

# *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*

Bijay K. Khajanchi,<sup>1</sup> Jian Sha,<sup>1</sup> Elena V. Kozlova,<sup>1</sup> Tatiana E. Erova,<sup>1</sup> Giovanni Suarez,<sup>1</sup> Johanna C. Sierra,<sup>1</sup> Vsevolod L. Popov,<sup>2</sup> Amy J. Horneman<sup>3</sup> and Ashok K. Chopra<sup>1</sup>

## Correspondence

Ashok K. Chopra  
achopra@utmb.edu

<sup>1</sup>Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555-1070, USA

<sup>2</sup>Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555-1070, USA

<sup>3</sup>Department of Medical and Research Technology, University of Maryland School of Medicine, Baltimore, MD 21201, USA

In this study, we delineated the role of *N*-acylhomoserine lactone(s) (AHLs)-mediated quorum sensing (QS) in the virulence of diarrhoeal isolate SSU of *Aeromonas hydrophila* by generating a double knockout  $\Delta$ *ahyRI* mutant. Protease production was substantially reduced in the  $\Delta$ *ahyRI* mutant when compared with that in the wild-type (WT) strain. Importantly, based on Western blot analysis, the  $\Delta$ *ahyRI* mutant was unable to secrete type VI secretion system (T6SS)-associated effectors, namely haemolysin coregulated protein and the valine-glycine repeat family of proteins, while significant levels of these effectors were detected in the culture supernatant of the WT *A. hydrophila*. In contrast, the production and translocation of the type III secretion system (T3SS) effector AexU in human colonic epithelial cells were not affected when the *ahyRI* genes were deleted. Solid surface-associated biofilm formation was significantly reduced in the  $\Delta$ *ahyRI* mutant when compared with that in the WT strain, as determined by a crystal violet staining assay. Scanning electron microscopic observations revealed that the  $\Delta$ *ahyRI* mutant was also defective in the formation of structured biofilm, as it was less filamentous and produced a distinct exopolysaccharide on its surface when compared with the structured biofilm produced by the WT strain. These effects of AhyRI could be complemented either by expressing the *ahyRI* genes *in trans* or by the exogenous addition of AHLs to the  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strain. In a mouse lethality experiment, 50% attenuation was observed when we deleted the *ahyRI* genes from the parental strain of *A. hydrophila*. Together, our data suggest that AHL-mediated QS modulates the virulence of *A. hydrophila* SSU by regulating the T6SS, metalloprotease production and biofilm formation.

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**Abbreviations:** AHL, *N*-acylhomoserine lactone; C4-HSL, *N*-3-butanoyl-DL-homoserine lactone; C6-HSL, *N*-3-hexanoyl-DL-homoserine lactone; CV, crystal violet; QS, quorum sensing; SEM, scanning electron microscopy; T2SS, type II secretion system; T3SS, type III secretion system; T6SS, type VI secretion system.

The GenBank/EMBL/DDBJ accession number for the *ahyRI* sequence of *A. hydrophila* SSU is DQ398101.

Four supplementary figures, showing cross streaks of *A. hydrophila* strains against a *Chromobacterium violaceum* CV026 reporter strain, Western blot analysis showing production of Act in the supernatant from *A. hydrophila* strains, measurement of biofilm mass by crystal violet staining formed on polystyrene by strains of *A. hydrophila*, and Western blot analysis showing production of Hcp2, are available with the online version of this paper.

## INTRODUCTION

*Aeromonas hydrophila* is a human pathogen that causes both intestinal and extraintestinal infections (Galindo *et al.*, 2006; Vila *et al.*, 2003). The organism is resistant to water chlorination and several antibiotics, specifically when it aggregates in a biofilm, posing a potential public health threat. Furthermore, *A. hydrophila* produces a wide battery of virulence factors, which function together to cause disease in the host (Chopra & Houston, 1999; Krovacek *et al.*, 1994; Sha *et al.*, 2002). We characterized three different enterotoxins from a diarrhoeal isolate SSU of *A. hydrophila* (Sha *et al.*, 2002). The most potent of these was

the cytotoxic enterotoxin (Act) (Ferguson *et al.*, 1997), which is secreted via the type II secretion system (T2SS) and functions as a haemolysin, a cytotoxin or an enterotoxin, depending upon the target cells (Chopra & Houston, 1999).

Recently, we showed the contribution of two other secretion systems, namely the type III secretion system (T3SS) and the type VI secretion system (T6SS), in the virulence of *A. hydrophila* SSU (Sha *et al.*, 2005, 2007; Sierra *et al.*, 2007; Suarez *et al.*, 2008), and reported the identification of a novel T3SS effector, AexU, which leads to the ADP-ribosylation of host cell proteins, resulting in their death via apoptosis (Sierra *et al.*, 2007). AexU also inhibits bacterial phagocytosis by macrophages and induces mouse lethality (Sha *et al.*, 2007). Likewise, the virulence-associated genes *vasH* and *vasK* are essential components of the T6SS, and our laboratory has provided evidence that isogenic mutants deleted for these genes are less virulent in a septicemic mouse model of *A. hydrophila* SSU infection (Suarez *et al.*, 2008). Recently, two T6SS-associated effectors, haemolysin co-regulated protein (Hcp) and the valine-glycine repeat G (VgrG) family of proteins, have been characterized, and their roles in the virulence of several pathogens established (Cascales, 2008; Mougous *et al.*, 2006; Pukatzki *et al.*, 2006, 2009).

We have demonstrated that deletion of the *vasH* gene prevents expression of the *hcp* gene, which indicates to us that the former is a regulator of the T6SS in *A. hydrophila* SSU (Suarez *et al.*, 2008). The genome of *Vibrio cholerae* contains three genes (*vgrG1*, *vgrG2* and *vgrG3*) that encode VgrG proteins, and mutations in the *hcp* gene block secretion of all of these VgrG proteins (Pukatzki *et al.*, 2006). Likewise, inactivation of the *vgrG* genes blocks secretion of Hcp (Pukatzki *et al.*, 2006). This mutual dependence for secretion of Hcp and VgrGs has also been reported in other pathogens, such as *Edwardsiella tarda* and enteroaggregative *Escherichia coli* (EAEC) (Dudley *et al.*, 2006; Pukatzki *et al.*, 2007; Zheng & Leung, 2007). It has been speculated that the VgrG proteins assemble into a trimeric complex that functions like a phage tail spike structure protein by forming a cell-puncturing device to deliver effector proteins into the host cells (Pukatzki *et al.*, 2007).

A cell-to-cell signalling system, known as quorum sensing (QS), may be implicated in controlling many of these virulence factors, including T3SS and T6SS effectors, at appropriate times, depending on the physiological conditions in the environment as well as in the host. In Gram-negative bacteria at least, three QS systems have been identified and designated LuxRI (AI-1), LuxS/AI-2 and AI-3 epinephrine/norepinephrine (Bassler & Losick, 2006; Reading & Sperandio, 2006; Waters & Bassler, 2006). The *N*-acylhomoserine lactone (AHL) QS system has been identified in both human (Kirwan *et al.*, 2006; Smith *et al.*, 2002; Swift *et al.*, 1999) and plant pathogens (Hussain *et al.*, 2008; Liu *et al.*, 2008). In different bacterial species, various

types of AHLs have been detected that differ in the number of carbon atoms (C4–C18) and substitution at the C3 of the acyl side chain (Kumari *et al.*, 2008).

An earlier study indicated that *A. hydrophila* produces two types of AHLs, namely *N*-3-butanoyl-DL-homoserine lactone (C4-HSL) and *N*-3-hexanoyl-DL-homoserine lactone (C6-HSL), of which C4-HSL was the predominant type (Swift *et al.*, 1997). Furthermore, AHLs that are synthesized by the LuxI protein family diffuse freely inside and outside of bacterial cells. At a certain critical threshold concentration, AHLs bind to their cognate receptors, in the LuxR protein family, and regulate the expression of the *luxI* gene for their synthesis, as well as control expression of many genes involved in biofilm formation, production of pigment and antibiotics, and virulence of the pathogen (de Kievit & Iglewski, 2000; Greenberg, 1997). Importantly, AhyR functions as both a negative and a positive regulator of the *ahyI* gene in *A. hydrophila* (Kirke *et al.*, 2004).

