009; Silverman *et al.*, 2012). The macromolecular structure of the T6SS has not yet been fully resolved, and it is not known how the T6SS machine assembles or delivers effectors. Based on recent studies (Ballister *et al.*, 2008; Basler *et al.*, 2012; Hood *et al.*, 2010; Leiman *et al.*, 2009; Pukatzki *et al.*, 2007), Hcps and VgrGs are believed to form a pilus that is displayed on the bacterial surface. The VgrG

Table 2. Phenotypic alterations of various T6SS mutants of *A. hydrophila* compared with the WT bacterium

	Secretion of Hcp	Motility	Protease activity	Biofilm formation	Virulence in mice
<i>Δhcp1</i>	No	Increased*	Increased*	No	Attenuated
<i>Δhcp2</i>	Increased	No	No	No	Attenuated
<i>ΔvgrG1</i>	No	No	No	No	Attenuated*
<i>ΔvgrG2</i>	Abrogated	Increased*	Decreased	Decreased*	Attenuated
<i>ΔvgrG3</i>	Abrogated	Decreased*	Decreased	Decreased	Attenuated
<i>ΔvgrG1/2</i>	Abrogated	No	No	Decreased	Attenuated
<i>ΔvgrG1/3</i>	Abrogated	No	No	No	Attenuated
<i>ΔvgrG2/3</i>	Increased	No	Decreased	Decreased	Attenuated
<i>ΔvgrG1/2/3</i>	Abrogated	No	No	Decreased	Attenuated
<i>ΔvasH</i>	Abrogated	No	No	No	Attenuated*
<i>ΔvasK</i>	Abrogated	No	No	Decreased*	Attenuated*

*Selected phenotypes that were examined for complementation. These phenotypes were successfully restored. No, No significant change compared with the WT bacterium.

molecules are assigned a dual function, both as membrane-puncturing tips at the end of an Hcp tube and, in the case of evolved VgrGs (e.g. the VgrG-1 from *V. cholerae* and *A. hydrophila*), as an effector molecule through the activity of the C-terminal effector domain (Pukatzki *et al.*, 2007, 2009; Suarez *et al.*, 2010a).

Despite the T6SS paralogues being highly conserved (or almost identical in the case of Hcp-1 and Hcp-2), their diverse genomic locations raised several questions. Do all the paralogues play an active role in T6SS function or other physiological processes? Do they have functional redundancies? To answer these questions, we generated various Hcp and VgrG mutants and assessed them for T6SS function, motility, protease activity, biofilm formation and virulence in mice. Our data clearly demonstrated that deleting *hcp* and *vgrG* paralogues altered the aforementioned bacterial functions.

Surprisingly, however, there was limited to no observed functional redundancy among and/or between the aforementioned T6SS effector paralogues in multiple assays. More specifically, the deletion of *hcp-2*, *vgrG-2* and *vgrG-3* altered the secretion function of the T6SS, while Hcp-1 and VgrG-1 did not influence this feature. This correlated well with their genetic locations; the *hcp* and *vgrG* paralogues within the T6SS cluster were more involved in forming T6SS structures, while Hcp-1 and VgrG-1 located outside the T6SS cluster mainly functioned as effectors, and their structural roles were observed only when their corresponding paralogues (i.e. Hcp-2 or VgrG-2 and VgrG-3) were deleted from the genome. Increased Hcp secretion in the *Δhcp2* and *ΔvgrG2/3* mutants (in which only Hcp-1 or VgrG-1 was produced) suggested that Hcp and VgrG paralogues might be regulated differently and may possess different regulatory elements (e.g. transcription factor binding sites in their respective promoters). To date, VgrG-1 is the sole effector physically shown to be translocated into eukaryotic cells through the T6SS in *V. cholerae* and *A. hydrophila* (Ma *et al.*, 2009; Pukatzki *et al.*,

2007; Suarez *et al.*, 2010a). The lack of functional redundancy of the VgrGs was also observed in the T6SS of *V. cholerae* in which deletion of *vgrG-1* or *vgrG-2* blocked the secretion of Hcp, while the deletion of *vgrG-3* had no effect on that feature (Pukatzki *et al.*, 2007).

