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# The two-component QseBC signalling system regulates *in vitro* and *in vivo* virulence of *Aeromonas hydrophila*

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We recently demonstrated that the *N*-acyl-homoserine lactone [autoinducer (AI)-1] and LuxS (AI-2)-based quorum-sensing (QS) systems exerted positive and negative regulation, respectively, on the virulence of a diarrhoeal isolate SSU of *Aeromonas hydrophila*. However, the role of a newly identified, two-component-based QseBC QS system in the regulation of bacterial virulence in general is not well understood, with only a limited number of studies showing its function in bacterial pathogenesis. In this report, we identified and characterized the QseBC QS system in *A. hydrophila* SSU and found that, as was the case with enterohaemorrhagic *Escherichia coli*, the open reading frames for the *qseB* (the response regulator) and *qseC* (the sensor histidine kinase) genes overlapped by 4 bp at the ATGA motif. Our data provide evidence that deletion of the *qseB* gene from *A. hydrophila* resulted in attenuation of bacterial virulence in a septicaemic mouse model of infection and diminished swimming and swarming motility, and the mutant bacteria formed denser biofilms compared with those from the parental strain of *A. hydrophila*. The decrease in the virulence of the *A. hydrophila* Δ*qseB* mutant correlated with reduced production of protease and the cytotoxic enterotoxin, which has associated haemolytic activity. The swimming and swarming motility, haemolytic activity, protease production and biofilm formation were restored in the *qseBC*-complemented strain to a level similar to that of the wild-type *A. hydrophila* SSU. Our study is the first, to our knowledge, to report a functional QseBC QS system in *A. hydrophila* which may be linked to AI-1 and AI-2 QS systems in modulating bacterial virulence, possibly through the cyclic diguanosine monophosphate.

## INTRODUCTION

In bacteria, two-component systems are widely used signal transduction mechanisms that facilitate in eliciting an adaptive response to various environmental stimuli, particularly through changes in gene transcription (Beier & Gross, 2006; Hoch, 2000). The two-component systems are typically composed of a membrane-associated sensor histidine kinase and a cytoplasmic transcriptional regulator (Beier & Gross, 2006; Hoch, 2000). In most cases, the stimuli sensed by these systems are transformed into a cellular signal via autophosphorylation of sensor kinase at the conserved histidine residue. The signal is then transmitted onto the response regulator, following phosphorylation at the aspartate residue, which results in its activation by undergoing conformational changes. The activated response regulator then exerts its regulation on the transcription of various target genes (Beier & Gross, 2006; Hoch, 2000).

One such two-component system that responds to a quorum sensing (QS) signal is QseBC (QseC, a sensor

Abbreviations: AI, autoinducer; CV, crystal violet; EHEC, enterohaemorrhagic *Escherichia coli*; gDNA, genomic DNA; QS, quorum sensing; RBC, red blood cell; SEM, scanning electron microscopy; T2SS, T3SS, T6SS, type X secretion system; WT, wild-type.

The GenBank/EMBL/DDBJ accession number for the *A. hydrophila* SSU *qseBC* gene is JF922013.

Five supplementary figures are available with the online version of this paper.

histidine kinase, and QseB, a response regulator), which was first discovered in enterohaemorrhagic *Escherichia coli* (EHEC) (Sperandio *et al.*, 2002). Later, this system was also found in other pathogens, such as *Salmonella enterica* serovar Typhimurium (Bearson & Bearson, 2008; Moreira *et al.*, 2010), *Edwardsiella tarda* (Wang *et al.*, 2011) and uropathogenic *E. coli* (UPEC) (Kostakioti *et al.*, 2009). In addition to responding to an autoinducer (AI)-3, QseC sensor kinase has been reported to also respond to eukaryotic hormones typified by epinephrine and/or norepinephrine (Clarke *et al.*, 2006; Waldor & Sperandio, 2007; Walters & Sperandio, 2006). Further, by using adrenergic receptor antagonists, it is possible to block the effects of AI-3 and epinephrine/norepinephrine, perhaps indicative of a similar structure for these molecules and a similar signalling pathway (Walters & Sperandio, 2006). The role of the QseBC system in the pathogenesis of EHEC, *Francisella tularensis* and *S.* Typhimurium was recently reported (Rasko *et al.*, 2008), as deletion of the QseC histidine kinase-encoding gene attenuated virulence of these bacteria (Rasko *et al.*, 2008). Further, a synthetic compound (LED209) that interferes with the QseC signalling inhibited the *in vitro* virulence of EHEC, *S.* Typhimurium and *F. tularensis*, and it also modulated *in vivo* virulence of the latter two pathogens (Rasko *et al.*, 2008). In UPEC, a recent study has shown that in addition to kinase activity, QseC has phosphatase activity that is critical in modulating the regulatory activity of QseB (Kostakioti *et al.*, 2009).