Microarray analyses have revealed the differential transcription of 26% of the genome in *Pectobacterium atrosepticum* (a plant pathogen) and 6% of the genome in *Pseudomonas aeruginosa* in EspI and LasI-RhII mutants, respectively, compared with their corresponding parental strains. These data suggest that AHL-mediated QS is a master regulator for many genes in these pathogens (Liu *et al.*, 2008; Schuster *et al.*, 2003). In addition, the role of AHL-mediated QS control in the T3SS and T6SS of *P. aeruginosa* and other pathogens has been investigated and reported (Bleves *et al.*, 2005; Gelhaus *et al.*, 2009; Henke & Bassler, 2004; Liu *et al.*, 2008; Schuster *et al.*, 2003). Although by microarray analysis it has been shown that the expression of the *hcp* gene is reduced in the *lasR-rhlR* mutant, when compared with that in WT *P. aeruginosa*, it was not known at the time that Hcp was an effector of the T6SS (Schuster *et al.*, 2003).

Specifically, we have shown that AHL production is significantly decreased when we delete two major virulence factor-encoding genes, such as *act* and an outer-membrane protein (*aopB*), an important component of the T3SS in *A. hydrophila* SSU (Sha *et al.*, 2005). We also observed that lactone production is modulated by regulatory genes such as *dam* (DNA adenine methyltransferase) and *gidA* (glucose-inhibited division A) in *A. hydrophila* SSU (Erova *et al.*, 2006a). These data prompted us to further investigate the regulatory role of AHL-mediated QS in the virulence of *A. hydrophila* SSU.

Our studies have been substantiated by previous reports showing that AHL-mediated QS regulates exoprotease production (Swift *et al.*, 1999) and biofilm formation (Lynch *et al.*, 2002) in *A. hydrophila*. In addition, the *ahyR* mutant of *A. hydrophila* J-1 is significantly attenuated in a fish infection model (Bi *et al.*, 2007). However, the role of AHL-mediated QS in clinical isolates of *A. hydrophila* has never been tested in a mouse model of infection, and the role of QS in modulating the T3SS and T6SS is largely unknown in this pathogen.

Consequently, in this study, we identified AhyRI (LuxRI homologue) in a clinical isolate SSU of *A. hydrophila* as a result of our annotation of the genome sequence of an environmental isolate ATCC 7966 of *A. hydrophila* (Seshadri *et al.*, 2006). We showed that disruption of the *ahyRI* genes in *A. hydrophila* SSU influenced metalloprotease production, secretion of the T6SS effectors (Hcp and VgrGs), biofilm formation, and mortality in a mouse model of infection. Our study is the first, to our knowledge, to document that AHL mediates the QS-regulated secretion of the newly discovered T6SS effectors in *A. hydrophila* SSU and, based on zymography, that the production of a metalloprotease is specifically reduced in the  $\Delta$ *ahyRI* mutant.

## METHODS

**Bacterial strains, plasmids, and chemicals.** The bacterial strains and plasmids used in this study are listed in Table 1. Chemically synthesized C4-HSL and C6-HSL were obtained from Fluka. Stock solutions (50 mM) of AHLs were prepared by dissolving them in acetonitrile (far-UV grade), and stocks were stored at  $-20^{\circ}\text{C}$ .

**Generation and characterization of the  $\Delta$ *ahyRI* mutant of *A. hydrophila* SSU.** Based on DNA sequences of the *ahyI/ahyR* genes (homologues of the *luxI/luxR* genes) in the *A. hydrophila* ATCC 7966 strain, two primers (*ahyR*, 5'-TTATTGCATCAGCTTGGGGAAG-3', and *ahyI*, 5'-TTATTCGGTGACCAGTTCGCG-3') were synthesized. By using these primers, a 1.5 kb DNA fragment was PCR-amplified from the genome of *A. hydrophila* SSU and subsequently cloned in the TA cloning vector pCR2.1 (Invitrogen). DNA sequence analysis revealed that this fragment was 1469 bp long and contained the entire *ahyRI* operon. By using restriction enzyme *StuI* (generating blunt ends), we removed a 443 bp DNA fragment from within the *ahyRI*

operon harboured in the TA cloning vector, and replaced it with the blunt-ended 2.0 kb  $\text{Sm}^{\text{R}}/\text{Sp}^{\text{R}}$  gene cassette from plasmid pHP45 $\Omega$  (Prentki & Krisch, 1984), which was obtained by *SmaI* restriction enzyme digestion. This strategy resulted in a 185 and a 196 bp deletion from the start codons of the *ahyI* and *ahyR* genes, respectively. The  $\text{Sm}^{\text{R}}/\text{Sp}^{\text{R}}$ -truncated *ahyRI* genes were removed from the pCR2.1 vector by *KpnI/XbaI* digestion and ligated to the pDMS197 suicide vector at compatible restriction enzyme sites. The resulting plasmid (pDMS197*ahyRI*Sm/Sp) was transformed into *E. coli* SM10, which contained  $\lambda$ *pir* (Edwards *et al.*, 1998). The recombinant *E. coli* (pDMS197*ahyRI*-Sm/Sp) cells were then conjugated with WT *A. hydrophila* SSU-R (Sha *et al.*, 2002). The transconjugants were selected based on resistance to appropriate antibiotics and sucrose and their genomic DNA (gDNA) subjected to Southern blot analysis for their correct identification, as described previously (Sha *et al.*, 2002).

**Complementation of the *A. hydrophila* SSU  $\Delta$ *ahyRI* mutant.** The *ahyRI* genes were PCR-amplified by using gDNA of *A. hydrophila* as a template and two primers (*ahyRI*-N/*SalI*, 5'-GGGGTCCGACAGC-AGCTTGTAATCCAACGC-3', and *ahyRI*-C/*EcoRI*, 5'-GGGGAA-TTCATGAACCGTCCAGCAGAGTGA-3'; restriction endonuclease sites underlined). We included 293 bp of the upstream and 210 bp of the downstream DNA flanking sequences containing potential promoter regions of divergent *ahyRI* genes for complementation studies. This DNA fragment (1972 bp) was cloned in the pBR322 vector ( $\text{Tc}^{\text{r}} \text{Ap}^{\text{r}}$ ) at *SalI*-*EcoRI* sites and transformed into the *E. coli* DH5 $\alpha$  strain. The pBR322/*ahyRI* ( $\text{Tc}^{\text{r}} \text{Ap}^{\text{r}}$ ) recombinant plasmid was isolated from the *E. coli* strain and electroporated into an *A. hydrophila*  $\Delta$ *ahyRI* mutant.

**Complementation of the *ahyR* gene in the *A. hydrophila* SSU  $\Delta$ *ahyRI* mutant.** To complement the *ahyR* gene in the double mutant  $\Delta$ *ahyRI* of *A. hydrophila*, the *ahyR* gene was amplified with the following primers: *ahyR*-N/*ScaI*, 5'-GGGAGTACTATGAAACAA-GACCAACTGCTT-3', and *ahyR*-C/*PstI*, 5'-GGGCTGCAGTTATTG-CATCAGCTTGGGGA-3'. The DNA fragment (783 bp) was cloned in

**Table 1.** Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<b><i>A. hydrophila</i> SSU strains</b>		
SSU-R	Rifampin-resistant ( $\text{Rif}^{\text{R}}$ ) strain of <i>A. hydrophila</i> SSU	CDC, Atlanta, GA, USA
$\Delta$ <i>ahyRI</i>	<i>ahyRI</i> gene deletion mutant of <i>A. hydrophila</i> SSU-R strain, $\text{Rif}^{\text{R}} \text{Sm}^{\text{R}} \text{Sp}^{\text{R}}$	Laboratory stock
$\Delta$ <i>ahyRI</i> /pBR322- <i>ahyRI</i>	<i>ahyRI</i> mutant complemented with <i>ahyRI</i> genes via pBR322, $\text{Rif}^{\text{R}} \text{Sm}^{\text{R}} \text{Sp}^{\text{R}} \text{Ap}^{\text{R}}$	This study
$\Delta$ <i>ahyRI</i> /pBR322- <i>ahyR</i>	<i>ahyRI</i> mutant complemented with <i>ahyR</i> gene via pBR322, $\text{Rif}^{\text{R}} \text{Sm}^{\text{R}} \text{Sp}^{\text{R}} \text{Tc}^{\text{R}}$	This study
<b><i>E. coli</i> strains</b>		
DH5 $\alpha$	Production of recombinant plasmids, <i>recA</i> , <i>gyrA</i>	Life Technologies
SM10	$\text{Km}^{\text{R}}$ , $\lambda$ <i>pir</i>	Edwards <i>et al.</i> (1998)
<b><i>C. violaceum</i> strain</b>		
CV026	ATCC 31532 derivative, <i>cvilI::Tn5xyIE</i> ; $\text{Km}^{\text{R}} \text{Sm}^{\text{R}}$	McClellan <i>et al.</i> (1997)
<b>Plasmids</b>		
pCR2.1	TA cloning vector, $\text{Ap}^{\text{R}} \text{Km}^{\text{R}}$	Invitrogen
pCR2.1/ <i>ahyRI</i>	TA cloning vector carrying <i>ahyRI</i> gene, $\text{Ap}^{\text{R}} \text{Km}^{\text{R}}$	This study
pHP45 $\Omega$	pHP45 plasmid containing a 2 kb $\text{Sm}^{\text{R}}/\text{Sp}^{\text{R}}$ gene cassette ( $\Omega$ element)	Prentki & Krisch (1984)
pCR2.1/ <i>ahyRI</i> -Sm/Sp	TA cloning vector harbouring <i>ahyRI</i> gene disrupted by $\Omega$ element, $\text{Ap}^{\text{R}} \text{Sm}^{\text{R}} \text{Sp}^{\text{R}}$	This study
pDMS197	Suicide vector, R6K <i>ori</i> , <i>sacB</i> , $\text{Tc}^{\text{R}}$	Edwards <i>et al.</i> (1998)
pDMS197/ <i>ahyRI</i> -Sm/Sp	Suicide vector containing <i>ahyRI</i> gene with $\Omega$ element, $\text{Tc}^{\text{R}} \text{Sm}^{\text{R}} \text{Sp}^{\text{R}}$	This study
pBR322	$\text{Ap}^{\text{R}} \text{Tc}^{\text{R}}$	Amersham
pBR322/ <i>ahyRI</i>	Contains <i>ahyRI</i> gene, $\text{Ap}^{\text{R}}$	This study
pBR322/ <i>ahyR</i>	Contains <i>ahyR</i> gene, $\text{Tc}^{\text{R}}$	This study