In addition to puncturing targeted eukaryotic cell membranes, the T6SS has also been reported to regulate gene expression involved in bacterial motility, biofilm formation and biofilm-specific antibiotic resistance (Aschtgen *et al.*, 2008; Das *et al.*, 2002; Enos-Berlage *et al.*, 2005; Weber *et al.*, 2009; Zhang *et al.*, 2011). Indeed, our current study also demonstrated that the Hcp and VgrG paralogues possess such regulatory functions. However, still, there is no functional redundancy among the paralogues. For example, deletion of *hcp-1* led to increased bacterial motility and protease production, while no such feature was observed in the *Δhcp2* mutant. With regard to the VgrG paralogues, VgrG-2 worked to negatively influence motility, while VgrG-3 worked to positively influence motility; VgrG-1 did not influence motility directly, but it was required for the actions of VgrG-2 and VgrG-3. As for the protease activity, VgrG-1 negatively influenced protease production in the absence of VgrG-2 and VgrG-3. With regard to biofilm formation, although all VgrGs played a role, the greatest reduction of biofilm formation occurred in *ΔvgrG2* and *ΔvgrG2/3* followed by *ΔvgrG1/2/3*, *ΔvgrG3* and *ΔvgrG1/2* mutants; there was no observed decrease in biofilm production in *ΔvgrG1* and *ΔvgrG1/3* mutant strains. Collectively, these data highlighted the lack of functional redundancy in these T6SS effector paralogues and emphasized the importance of interaction of the paralogues in regulating bacterial-virulence-associated features. Indeed, it was reported that only VgrG-2 of *V. cholerae* was essential for Hcp secretion as well as killing of amoebae and bacteria, while VgrG-1 was essential for Hcp secretion and amoebae killing, but not required for *E. coli* killing. The interactions of VgrGs were believed to be responsible for these functional variations (Zheng *et al.*, 2011); however, the exact mechanisms

associated with such functional variations of Hcp and VgrG paralogues need to be further explored and elucidated.

Although the T6SS function was abrogated in the $\Delta vasH$ mutant resulting in an attenuated phenotype in mice (Suarez *et al.*, 2008), surprisingly, no alterations of the aforementioned *in vitro* phenotypes (i.e. motility, protease production or biofilm formation) were observed in the $\Delta vasH$ mutant compared to the WT bacterium (Fig. 9a–c). Since VasH is a σ_{54} -dependent transcriptional regulator, it could play a regulatory role beyond the T6SS. Another possible explanation is that deletion of the *vasH* gene compensates for the regulatory effects associated with specific deletions of *hcp* and *vgrG* paralogues, as all of them were equally affected in the $\Delta vasH$ mutant. With regard to the $\Delta vasK$ mutant, it displayed significantly less biofilm formation but unaltered protease production and swimming motility compared with the WT bacterium (Fig. 9). The $\Delta vasK$ mutant was attenuated in mice, and, interestingly, the Hcp secretion was also blocked *in vitro* in this mutant (Suarez *et al.*, 2008). Therefore, the phenotypic alterations displayed by the $\Delta vasK$ mutant exactly mirrored those seen in the $\Delta vgrG1/2/3$ mutant (Figs 2, 3, 4, 5 and 7). Considering the structural roles played by VasK and the VgrGs, the T6SS may have a direct influence on bacterium biofilm formation, while other *in vitro* phenotypic alterations (such as protease production and swimming motility associated with individual Hcp or VgrG null mutants) could be due to indirect effects that the absence of corresponding Hcp or VgrG proteins have on T6SS function. Interestingly, in Fig. 9a, we noted that the biofilm formation by the $\Delta vgrG2$ mutant was even more severely impaired compared with the $\Delta vasK$ mutant. In the future, we will delete the *vgrG2* gene from the $\Delta vasK$ background strain to discern whether the effects of VasK and VgrG2 are T6SS-dependent or are regulatory in nature.

One of our most intriguing findings was that the single deletion of *vgrG-1* led to the most significant attenuation in mice. This was interesting since, unlike in other *vgrG* mutants, the deletion of *vgrG-1* led to no or limited effect on motility (Fig. 3b), protease activity (Fig. 4b) and biofilm formation (Fig. 5b). Furthermore, T6SS function seemed unaffected in $\Delta vgrG1$ (Fig. 2a). However, considering the primary role of VgrG-1 as the chief T6SS effector, the loss of T6SS function was likely to be the main cause for the attenuation in *A. hydrophila* SSU. Similarly, the attenuation observed in the $\Delta hcp1$ mutant was also likely to be due to its nature as a T6SS effector; however, since $\Delta hcp1$ still produced VgrG-1 and exhibited increased motility and protease production, the $\Delta hcp1$ mutant was less attenuated than the other tested mutants. Interestingly, secretion of Hcp was increased in the $\Delta hcp2$ and $\Delta vgrG2/3$ mutants, despite the two mutants being avirulent in mice. Perhaps in the two aforementioned mutants, the dysregulation of the T6SS led to the uncontrolled secretion of Hcp and ultimate dysfunction of the system as a whole. Alternatively, it is also plausible that the mutants could have regulatory effects that were independent of the T6SS.