Initially, only four *Aeromonas* species were recognized; however, through developments in the molecular post-genomic era, there are now 30 known species of *Aeromonas* (http://www.bacterio.cict.fr/). Among these, *A. hydrophila*, *A. caviae* and *A. veronii* biovar sobria are the most common species known to cause the majority of human infections (Janda, 1991). Our case-control study reported the presence of aeromonads in 7.2 % of children suffering from diarrhoea in Bangladesh, with only 3.3 % of the healthy children excreting aeromonds in their stools (Albert *et al.*, 2000). Likewise, in our 27 month prospective study, we showed a similar isolation rate of this organism from the paediatric population suffering from diarrhoea (7.3 %) at the Mercy Hospital in Chicago, with only 2.2 % of the control healthy children excreting aeromonads in the stools (Challapalli *et al.*, 1988). In addition, our recent study has clearly shown transmission of *Aeromonas* species from water to humans (Khajanchi *et al.*, 2010). A PubMed search using the word '*Aeromonas*' generated approximately 663 citations between 1980 and 1994 (Janda & Abbott, 2010). However, to date, this number has increased to 5398, which illustrates that over the years there has been an enormous increase in studying the genus *Aeromonas* by the scientific and medical community.

In humans, *Aeromonas* species cause diarrhoea and various extra-intestinal infections (Galindo *et al.*, 2006; Janda, 2002; Vila *et al.*, 2003), which include septicaemia, cellulitis, wound infections, urinary tract infections, soft tissue infections and, occasionally, meningitis and peritonitis (Galindo *et al.*, 2006; Horneman *et al.*, 2007; Vila *et al.*, 2003). This pathogen can cause haemolytic uraemic syndrome and necrotizing fasciitis, particularly in children with a compromised immune status (Abuhammour *et al.*, 2006; Figueras *et al.*, 2007). In addition, this waterborne pathogen receives significant attention during natural disasters, as aeromonads were most frequently isolated from wounds of patients following the 2004 tsunami in Thailand (Hiransuthikul *et al.*, 2005). Further, an increased isolation rate of *Aeromonas* species was noted in floodwater samples after Hurricane Katrina in New Orleans (Presley *et al.*, 2006).

In addition to causing human diseases, aeromonads are also associated with various severe diseases in both cold- and warm-blooded animals, and these include furunculosis in fish (commonly caused by *A. salmonicida*), ulcerative stomatitis in snakes and lizards, 'red leg' disease in frogs, septicaemia in dogs and septic arthritis in calves (Janda & Abbott, 2010).

*A. hydrophila* produces a number of virulence factors which function together to cause diseases in the host (Chopra & Houston, 1999; Khajanchi *et al.*, 2010; Krovacek *et al.*, 1994; Sha *et al.*, 2002). In our laboratory, we have identified and characterized new virulence factors/mechanisms that contribute to virulence in the diarrhoeal isolate SSU of *A. hydrophila*. Some of the key virulence factors that have been characterized are: (i) a cytotoxic enterotoxin (Act), a type 2 secretion system (T2SS) toxin that functions as a haemolysin, a cytotoxin or an enterotoxin, depending upon the target cells (Chopra *et al.*, 2000; Galindo *et al.*, 2004; Sha *et al.*, 2002); (ii) a new T3SS effector, AexU, which leads to ADP-ribosylation of the host cell proteins, resulting in their death via apoptosis (Sierra *et al.*, 2007, 2010); and (iii) two T6SS effectors, such as haemolysin-coregulated protein (Hcp) and the valine glycine repeat G (VgrG) family of proteins (Suarez *et al.*, 2008). VgrG1 of *A. hydrophila* possesses actin ADP-ribosylating activity associated with its carboxyl-terminal vegetative insecticidal protein-2 (VIP-2) domain that induces cell rounding followed by host cell apoptosis (Suarez *et al.*, 2010a). Hcp, on the other hand, modulates innate immunity by inhibiting phagocytosis of *A. hydrophila*, thus allowing bacterial multiplication and spread to different organs of mice, resulting in their death (Suarez *et al.*, 2010b).