the pBR322 vector (Tc<sup>R</sup> Ap<sup>R</sup>) at *ScaI/PstI* sites and transformed into *E. coli* strain DH5 $\alpha$ . The pBR322/*ahyR* (Tc<sup>R</sup> Ap<sup>S</sup>) recombinant plasmid was isolated from the *E. coli* strain and electroporated into the *A. hydrophila*  $\Delta$ *ahyRI* mutant.

**Lactone production.** AHL production was detected by cross-streaking of the WT,  $\Delta$ *ahyRI* mutant and complemented strains of *A. hydrophila* SSU on LB agar medium against the biosensor strain *Chromobacterium violaceum* CV026, as described elsewhere (McClellan *et al.*, 1997).

**Measurement of protease activity.** Protease activity was measured in culture filtrates of overnight-grown cultures of WT *A. hydrophila*,  $\Delta$ *ahyRI* mutant and the complemented strains ( $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> and  $\Delta$ *ahyRI/ahyR*<sup>+</sup>) as described previously (Erova *et al.*, 2006b). The protease activity was calculated per millilitre of culture filtrate per 10<sup>8</sup> c.f.u. To determine the nature of proteases, we used metallo and serine protease inhibitors EDTA and PMSF at final concentrations of 100 and 10 mM, respectively. The proteases were inactivated with their corresponding inhibitors for 2 h at 37 °C before measuring the activity. To characterize the nature of protease(s) and to delineate which specific protease(s) was affected in the  $\Delta$ *ahyRI* mutant, we performed casein zymography using established procedures (Nicodème *et al.*, 2005; Swift *et al.*, 1999).

**Measurement of haemolytic activity.** The haemolytic activity associated with Act of WT *A. hydrophila* SSU and its  $\Delta$ *ahyRI* mutant strain was measured as previously described (Sha *et al.*, 2002). For the neutralization assay, culture filtrates of WT and  $\Delta$ *ahyRI* mutant strains were mixed with either pre-immune (control) or hyperimmune rabbit sera (laboratory stock, 1:10 dilution) containing antibodies to Act (Erova *et al.*, 2007).

**Measurement of cytotoxic activity.** RAW 264.7 murine macrophages were infected at an m.o.i. of 5 with the WT *A. hydrophila* SSU and its  $\Delta$ *ahyRI* mutant strain. During infection, cell morphology was monitored, and at various time points after infection, host cell cytotoxicity associated with Act or T3SS and T6SS effectors was measured in the tissue culture supernatant by the release of lactate dehydrogenase (LDH) enzyme using a CytoTox 96 kit (Promega) (Sha *et al.*, 2005).

**Swimming and swarming motility assay.** LB medium with 0.3 % Difco Bacto-agar (Difco Laboratories) was used to characterize the swimming motility (Kozlova *et al.*, 2008), while Difco nutrient broth with 0.5 % Eiken agar (Eiken Chemical) was employed for measuring swarming motility (Kirov *et al.*, 2002) of WT *A. hydrophila* SSU and its  $\Delta$ *ahyRI* mutant strain. The *A. hydrophila* ATCC 7966 strain (Seshadri *et al.*, 2006), which does not possess genes encoding the lateral flagellum, was used as a negative control in the swarming motility assay.

**Crystal violet (CV) biofilm assay.** As a modification of the biofilm ring assay (O'Toole & Kolter, 1998), the WT,  $\Delta$ *ahyRI* mutant and  $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> and  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strains of *A. hydrophila* were grown directly from the -80 °C stock in 3 ml LB medium contained in polystyrene tubes at 37 °C for 24 h with shaking. The  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strain was grown in the presence of 20  $\mu$ M C4-HSL. Biofilm formation was quantified according to the procedure described elsewhere (Morohoshi *et al.*, 2007). Finally, the biofilm formation results were normalized to 1  $\times$  10<sup>9</sup> c.f.u. to account for any differences in the growth rates of various bacterial strains used. The experiment was repeated independently three times.

**Scanning electron microscopy (SEM) biofilm experiments.** SEM on biofilm formation was performed using 13 mm diameter

Thermanox plastic coverslips. After 48 h incubation, unattached cells were removed, the coverslips were fixed and stained with ruthenium red, and samples were examined in a Hitachi S4700 field emission scanning electron microscope (Hitachi High Technologies America) according to the procedure described in our previous study (Kozlova *et al.*, 2008).

**Western blot analysis.** Overnight cultures of the WT,  $\Delta$ *ahyRI* mutant and complemented strains were diluted 1:20 in fresh LB medium and grown for 2 h (OD<sub>600</sub> ~0.8) and 4 h (OD<sub>600</sub> ~1.4) at 37 °C with shaking at 180 r.p.m. The  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strain was grown in the presence of 20  $\mu$ M C4-HSL. To measure protein levels in the cells, 2 ml culture was pelleted (10 000 g for 10 min at 4 °C) and dissolved in 200  $\mu$ l 2  $\times$  sample buffer. To measure secretion of the proteins in the LB medium, 2 ml supernatants were separated from the pellet and filtered through a 0.22  $\mu$ m pore-size membrane filter. Proteins present in the supernatant fraction were precipitated with TCA (10 % final concentration) and pelleted by high-speed centrifugation at 14 000 g for 15 min at 4 °C. The pellet was resuspended in 50  $\mu$ l 2  $\times$  sample buffer.

Equal amounts of samples were subjected to SDS-PAGE, and Western blot analysis was performed using polyclonal antisera against Hcp2 and VgrG2 [1:1000 dilution in Tris-buffered saline (TBS)/0.5 % skimmed milk], as described previously (Suarez *et al.*, 2008). We used antibodies to bacterial DnaK (bacterial cytosolic protein) as a control in Western blot analysis to demonstrate that the T6SS effectors in the culture supernatant were not released as a result of bacterial lysis (Sha *et al.*, 2007).

We used recombinant VgrG2 for antibody production, and the immune sera obtained did not differentiate between VgrG2 and VgrG3 proteins of *A. hydrophila* SSU due to the high homology (~90 %) between them and their similar sizes on Western blots.

To measure the expression and translocation of AexU, a T3SS effector, in HT-29 human colonic epithelial cells, we followed the methodology that we described previously (Sha *et al.*, 2007).

**Animal experiments.** Groups of 10 Swiss Webster mice (Taconic Farms) were infected by the intraperitoneal (i.p.) route with 3  $\times$  10<sup>7</sup> c.f.u. (WT or its  $\Delta$ *ahyRI* mutant) in accordance with the approved animal care protocol. One group of mice was inoculated with Dulbecco's phosphate buffered saline (DPBS) ( $n$ =10) and served as a control. Deaths were recorded for 16 days post-infection. This bacterial dose used represented approximately 2  $\times$  LD<sub>50</sub> of WT *A. hydrophila* (Xu *et al.*, 1998).

**Statistics.** Wherever applicable, at least three independent experiments were performed, and the data analysed by using Student's *t* test, with *P* values of  $\leq$ 0.05 considered significant. The animal data were analysed by using Fisher's exact test.