In this study, we sought to expand our understanding of the *A. hydrophila* T6SS by dissecting the roles played by the three VgrG and two Hcp paralogues in T6SS function, motility, protease activity, biofilm formation and virulence in mice. As it turned out, there was limited to no observed functional redundancy among and/or between the aforementioned T6SS effector paralogues. Although deletion of Hcp and VgrG paralogues affected a number of virulence-associated features, the dysfunction of the T6SS was still the major contributor to the attenuation of these mutants observed in mice, emphasizing the importance of the T6SS in the pathogenesis of *Aeromonas* infections.

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REFERENCES

- Altwegg, M., Martinetti Lucchini, G., Lüthy-Hottenstein, J. & Rohrbach, M. (1991). *Aeromonas*-associated gastroenteritis after consumption of contaminated shrimp. *Eur J Clin Microbiol Infect Dis* **10**, 44–45.
- Aschtgen, M. S., Bernard, C. S., De Bentzmann, S., Lloubès, R. & Cascales, E. (2008). SciN is an outer membrane lipoprotein required for type VI secretion in enteroaggregative *Escherichia coli*. *J Bacteriol* **190**, 7523–7531.
- Ballister, E. R., Lai, A. H., Zuckermann, R. N., Cheng, Y. & Mougous, J. D. (2008). *In vitro* self-assembly of tailorable nanotubes from a simple protein building block. *Proc Natl Acad Sci U S A* **105**, 3733–3738.
- Basler, M., Pilhofer, M., Henderson, G. P., Jensen, G. J. & Mekalanos, J. J. (2012). Type VI secretion requires a dynamic contractile phage tail-like structure. *Nature* **483**, 182–186.
- Bröms, J. E., Meyer, L., Lavander, M., Larsson, P. & Sjöstedt, A. (2012). DotU and VgrG, core components of type VI secretion systems, are essential for *Francisella* LVS pathogenicity. *PLoS ONE* **7**, e34639.
- Brouqui, P. & Raoult, D. (2001). Endocarditis due to rare and fastidious bacteria. *Clin Microbiol Rev* **14**, 177–207.
- Cascales, E. (2008). The type VI secretion toolkit. *EMBO Rep* **9**, 735–741.
- Castillo, M. (2012). Flesh-eating infection victim Aimee Copeland recovering in rehab with positive outlook. http://www.cbsnews.com/8301-504763_162-57466495-10391704/flesh-eating-infection-victim-aimee-copeland-recovering-in-rehab-with-positive-outlook/.
- Chopra, A. K. & Houston, C. W. (1999). Enterotoxins in *Aeromonas*-associated gastroenteritis. *Microbes Infect* **1**, 1129–1137.
- Chopra, A. K., Houston, C. W., Peterson, J. W. & Jin, G. F. (1993). Cloning, expression, and sequence analysis of a cytolytic enterotoxin gene from *Aeromonas hydrophila*. *Can J Microbiol* **39**, 513–523.
- Chopra, A. K., Peterson, J. W., Xu, X. J., Coppenhaver, D. H. & Houston, C. W. (1996). Molecular and biochemical characterization