In bacteria, the cell-to-cell signalling system, known as QS, has been identified as a global regulator which controls virulence mechanisms at appropriate times, depending on the physiological conditions in the environment as well as in the host. In Gram-negative bacteria, three QS circuits have been identified, of which AI-3/QseBC is the least studied. Recently, we characterized the role of *N*-acyl-homoserine lactone-mediated (AI-1) and LuxS-based (AI-2) QS systems in the regulation of virulence factors in *A. hydrophila* SSU (Khajanchi *et al.*, 2009; Kozlova *et al.*, 2008). We demonstrated that the *ahyRI*-based AI-1 QS

system of *A. hydrophila* was a positive regulator of bacterial virulence, as disruption of the *ahyRI* genes reduced metalloprotease production, biofilm formation, secretion of the type T6SS effectors, e.g. Hcp and VgrG family of proteins, and mortality in a septicaemic mouse model of infection (Khajanchi *et al.*, 2009). In contrast, we showed that the LuxS-based QS system (AI-2) negatively regulated the virulence of this pathogen, as the *luxS* mutant resulted in increased biofilm formation and enhanced mortality in an animal model compared with the wild-type (WT) bacterium (Kozlova *et al.*, 2008). We also provided data showing that the bacterial second messenger cyclic-diguanosine monophosphate (c-di-GMP) (Hengge, 2009; Römling & Simm, 2009) affected the virulence-associated phenotype in WT and an *ahyRI* mutant of *A. hydrophila* SSU (Kozlova *et al.*, 2011).

By our sequence annotation of *A. hydrophila* ATCC 7966 strain (Seshadri *et al.*, 2006), we identified *qseB* and *qseC* genes in *A. hydrophila* SSU, which exhibited a 99 and 96 % homology at the amino acid level with the corresponding genes of strain ATCC 7966. A 4 bp overlap with the ATGA motif was found in the open reading frames (ORFs) for *qseB* and *qseC* genes in which the translation stop codon of QseB overlapped with the translation start codon for QseC. A similar genomic organization for *qseBC* genes was identified in EHEC (Clarke & Sperandio, 2005b); however, QseB exhibited 51 % and QseC showed only 31 % identity with the corresponding proteins of *E. coli*.

In this study, we identified and characterized the role of the QseBC system in the regulation of virulence in *A. hydrophila* SSU by generating a Δ*qseB* isogenic mutant. We demonstrated that the QseBC system in *A. hydrophila* positively regulated both swimming and swarming motility, and haemolytic activity of Act and protease production, while negatively modulating the biofilm formation. Since QseBC functions as both a positive and negative regulator in controlling *in vitro* virulence of *A. hydrophila*, we indeed observed a marginal, but statistically significant, attenuation of virulence in an *in vivo* septicaemic mouse model when the *qseB* gene was deleted from the WT strain. As mentioned above, QseBC has been studied recently in other bacterial species (Bearson & Bearson, 2008; Kostakioti *et al.*, 2009; Sperandio *et al.*, 2002; Wang *et al.*, 2011), and this regulatory system controls different sets of virulence genes in various bacteria. Consequently, more information is needed as to how this regulatory system may be modulating bacterial virulence in general. We believe *Aeromonas* represents a model organism to study the QseBC system, as it has characteristics of *E. coli*, *Vibrio* and *Pseudomonas* (Kozlova *et al.*, 2011). In addition, it possesses functional type 2, 3 and 6 secretion systems through which bacteria secrete a variety of virulence factors. The presence of this network of virulence factors results in very complex regulatory mechanisms that turn virulence genes 'off' and 'on' based on QS signalling molecules present in a given environment. Therefore, our study is timely and important in better understanding the functional role of QseBC QS in the regulation of virulence in *A. hydrophila*, as currently there are no data available, to our knowledge, on the QseBC system of *A. hydrophila*.