## RESULTS

### Characterization of the *AhyRI* QS regulon in *A. hydrophila* SSU

In an earlier study (Swift *et al.*, 1997), a LuxRI homologue was identified in *A. hydrophila* A1 strain and designated *AhyRI* (GenBank accession no. X89469). By our sequence annotation of *A. hydrophila* ATCC 7966 (Seshadri *et al.*, 2006), we identified *ahyR* and *ahyI* genes in a clinical isolate, *A. hydrophila* SSU, which had a 97 and 94 % sequence homology with the corresponding genes of the *A.*

*hydrophila* A1 strain, respectively. The *ahyR* and *ahyI* genes in *A. hydrophila* SSU were oppositely oriented with a 59 bp intergenic region. Furthermore, a Pfam protein sequence search (<http://pfam.sanger.ac.uk/>) revealed that AhyR had two domains, one for autoinducer binding and the other a regulatory domain (Lux family).

To characterize the role of AHL-mediated QS in the regulation of virulence in *A. hydrophila* SSU, we deleted both the *ahyR* and *ahyI* genes by double crossover homologous recombination and subsequently generated a complemented strain with both of the *ahyRI* genes ( $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup>). For further confirmation of interaction between AhyR and signalling molecules (AHLs) in modulating virulence factors, we also complemented the *ahyR* gene in the  $\Delta$ *ahyRI* mutant strain ( $\Delta$ *ahyRI/ahyR*<sup>+</sup>) and examined the restoration of phenotypic changes by the exogenous addition of AHLs. As expected, the  $\Delta$ *ahyRI* mutant was unable to produce AHLs, which was examined by using *C. violaceum* CV026 as a biosensor strain (Supplementary Fig. S1a). Lactone production was restored in the  $\Delta$ *ahyRI* complemented strain (Supplementary Fig. S1b).

### The $\Delta$ *ahyRI* mutant produced a reduced level of protease

Earlier studies had indicated that the pathogenic and virulence characteristics of *A. hydrophila* are associated with the production of exoenzymes (e.g. proteases and lipases) (Chopra & Houston, 1999; Janda & Abbott, 1998). Consequently, we measured protease production, and the  $\Delta$ *ahyRI* mutant strain produced a significantly reduced level of protease compared with that of the WT *A. hydrophila* strain (Table 2). Furthermore, the protease production was restored to the WT level in the  $\Delta$ *ahyRI* complemented strain ( $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup>) (Table 2). For further confirmation of complementation, protease production was also measured in the  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strains when they were simultaneously supplied with two different exogenous AHLs (C4-HSL and C6-HSL). We observed that addition of both of the exogenous lactones restored protease production (Table 2), which suggested to us that AHL molecules interacted with AhyR to control protease production in *A. hydrophila* SSU.

Based on casein zymography, we identified three protein bands with protease activity (with sizes of 61, 52 and 19 kDa) in the culture filtrates of WT *A. hydrophila* SSU and its  $\Delta$ *ahyRI* complemented strain ( $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup>). Importantly, 61 kDa and 52 kDa protease-associated protein bands were missing in the  $\Delta$ *ahyRI* mutant (Fig. 1). Furthermore, we noted that the 61 kDa band represented a metalloprotease, as treatment of the culture supernatants from WT *A. hydrophila* and its  $\Delta$ *ahyRI* complemented strain with EDTA resulted in complete disappearance of this band (Fig. 1). The nature of the proteases associated with the 52 and 19 kDa bands is unknown. Based on our enzyme assay, EDTA resulted in 81% loss of the total protease activity, while PMSF had minimal effect on the protease activity.

**Table 2.** Measurement of protease activity in culture supernatants and biofilm mass on polystyrene plastic of *A. hydrophila* SSU, the  $\Delta$ *ahyRI* mutant and complemented strains

ND, Not determined.

Strain	Protease activity (OD <sub>595</sub> ml <sup>-1</sup> per 10 <sup>8</sup> c.f.u.) (mean $\pm$ SD)	Biofilm formation ( <i>A</i> <sub>570</sub> ) (mean $\pm$ SD)
<i>A. hydrophila</i> SSU	1.20 $\pm$ 0.23	3.70 $\pm$ 0.28
$\Delta$ <i>ahyRI</i>	0.11 $\pm$ 0.04*‡	0.55 $\pm$ 0.07*#
$\Delta$ <i>ahyRI/ahyR</i> <sup>+</sup> <i>ahyI</i> <sup>+</sup>	1.98 $\pm$ 0.40*§	2.50 $\pm$ 0.28*††
$\Delta$ <i>ahyRI/ahyR</i> <sup>+</sup> + C4-HSL	1.41 $\pm$ 0.18*	3.90 $\pm$ 0.14*‡‡
$\Delta$ <i>ahyRI/ahyR</i> <sup>+</sup> + C6-HSL	0.56 $\pm$ 0.03*¶	ND

Protease activity: \*‡: statistically significant differences between the  $\Delta$ *ahyRI* mutant and the WT bacteria by Student's *t* test ( $P=0.001$ ); \*§: between the  $\Delta$ *ahyRI* mutant and  $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> complemented strain ( $P=0.001$ ); \*||, ¶: between the  $\Delta$ *ahyRI* mutant and  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strain with exogenous supply of C4-HSL or C6-HSL ( $P<0.001$ ).

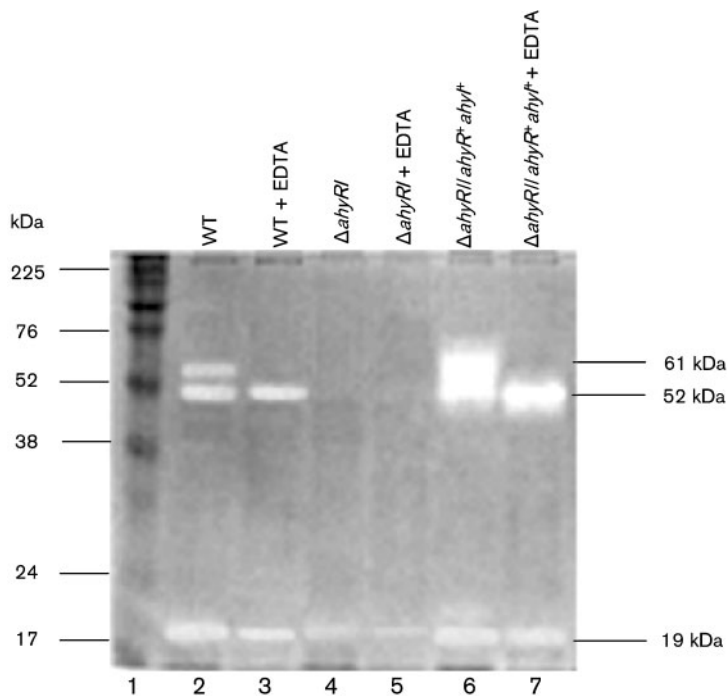
Biofilm formation: \*#: statistically significant differences between the  $\Delta$ *ahyRI* mutant and the WT bacteria ( $P=0.004$ ); \*††: between the  $\Delta$ *ahyRI* mutant and  $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> complemented strain ( $P=0.01$ ); \*‡‡: between the  $\Delta$ *ahyRI* mutant and  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strain with exogenous supply of C4-HSL ( $P=0.001$ ).

### Haemolytic and cytotoxic activities associated with Act were unaltered in the $\Delta$ *ahyRI* mutant

The level of haemoglobin release from rabbit erythrocytes was identical in both the WT and the  $\Delta$ *ahyRI* mutant strain of *A. hydrophila* SSU. To demonstrate that the lack of detection in reduction of Act-associated haemolytic activity was not due to the upregulation of other genes encoding haemolysin, we performed a haemolytic activity assay after neutralization of Act in the culture supernatant using specific antibodies. We noted that the level of residual haemolytic activity in the WT versus  $\Delta$ *ahyRI* mutant strains remained unchanged, indicating that the expression of other haemolysin genes, in general, was not altered by deletion of the *ahyRI* genes. In addition, based on Western blot analysis, similar levels of Act were noted in the WT and the  $\Delta$ *ahyRI* mutant strain of *A. hydrophila* SSU (Supplementary Fig. S2). Likewise, the  $\Delta$ *ahyRI* mutant produced cytotoxic activity at a level similar to that of the WT bacteria (data not shown). These data suggested to us that the AhyRI QS regulon had no effect on Act-mediated biological activities in *A. hydrophila* SSU.