- of a heat-labile cytotoxic enterotoxin from *Aeromonas hydrophila*. *Microb Pathog* **21**, 357–377.
- Das, S., Chakraborty, A., Banerjee, R. & Chaudhuri, K. (2002). Involvement of in vivo induced *icmF* gene of *Vibrio cholerae* in motility, adherence to epithelial cells, and conjugation frequency. *Biochem Biophys Res Commun* **295**, 922–928.
- Edberg, S. C., Browne, F. A. & Allen, M. J. (2007). Issues for microbial regulation: *Aeromonas* as a model. *Crit Rev Microbiol* **33**, 89–100.
- Edwards, R. A., Keller, L. H. & Schifferli, D. M. (1998). Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression. *Gene* **207**, 149–157.
- Enos-Berlage, J. L., Guvener, Z. T., Keenan, C. E. & McCarter, L. L. (2005). Genetic determinants of biofilm development of opaque and translucent *Vibrio parahaemolyticus*. *Mol Microbiol* **55**, 1160–1182.
- Erova, T. E., Pillai, L., Fadl, A. A., Sha, J., Wang, S., Galindo, C. L. & Chopra, A. K. (2006). DNA adenine methyltransferase influences the virulence of *Aeromonas hydrophila*. *Infect Immun* **74**, 410–424.
- Galindo, C. L., Sha, J., Fadl, A. A., Pillai, L. & Chopra, A. K. (2006). Host immune responses to *Aeromonas* virulence factors. *Curr Immunol Rev* **2**, 13–26.
- Hachani, A., Lossi, N. S., Hamilton, A., Jones, C., Bleves, S., Albesa-Jové, D. & Filloux, A. (2011). Type VI secretion system in *Pseudomonas aeruginosa*: secretion and multimerization of VgrG proteins. *J Biol Chem* **286**, 12317–12327.
- Hood, R. D., Singh, P., Hsu, F., Güvener, T., Carl, M. A., Trinidad, R. R., Silverman, J. M., Ohlson, B. B., Hicks, K. G. & other authors (2010). A type VI secretion system of *Pseudomonas aeruginosa* targets a toxin to bacteria. *Cell Host Microbe* **7**, 25–37.
- Janda, J. M., Guthertz, L. S., Kokka, R. P. & Shimada, T. (1994). *Aeromonas* species in septicemia: laboratory characteristics and clinical observations. *Clin Infect Dis* **19**, 77–83.
- Khajanchi, B. K., Sha, J., Kozlova, E. V., Erova, T. E., Suarez, G., Sierra, J. C., Popov, V. L., Horneman, A. J. & Chopra, A. K. (2009). *N*-acylhomoserine lactones involved in quorum sensing control the type VI secretion system, biofilm formation, protease production, and *in vivo* virulence in a clinical isolate of *Aeromonas hydrophila*. *Microbiology* **155**, 3518–3531.
- Kirov, S. M. (1993). The public health significance of *Aeromonas* spp. in foods. *Int J Food Microbiol* **20**, 179–198.
- Kozlova, E. V., Popov, V. L., Sha, J., Foltz, S. M., Erova, T. E., Agar, S. L., Horneman, A. J. & Chopra, A. K. (2008). Mutation in the *S*-ribosylhomocysteinase (*luxS*) gene involved in quorum sensing affects biofilm formation and virulence in a clinical isolate of *Aeromonas hydrophila*. *Microb Pathog* **45**, 343–354.
- Kühn, I., Albert, M. J., Ansaruzzaman, M., Bhuiyan, N. A., Alabi, S. A., Islam, M. S., Neogi, P. K., Huys, G., Janssen, P. & other authors (1997). Characterization of *Aeromonas* spp. isolated from humans with diarrhea, from healthy controls, and from surface water in Bangladesh. *J Clin Microbiol* **35**, 369–373.
- Leiman, P. G., Basler, M., Ramagopal, U. A., Bonanno, J. B., Sauder, J. M., Pukatzki, S., Burley, S. K., Almo, S. C. & Mekalanos, J. J. (2009). Type VI secretion apparatus and phage tail-associated protein complexes share a common evolutionary origin. *Proc Natl Acad Sci U S A* **106**, 4154–4159.
- Ma, A. T., McAuley, S., Pukatzki, S. & Mekalanos, J. J. (2009). Translocation of a *Vibrio cholerae* type VI secretion effector requires bacterial endocytosis by host cells. *Cell Host Microbe* **5**, 234–243.
- Merino, S., Rubires, X., Knochel, S. & Tomas, J. M. (1995). Emerging pathogens: *Aeromonas* spp. *Int J Food Microbiol* **28**, 157–168.
- Morohoshi, T., Shiono, T., Takidouchi, K., Kato, M., Kato, N., Kato, J. & Ikeda, T. (2007). Inhibition of quorum sensing in *Serratia marcescens* AS-1 by synthetic analogs of *N*-acylhomoserine lactone. *Appl Environ Microbiol* **73**, 6339–6344.
- Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A., Goodman, A. L., Joachimiak, G., Ordoñez, C. L. & other authors (2006). A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. *Science* **312**, 1526–1530.
- Palú, A. P., Gomes, L. M., Miguel, M. A., Balassiano, I. T., Queiroz, M. L., Freitas-Almeida, A. C. & de Oliveira, S. S. (2006). Antimicrobial resistance in food and clinical *Aeromonas* isolates. *Food Microbiol* **23**, 504–509.
- Podladchikova, O., Antonenka, U., Heesemann, J. & Rakin, A. (2011). *Yersinia pestis* autoagglutination factor is a component of the type six secretion system. *Int J Med Microbiol* **301**, 562–569.
- Pukatzki, S., Ma, A. T., Sturtevant, D., Krastins, B., Sarracino, D., Nelson, W. C., Heidelberg, J. F. & Mekalanos, J. J. (2006). Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. *Proc Natl Acad Sci U S A* **103**, 1528–1533.
- Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D. & Mekalanos, J. J. (2007). Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. *Proc Natl Acad Sci U S A* **104**, 15508–15513.
- Pukatzki, S., McAuley, S. B. & Miyata, S. T. (2009). The type VI secretion system: translocation of effectors and effector-domains. *Curr Opin Microbiol* **12**, 11–17.
- Rabaan, A. A., Gryllos, I., Tomás, J. M. & Shaw, J. G. (2001). Motility and the polar flagellum are required for *Aeromonas caviae* adherence to HEp-2 cells. *Infect Immun* **69**, 4257–4267.
- Records, A. R. (2011). The type VI secretion system: a multipurpose delivery system with a phage-like machinery. *Mol Plant Microbe Interact* **24**, 751–757.
- Seshadri, R., Joseph, S. W., Chopra, A. K., Sha, J., Shaw, J., Graf, J., Haft, D., Wu, M., Ren, Q. & other authors (2006). Genome sequence of *Aeromonas hydrophila* ATCC 7966^T: jack of all trades. *J Bacteriol* **188**, 8272–8282.
- Sha, J., Agar, S. L., Baze, W. B., Olano, J. P., Fadl, A. A., Erova, T. E., Wang, S., Foltz, S. M., Suarez, G. & other authors (2008). Braun lipoprotein (Lpp) contributes to virulence of *Yersinia*: potential role of Lpp in inducing bubonic and pneumonic plague. *Infect Immun* **76**, 1390–1409.
- Sierra, J. C., Suarez, G., Sha, J., Foltz, S. M., Popov, V. L., Galindo, C. L., Garner, H. R. & Chopra, A. K. (2007). Biological characterization of a new type III secretion system effector from a clinical isolate of *Aeromonas hydrophila*—part II. *Microb Pathog* **43**, 147–160.
- Sierra, J. C., Suarez, G., Sha, J., Baze, W. B., Foltz, S. M. & Chopra, A. K. (2010). Unraveling the mechanism of action of a new type III secretion system effector AexU from *Aeromonas hydrophila*. *Microb Pathog* **49**, 122–134.
- Sierra, J. C., Suarez, G. & Chopra, A. K. (2011). An intriguing emerging human pathogen *Aeromonas hydrophila*: pathogenesis and vaccines. In *Vaccines Against Bacterial Biothreat Pathogens*. pp. 1–34. Edited by V. A. Feodorova & V. L. Motin. Kerala, India: Research Signpost.
- Silver, A. C., Rabinowitz, N. M., Küffer, S. & Graf, J. (2007). Identification of *Aeromonas veronii* genes required for colonization of the medicinal leech, *Hirudo verbana*. *J Bacteriol* **189**, 6763–6772.
- Silverman, J. M., Brunet, Y. R., Cascales, E. & Mougous, J. D. (2012). Structure and regulation of the Type VI secretion system. *Annu Rev Microbiol* **66**, 453–472.
- Suarez, G., Sierra, J. C., Sha, J., Wang, S., Erova, T. E., Fadl, A. A., Foltz, S. M., Horneman, A. J. & Chopra, A. K. (2008). Molecular