### Swimming and swarming motility of the $\Delta$ *ahyRI* mutant was unaffected

*A. hydrophila* SSU WT strain had both swimming and swarming motility; however, the  $\Delta$ *ahyRI* mutant migrated in a manner similar to that of the parental strain on the



**Fig. 1.** Casein zymogram of supernatant proteins of WT *A. hydrophila* SSU, the  $\Delta$ *ahyRI* mutant and its complemented strain. After incubation of the SDS-polyacrylamide gel (12%) in 2% casein solution, Coomassie blue staining showed activity associated with proteases, which appeared as clear bands due to hydrolysis of casein on a blue background. Lanes: 1, molecular mass standard; 2, supernatant from WT *A. hydrophila* SSU; 3, supernatant from WT *A. hydrophila* SSU treated with EDTA; 4, supernatant from the  $\Delta$ *ahyRI* mutant; 5, supernatant from the  $\Delta$ *ahyRI* mutant treated with EDTA; 6, supernatant from the complemented strain ( $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup>); 7, supernatant from the complemented strain ( $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup>) treated with EDTA.

swimming and swarming agar plates (data not shown), which indicated that the swimming and swarming motility was not regulated by the AHL-mediated QS in *A. hydrophila* SSU.

### CV staining biofilm assay demonstrated attachment deficiency in the $\Delta$ *ahyRI* mutant

To measure the solid surface-associated biofilm formation, we performed a CV staining assay after 24 h growth of the WT,  $\Delta$ *ahyRI* mutant, and  $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> and  $\Delta$ *ahyRI/ahyR*<sup>+</sup> (with exogenous AHLs) complemented strains in LB medium (Table 2). The  $\Delta$ *ahyRI* mutant formed a significantly decreased solid surface-associated biofilm in polystyrene tubes (Table 2), with an 86% reduction in the CV staining when compared with that of the WT *A. hydrophila* SSU strain (Supplementary Fig. S3). The  $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> complemented strain adhered to the polystyrene tube in a manner similar to that of the WT bacteria (Supplementary Fig. S3). Addition of exogenous C4-HSL (20  $\mu$ M) also restored biofilm formation in the  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strain (Table 2). These data suggested to us that AHL QS had a positive regulatory role on the biofilm formation in *A. hydrophila* SSU.

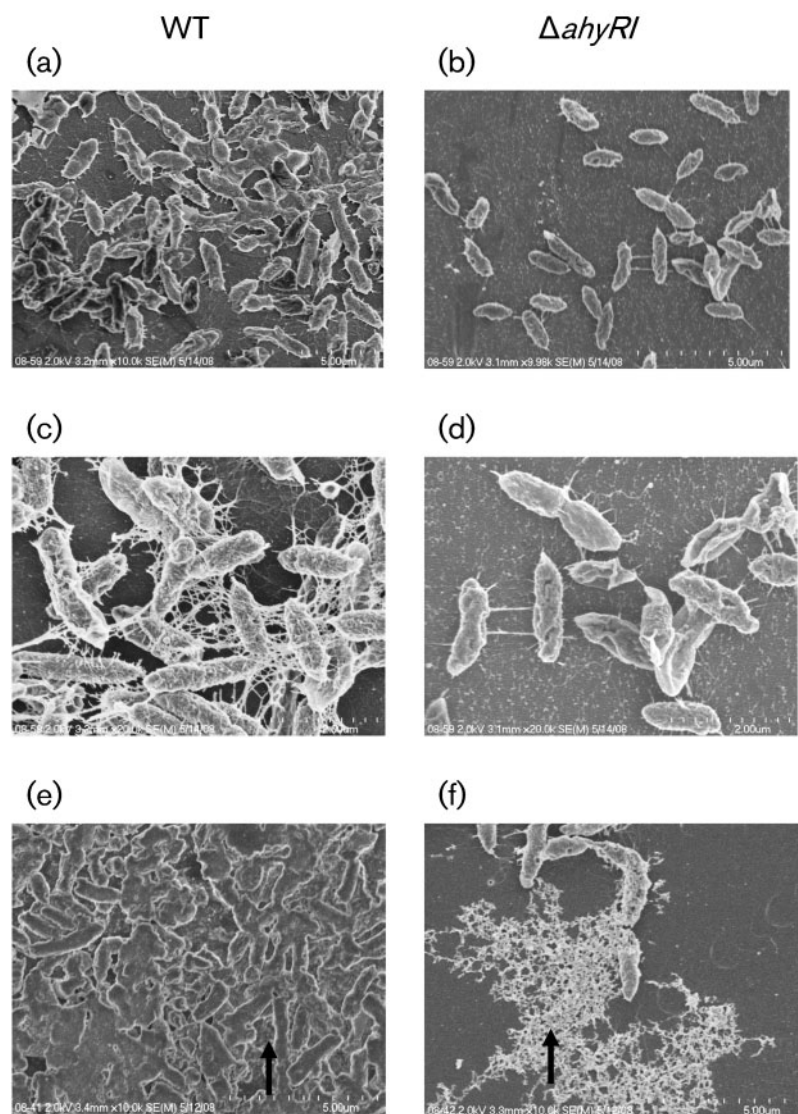
### A distinct architecture of biofilm was observed in the $\Delta$ *ahyRI* mutant when examined by SEM

Exopolysaccharide (EPS), which consists of polysaccharides, DNA and proteins, plays an important role in determining biofilm architecture (de Kievit, 2009). To investigate the surface architecture of bacterial cells aggregated in biofilm formed by the WT and  $\Delta$ *ahyRI*

mutant, we performed SEM. Ruthenium red staining is an excellent method for visualization of surface properties of bacteria (Fassel & Edmiston, 1999), and hence we stained bacterial aggregates with ruthenium red. As expected, SEM results revealed that WT bacteria formed a structured biofilm in which cells were well connected by filaments (Fig. 2a, c and e). In contrast, the  $\Delta$ *ahyRI* mutant was less filamentous, and it was not properly aggregated as was the parental strain (Fig. 2b, d and f). In addition, EPS produced by WT bacteria (indicated by arrow in Fig. 2e) was thick and tightly attached to the surface of the bacteria. On the other hand, EPS produced by the  $\Delta$ *ahyRI* mutant (indicated by arrow in Fig. 2f) was loosely bound to bacterial cells. The  $\Delta$ *ahyRI* complemented strains produced biofilms similar to that of the WT bacteria (data not shown). Overall, SEM images indicated that the  $\Delta$ *ahyRI* mutant was defective in the production of mature biofilm compared with the parental strain, which suggested to us that AHL-mediated QS has a regulatory role in biofilm development of *A. hydrophila* SSU.

### AHL mediated the QS-controlled secretion of Hcp, a T6SS effector

The T6SS is a novel secretion mechanism, and very little is known as to how effector proteins are secreted and translocated to host cells through this system. We recently demonstrated that secreted Hcp could bind to murine RAW 264.7 macrophages from outside and that it could also translocate into host cells, resulting in their apoptosis (Suarez *et al.*, 2008). In this study, to examine the role of AHL-mediated QS in the regulation of the T6SS, we performed Western blot analysis to determine the produc-



**Fig. 2.** SEM images of biofilm formation by WT *A. hydrophila* SSU and its  $\Delta$ *ahyRI* mutant after 48 h cultivation at 37 °C on Thermanox coverslips stained with ruthenium red. (a, c) Compact aggregation; cells were well connected with filaments in biofilm formed by the WT strain. (b, d) Less aggregated cells in the  $\Delta$ *ahyRI* mutant biofilm, which were connected with fewer filaments compared with the biofilm formed by the WT bacteria. (e) A thick EPS was produced by the WT strain that was tightly bound to the surface of the cells (indicated by an arrow). (f) The  $\Delta$ *ahyRI* mutant produced a distinct type of EPS compared with that of the WT strain, and the EPS was loosely bound to the surface of the cells (indicated by an arrow).

tion and secretion of Hcp at different time points (2 h and 4 h) in WT,  $\Delta$ *ahyRI* mutant and the  $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> and  $\Delta$ *ahyRI/ahyR*<sup>+</sup> (with C4-HSL) complemented strains of *A. hydrophila* SSU. We only show data on expression and secretion of Hcp in the WT, mutant and complemented strains at the 4 h time point, and the data at the 2 h time point are included in a supplementary figure.

We found that Hcp2 could be detected in bacterial cell pellets in both the WT and its  $\Delta$ *ahyRI* mutant at 2 h (Supplementary Fig. S4, lanes 2 and 4) and 4 h (Fig. 3, lanes 2 and 4). However, in the WT bacteria, a significant amount of Hcp2 was secreted into the medium at both 2 h (Supplementary Fig. S4, lane 1) and 4 h (Fig. 3, lane 1). In contrast, the  $\Delta$ *ahyRI* mutant was unable to secrete Hcp2 at 2 h (Supplementary Fig. S4, lane 3) and 4 h (Fig. 3, lane 3). The  $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> complemented strain secreted Hcp2 to the WT level (Fig. 3, lane 5). Also, addition of C4-HSL to the  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strain restored Hcp2 secretion (Fig. 3, lane 7), which suggested to us that

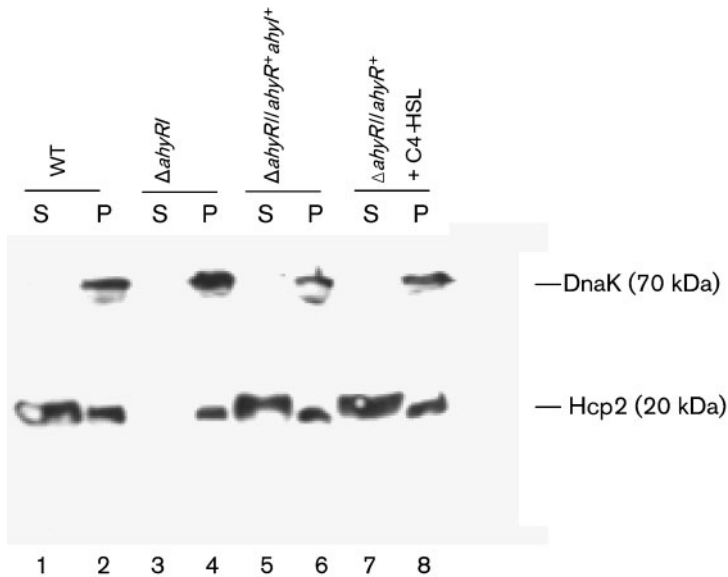
AHL mediated the QS-regulated secretion of Hcp in *A. hydrophila* SSU.

#### AHL-mediated QS also regulated secretion of another T6SS effector, VgrG

As in *V. cholerae* (Pukatzki *et al.*, 2006), there were three copies of the *vgrG*-encoded effector proteins, namely VgrG1, VgrG2 and VgrG3 in *A. hydrophila* SSU (Suarez *et al.*, 2008). Furthermore, the role of VgrGs in cytotoxicity and virulence has recently been reported in *V. cholerae* (Pukatzki *et al.*, 2006, 2009). However, the regulation of these VgrG effector molecules is currently unknown. In the present study, we examined whether AHL-mediated QS regulated the production and secretion of these VgrGs in *A. hydrophila* SSU.

In Western blot analysis, the production of VgrG2 effector was noted both in the WT and in the  $\Delta$ *ahyRI* mutant of *A. hydrophila* SSU (Fig. 4, lanes 2 and 4, lower band). A

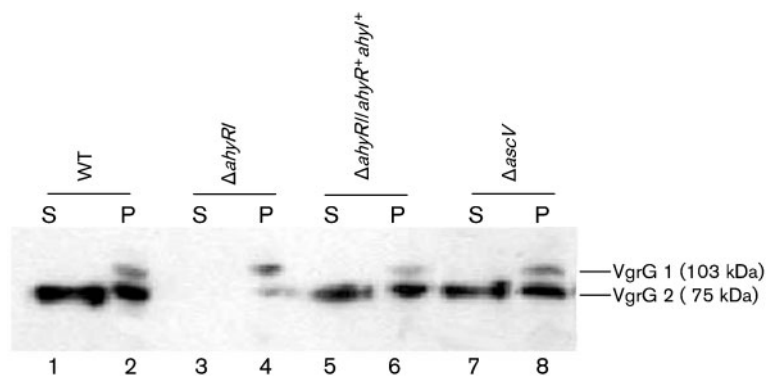




**Fig. 3.** Western blot analysis showing production of Hcp2 in the cell pellet (P) and secretion of Hcp2 in the culture supernatants (S) of WT *A. hydrophila* SSU, the  $\Delta$ *ahyRI* mutant and complemented strains. Lanes: 1, supernatant of WT strain; 2, cell pellet of WT strain; 3, supernatant of the  $\Delta$ *ahyRI* mutant; 4, cell pellet of the  $\Delta$ *ahyRI* mutant; 5, supernatant of the  $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> complemented strain; 6, cell pellet of the  $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> complemented strain; 7, supernatant of the  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strain; 8, cell pellet of the  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strain. The  $\Delta$ *ahyRI/ahyR*<sup>+</sup> complemented strain was grown in LB medium supplemented with 20  $\mu$ M C4-HSL. Polyclonal antibody against Hcp2 (at 1 : 1000 dilution) and secondary antibody (1 : 10 000 dilution, goat anti-mouse IgG) conjugated with horseradish peroxidase (HRP) were used. The blots were developed after reaction with SuperSignal West Pico chemiluminescence agent (Pierce) followed by X-ray film exposure. Anti-DnaK antibodies were used to measure the intactness of bacterial cells. Three independent experiments were performed.

significant level of VgrG2 effector protein was secreted in the supernatant collected from the WT *A. hydrophila* SSU strain (Fig. 4, lane 1); however, the  $\Delta$ *ahyRI* mutant was unable to secrete VgrG2 into the medium (Fig. 4, lane 3). Importantly, secretion of VgrG2 effector protein was restored in the  $\Delta$ *ahyRI* complemented strain (Fig. 4, lane

5). Deletion of the *ascV* gene, which encodes an inner membrane component of the T3SS channel from *A. hydrophila* SSU, had no effect on the expression and secretion of these effector proteins (Fig. 4, lanes 7 and 8), which suggested that production and secretion of VgrGs were T3SS-independent. Overall, these results suggested to



**Fig. 4.** Western blot analysis showing VgrG1 and VgrG2 production in the cell pellet (P) and secretion of VgrGs in the culture supernatants (S) of WT *A. hydrophila* SSU, the  $\Delta$ *ahyRI* mutant and complemented strains. Lanes: 1, supernatant of the WT strain; 2, cell pellet of the WT strain; 3, supernatant of the  $\Delta$ *ahyRI* mutant; 4, cell pellet of the  $\Delta$ *ahyRI* mutant; 5, supernatant of the  $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> complemented strain; 6, cell pellet of the  $\Delta$ *ahyRI/ahyR*<sup>+</sup>*ahyI*<sup>+</sup> complemented strain; 7, supernatant of the  $\Delta$ *ascV* mutant; 8, cell pellet of the  $\Delta$ *ascV* mutant. The *ascV* gene constitutes a component of the T3SS and was used as a control. Polyclonal antibody against VgrG2 (at 1 : 1000 dilution) and secondary antibody (1 : 10 000 dilution, goat anti-mouse IgG) conjugated with HRP were used. The blots were developed after reaction with West Femo chemiluminescence substrate (Pierce) followed by X-ray film exposure. Results were reproduced through three independent experiments.



us that AHL mediated the QS-controlled secretion of VgrG2 effector of T6SS in *A. hydrophila* SSU.

### Expression and translocation of AexU, a T3SS effector, was not affected in the $\Delta$ ahyRI mutant of *A. hydrophila* SSU

To demonstrate regulation of the AHL-mediated QS on T3SS effector translocation, we examined expression of the *aexU* gene in bacterial cell pellets and translocation of AexU in human colonic epithelial (HT-29) cells in the WT and  $\Delta$ ahyRI mutant grown in Dulbecco's Modified Eagle's Medium (DMEM). We found that the  $\Delta$ ahyRI mutant had levels of production of AexU similar to those of WT bacteria (data not shown). Similarly, no difference in the translocation of AexU in HT-29 cells was noted after infection of the latter with the  $\Delta$ ahyRI mutant and WT bacteria (data not shown). These data indicated that AHL-mediated QS had no effect on the expression and translocation of the T3SS effector AexU.