characterization of a functional type VI secretion system from a clinical isolate of *Aeromonas hydrophila*. *Microb Pathog* **44**, 344–361.

Suarez, G., Sierra, J. C., Erova, T. E., Sha, J., Horneman, A. J. & Chopra, A. K. (2010a). A type VI secretion system effector protein, VgrG1, from *Aeromonas hydrophila* that induces host cell toxicity by ADP ribosylation of actin. *J Bacteriol* **192**, 155–168.

Suarez, G., Sierra, J. C., Kirtley, M. L. & Chopra, A. K. (2010b). Role of Hcp, a type 6 secretion system effector, of *Aeromonas hydrophila* in modulating activation of host immune cells. *Microbiology* **156**, 3678–3688.

Weber, B., Hasic, M., Chen, C., Wai, S. N. & Milton, D. L. (2009). Type VI secretion modulates quorum sensing and stress response in *Vibrio anguillarum*. *Environ Microbiol* **11**, 3018–3028.

Zhang, L., Hinz, A. J., Nadeau, J. P. & Mah, T. F. (2011). *Pseudomonas aeruginosa* *tssC1* links type VI secretion and biofilm-specific antibiotic resistance. *J Bacteriol* **193**, 5510–5513.

Zheng, J., Ho, B. & Mekalanos, J. J. (2011). Genetic analysis of anti-amoebae and anti-bacterial activities of the type VI secretion system in *Vibrio cholerae*. *PLoS ONE* **6**, e23876.

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