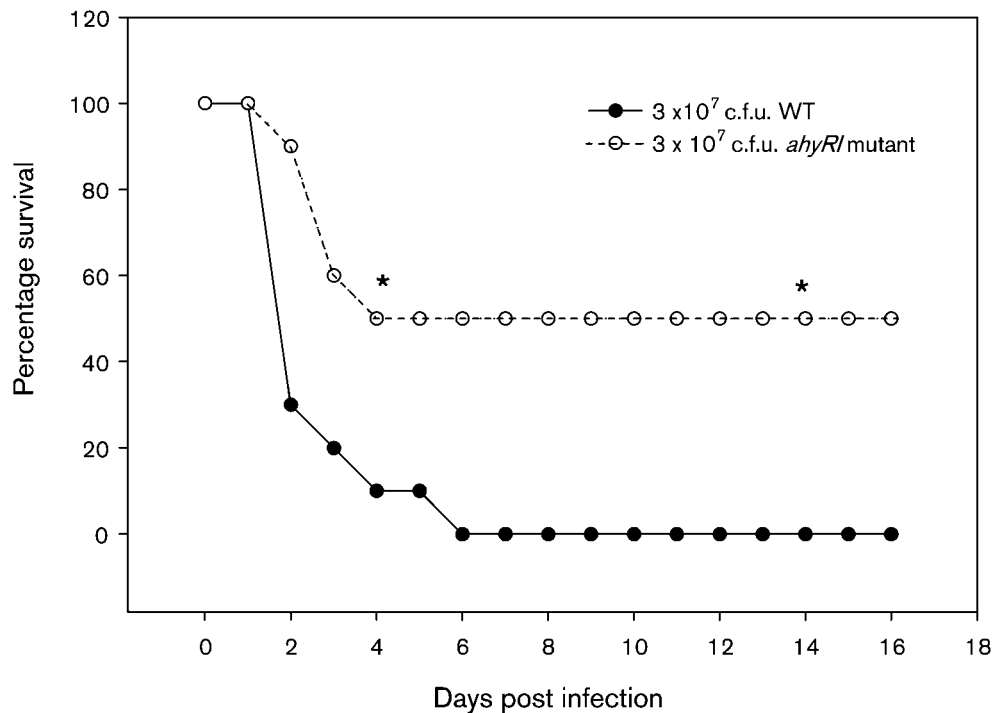
### The $\Delta$ ahyRI mutant showed decreased virulence in an animal model

By using *in vitro* experiments, we demonstrated that deletion of the *ahyRI* genes from *A. hydrophila* SSU

resulted in the decreased production of protease and prevented secretion of T6SS effectors, such as Hcp and VgrGs, and that the mutant was unable to produce mature biofilms. To examine whether these changes in virulence factors regulated by AHL-mediated QS had any influence on *in vivo* virulence of *A. hydrophila* SSU, we injected mice intraperitoneally with the  $\Delta$ ahyRI mutant and the WT strain of *A. hydrophila* at a lethal dose of  $3 \times 10^7$  c.f.u. (Fig. 5). We noted that 100% of the animals infected with the WT *A. hydrophila* died within 6 days. However, mice infected with the  $\Delta$ ahyRI mutant strain exhibited significantly lower mortality (only 50%) over a tested period of 16 days, which suggested to us that bacterial attenuation occurred when we deleted the *ahyRI* genes from *A. hydrophila* SSU.

## DISCUSSION

In the present study, we examined the regulation of AHL-mediated QS in modulating various virulence factors, including the T6SS and biofilm formation in a clinical isolate of *A. hydrophila* SSU. Interestingly, we showed that deletion of the *luxS* gene (AI-2-mediated QS) increased the overall virulence of *A. hydrophila* SSU (Kozlova *et al.*, 2008). In contrast, deletion of the *ahyRI* genes decreased



**Fig. 5.** The AhyRI QS regulon of *A. hydrophila* SSU contributes to the virulence of the bacterium. Swiss Webster mice ( $n=10$  per group) were injected intraperitoneally with two 50% lethal doses of WT *A. hydrophila* SSU. The same dose was used to infect mice with the  $\Delta$ ahyRI mutant, and both groups were monitored for death over a 16 day period. The data were statistically analysed by using Fisher's exact test. Three independent experiments were performed, and data from a typical experiment are shown. The asterisks denote statistically significant differences between the *ahyRI* mutant and WT bacteria ( $P \leq 0.05$ ).

the virulence of this pathogen. Therefore, in *A. hydrophila* SSU, QS systems have both a positive and negative effect on regulation of virulence.

A role for protease in *Aeromonas*-associated tissue damage has been reported (Sakai, 1985), and in experimental animal models, the protease null mutants exhibit a decreased virulence both with *A. hydrophila* and *Aeromonas salmonicida* (Leung & Stevenson, 1988; Sakai, 1985) compared with that of the WT bacteria. Moreover, the early expression of exoprotease may stimulate host defence (Swift *et al.*, 1999), and therefore regulation of protease production by QS could be an important step in modulating host defence as well as in establishing an infection.

We observed, in agreement with earlier studies (Bi *et al.*, 2007; Swift *et al.*, 1999), that AHL mediated QS-regulated protease production in *A. hydrophila* SSU.

Based on casein zymogram analysis, three protein bands of sizes 61, 52 and 19 kDa were associated with protease activity of *A. hydrophila* SSU, and two of these higher molecular mass bands were missing from the  $\Delta$ ahyRI mutant strain. We provided evidence that the 61 kDa metalloprotease contributed to the majority of the protease activity (81 %) in *A. hydrophila* SSU. In contrast, a previous study showed that serine protease contributed approximately 60 % and metalloprotease accounted for approximately 30 % of the total protease activity in the *A. hydrophila* AH-1N strain (Swift *et al.*, 1999). By zymogram analysis, it was demonstrated that the  $\Delta$ ahyI mutant did not produce the serine protease band (Swift *et al.*, 1999). Interestingly, in our study, addition of serine protease inhibitor minimally affected the total protease activity of *A. hydrophila* SSU. The nature of the other two proteases needs to be further elucidated.

We provided evidence that deletion of the *act* gene, which is secreted through the T2SS in *A. hydrophila* SSU, decreased the production of AHL molecules (Sha *et al.*, 2005). Consequently, we tested whether AHL-mediated QS regulated the function of Act. However, our study did not reveal any role of this AI-1 QS system in controlling the haemolytic and cytotoxic activities of this toxin in *A. hydrophila* SSU. We demonstrated that Act and T3SS- and T6SS-associated effectors contributed to cytotoxicity in host cells in *A. hydrophila* SSU (Sha *et al.*, 2005; Suarez *et al.*, 2008). Although, AHL mediated the QS-regulated T6SS, we could not differentiate between the level of cytotoxicity in the  $\Delta$ ahyRI mutant and that in the parental bacterium. These results indicated to us that cytotoxicity associated with Act and T3SS effectors could be masking T6SS-associated cytotoxic effects. In future studies, we will determine the cytotoxicity of the  $\Delta$ ahyRI mutant in *act* and *ascV* mutant strains of *A. hydrophila* SSU to discern the effect of T6SS on host cell toxicity. It will also be important to evaluate how Act might be modulating AHL levels in *A. hydrophila* SSU.

Motility is an important virulence factor of Gram-negative bacterial pathogens, as it helps them to reach the target

host tissue to colonize and cause disease (Galindo *et al.*, 2006). We noted a significantly decreased swimming motility in the  $\Delta$ luxS mutant of *A. hydrophila* SSU compared with that in the WT strain (Kozlova *et al.*, 2008). However, deletion of the *ahyRI* QS regulon did not affect the swimming and swarming motility of *A. hydrophila* SSU, which was in accordance with an earlier study on *Pseudomonas syringae* in which the AHL-mediated QS regulon did not affect motility in this plant pathogen (Kinscherf & Willis, 1999). In contrast, in other pathogens (e.g. *Erwinia chrysanthemi* and *Yersinia enterocolitica*), deletion of the *luxRI* homologues produces either enhanced motility (Hussain *et al.*, 2008) or decreased motility (Atkinson *et al.*, 2006).

Biofilms are adherent aggregates of bacterial cells growing on biotic and abiotic surfaces. Biofilm-forming bacteria are less susceptible to host immune responses and various antimicrobial agents (Costerton *et al.*, 1999). Importantly, biofilms are often associated with chronic infection, such as cystic fibrosis, caused by *P. aeruginosa* and catheter-associated biofilms of *Staphylococcus epidermidis* (Zhu & Mekalanos, 2003). In biofilms, EPS is a key component that determines physicochemical and biological properties (Laue *et al.*, 2006). Indeed, EPS is required for the initial attachment of *V. cholerae* (Watnick & Kolter, 1999) and *S. epidermidis* to a solid surface (McKenney *et al.*, 1998).

In the present study, a CV binding assay showed that the  $\Delta$ ahyRI mutant of *A. hydrophila* SSU was defective in solid surface attachment, and SEM images further confirmed that the  $\Delta$ ahyRI mutant strain produced a defective EPS on its surface which resulted in the formation of unstructured biofilms. Similar results have been reported in *P. aeruginosa* (Davies *et al.*, 1998) and other pathogens, e.g. *Serratia liquefaciens* (Labbate *et al.*, 2004), in which deletion of the homologous genes of the LuxRI QS system shows unstructured and frail biofilm formation.

More importantly, we noted that the  $\Delta$ ahyRI mutant of *A. hydrophila* SSU was unable to secrete Hcp and that it was also defective in biofilm formation, suggesting that the secretion of this protein plays an important role in the development of biofilm. Indeed, a role for Hcp in biofilm development has been noted in *P. aeruginosa* (Southey-Pillig *et al.*, 2005) and *V. parahaemolyticus* (Enos-Berlage *et al.*, 2005). When these studies were performed, it was not known that Hcp is a T6SS effector. However, the underlying mechanism(s) that modulates biofilm formation through the T6SS effector Hcp is far from clear and needs further in-depth studies, not only in *A. hydrophila* SSU, but also in other pathogens.

Although the T6SS has recently been identified in several Gram-negative bacterial pathogens (Cascales, 2008; Mougous *et al.*, 2006; Pukatzki *et al.*, 2006, 2009), the identification of the mechanism(s) of secretion and translocation of its effectors is still in its infancy. Thus far, only the Hcp and VgrG family of proteins have been shown to be secreted and translocated into eukaryotic cells

by this T6SS (Ma *et al.*, 2009). Our recent study showed that *A. hydrophila* SSU possesses a functional T6SS, and that the effector protein Hcp is translocated into eukaryotic cells through this system and plays an important role in the virulence of this pathogen (Suarez *et al.*, 2008).

VgrG proteins have different C-terminal extensions, which contain domains with different activities. For example, VgrG1 and VgrG3 from *V. cholerae* carry a repeat in structural toxin A (RtxA) and peptidoglycan-binding domains, respectively, while VgrG from *P. aeruginosa* carries a zinc metalloprotease domain (Pukatzki *et al.*, 2007). Furthermore, it has been reported that VgrG1 of *V. cholerae* has actin cross-linking activities in eukaryotic cells which are associated with cell-rounding phenotypes (Ma *et al.*, 2009; Pukatzki *et al.*, 2006, 2007). Since T6SS has complex regulatory machinery, an optimal timing of gene expression of this cluster is necessary for its optimal function. We believe that AHL-mediated QS plays a crucial role in controlling this complex secretion machinery in *A. hydrophila* SSU.

We noted that the secretion of Hcp2 and VgrG2 in the supernatant was impaired when we deleted the AhyRI QS regulon from *A. hydrophila* SSU, which suggested to us that AHL mediated the QS-regulated secretion of these effector proteins. However, these effectors could still be detected in the cell pellet of the  $\Delta$ ahyRI mutant strain at a similar level to that of the WT *A. hydrophila* SSU. We speculated that the absence of intercellular accumulation of T6SS effectors in the  $\Delta$ ahyRI mutant strain could be due either to their rapid degradation and/or alternatively to the reduced expression of the corresponding genes, and this needs to be further investigated.

Furthermore, there are two copies of the *hcp* gene (*hcp1* and *hcp2*) in the genome of *A. hydrophila* SSU, and because they are nearly identical (98% homology) and similar in size, it is possible that we could also be detecting Hcp1 on the Western blots when using polyclonal antibodies against Hcp2. Likewise, VgrG2 has high homology with VgrG3, and they are similar in size; consequently, we might be detecting VgrG3 along with VgrG2 on the Western blots by using VgrG2 antibodies. However, it is not known whether genes encoding *hcp1* and *2* are expressed and regulated similarly, as the *hcp1* gene is not located within the T6SS gene cluster (Suarez *et al.*, 2008). However, both *vgrG2* and *vgrG3* are located within the T6SS gene cluster (Suarez *et al.*, 2008). In *A. hydrophila* ATCC 7966 strain, the AHA gene designations are as follows: *hcp1* (AHA\_1118); *hcp2* (AHA\_1826); *vgrG1* (AHA\_1119); *vgrG2* (AHA\_1827); and *vgrG3* (AHA\_1848).

In a recent study on the plant pathogen *Pectobacterium atrosepticum*, it was shown for the first time that AHL mediates QS-regulated T6SS and its putative effectors Hcp and VgrGs (Liu *et al.*, 2008). In addition, it was shown by microarray analysis that 11 of the 18 genes of the T6SS cluster are expressed at significantly lower levels in the *expl* mutant than in the WT *Pectobacterium atrosepticum* (Liu

*et al.*, 2008). This study is very provocative and provides the first clue that the AhyRI regulon might affect bacterial virulence by modulating the T6SS.

Since VgrGs have a high homology between them, we detected VgrG1 in the bacterial cell pellet by using antibodies against VgrG2 (Fig. 4, lanes 2 and 4, upper band). However, we could not detect VgrG1 in the culture supernatant of either the WT or its  $\Delta$ ahyRI mutant strain (Fig. 4, lanes 1 and 3). Nonetheless, the production of VgrG1 was similar in the WT versus its  $\Delta$ ahyRI mutant strain in bacterial pellets. It was recently shown that deletion of the *clpV* gene, an ATPase which provides energy for the secretion of T6SS effectors, inhibits their secretion, while the expression and production of these effector proteins are unaltered (Mougous *et al.*, 2006). Likewise, we demonstrated in the *vasK* mutant that the secretion, but not the expression/production and translocation of Hcp, were affected, and that the *vasK* mutant was highly attenuated in a septicemic mouse model of *A. hydrophila* infection.

In addition, we showed that mice infected with WT *A. hydrophila* SSU had circulating antibodies to Hcp, and animals immunized with recombinant Hcp were protected from subsequent challenge with WT bacterium (Suarez *et al.*, 2008). These data clearly suggested to us that secreted Hcp played an important role in the virulence of this pathogen (Suarez *et al.*, 2008). Therefore, we speculate that AHL-mediated QS regulates secretion of T6SS effectors by modulating other T6SS components, such as the *vasK* and *clpV* genes. However, further detailed studies are needed to delineate the mechanistic basis of how AHL-mediated QS regulates the T6SS in *A. hydrophila* SSU.

The T3SS has been identified in several *Aeromonas* species, and its role in the establishment of infection in the host determined (Burr *et al.*, 2003; Sha *et al.*, 2005, 2007; Vilches *et al.*, 2004). Furthermore, studies have shown that QS modulates the T3SS either positively in enteropathogenic and enterohaemorrhagic *E. coli* (Sperandio *et al.*, 1999) or negatively in *P. aeruginosa*, *Y. pestis*, *Vibrio harveyi* and *Vibrio parahaemolyticus* (Blevins *et al.*, 2005; Gelhaus *et al.*, 2009; Henke & Bassler, 2004). However, in the present study, we noted that the AHL-mediated QS system had no role in the regulation of the T3SS effector AexU in *A. hydrophila* SSU. Similarly, we showed that deletion of the *luxS* gene from *A. hydrophila* SSU had no effect on the expression and translocation of AexU (Kozlova *et al.*, 2008). In enterohaemorrhagic *E. coli*, the T3SS is regulated by the AI-3-mediated QS (Sperandio *et al.*, 1999), and recently we identified the AI-3 QS in *A. hydrophila* SSU (unpublished data). In the future, we will examine the role of AI-3-mediated QS in regulating T3SS genes in *A. hydrophila* SSU.

There is limited information on the role of an AHL-mediated QS *in vivo* model of infection. Our study on the septicemic model of mouse infection indicated that the  $\Delta$ ahyRI mutant of *A. hydrophila* SSU is significantly less

virulent than the WT bacterium, which suggested to us that this AHL-mediated QS regulon contributes to the virulence of *A. hydrophila* SSU. The *in vivo* role of AHL-mediated QS has been well established in a report of an acute lung infection model of *P. aeruginosa* (Smith *et al.*, 2002). Furthermore, AHLs have been detected in lung tissues of mice infected with *P. aeruginosa* (Wu *et al.*, 2000), which is further evidence that these signalling molecules play a role in bacterial pathogenesis. In addition, deletion of LasRI and/or RhlRI QS systems from *P. aeruginosa* was found to result in significant attenuation in terms of its ability to colonize hosts, induce inflammation and cause mortality (Pearson *et al.*, 2000; Rumbaugh *et al.*, 1999; Smith *et al.*, 2002; Tang *et al.*, 1996; Wu *et al.*, 2001). In a future study, we will delineate the direct role of AHL molecules in the pathogenesis of *A. hydrophila* SSU in a mouse model of infection.

In conclusion, we demonstrated that AHL-mediated QS plays a crucial role in modulating the virulence of *A. hydrophila* SSU, and these findings validated the notion that interference by AHL-mediated QS would be a promising target for the development of new-generation antimicrobial therapeutics.

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