## METHODS

**Bacterial strains, plasmids and chemicals.** The bacterial strains and plasmids used in this study are listed in Table 1. LB medium was supplemented with L-arabinose (0.2 %) when the *ggdef*-domain-containing gene (GGDEF-domain-containing proteins increase c-di-GMP within bacterial cells) was expressed from the pBAD/Myc-HisB::*ggdef* plasmid (Table 1) under the control of an arabinose-inducible pBAD promoter (Kozlova *et al.*, 2011). The kinetic growth data showed no difference in the growth rates of WT *A. hydrophila* SSU versus the *qseB* mutant strain (data not shown). However, we always normalized the data with the same c.f.u. of bacteria for any minor variations that we might observe in the growth rates among the strains tested.

**Generation of an isogenic *qseB* mutant of *A. hydrophila* SSU.** The *qseB* single-knockout mutant of *A. hydrophila* SSU was generated by using a double crossover homologous recombination method. The *qseB* gene (690 bp) was amplified by PCR employing genomic DNA (gDNA) of *A. hydrophila* SSU-R (Table 1) as the template, and a pair of primers (qseB-N, 5′-ATGCGGATCCTGTTGGTGGAAGA-3′ and qseB-C, 5′-CATGCCCGGTGGTCCCGGCGCTG-3′). The PCR product was then cloned in the TA cloning vector pCR2.1 (Invitrogen). Subsequently, the pCR2.1-*qseB* recombinant plasmid (r-plasmid) was transformed into *E. coli* DH5α (Table 1). Within the *qseB* coding region, a unique *Blp*I restriction enzyme site exists; the pCR2.1-*qseB* r-plasmid was thus linearized by *Blp*I digestion. A kanamycin resistance ($Km^r$) gene cassette flanked by the *Blp*I restriction site from the plasmid pUC4K (GE Healthcare) was inserted at the *Blp*I site of pCR2.1-*qseB* r-plasmid to generate a pCR2.1-*qseB*-Km plasmid (Table 1). After digestion with the *Kpn*I/*Xba*I restriction enzymes, the DNA fragment from the pCR2.1-*qseB*-Km r-plasmid was ligated to a pDMS197 suicide vector at the *Kpn*I/*Xba*I sites, and the resulting plasmid (pDMS197-*qseB*-Km) was transformed into an *E. coli* SM10 *λpir* strain (Edwards *et al.*, 1998) (Table 1). The recombinant *E. coli* (pDMS197-*qseB*-Km) was then conjugated with the WT *A. hydrophila* SSU-R. The transconjugants were selected based on resistance to appropriate antibiotics and sucrose and subjected to further analysis (Sha *et al.*, 2002). The identity of the *qseB* mutant was confirmed by Southern blot analysis.

**Southern blot analysis.** From the WT *A. hydrophila* SSU and its *qseB* mutant strain, the gDNA was isolated, digested with *Kpn*I/*Xba*I restriction enzymes and subjected to 0.8 % agarose gel electrophoresis. Southern blot analysis was performed as described previously (Sha *et al.*, 2002; Xu *et al.*, 1998). The PCR products from the *qseB* gene, $Km^r$ gene cassette, and the pDMS197 vector digested by using *Xba*I–*Kpn*I were employed as probes.

**Complementation of the *A. hydrophila* SSU Δ*qseB* mutant.** The *qseBC* genes were amplified by PCR using gDNA of *A. hydrophila* ATCC 7966 as the template and two primers QseBCN-*Hin*dIII (5′-GGGAAGCTTGCATCGACCCCAACTTCTTCT-3′) and QseBCC-*Nhe*I (5′-GGGGCTAGCTGGAGCACATGGTGACGGT-3′; the restriction endonuclease sites are underlined in both primers). Since *qseBC* genes are highly homologous between *A. hydrophila* strains SSU and ATCC 7966, we used *qseBC* from the latter strain for complementation, as we annotated the genome of this strain and the upstream sequences containing the promoter regions for these genes were available (Seshadri *et al.*, 2006). We included a 249 bp flanking

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

Abbreviations: Rif, rifampicin; Km, kanamycin; Sm, streptomycin; Sp, spectinomycin; Ap, ampicillin; Tc, tetracycline.

| Strain or plasmid | Relevant characteristic(s) | Source or reference |
|---|---|---|
| ***A. hydrophila* SSU** | | CDC |
| ATCC 7966 | Environmental isolated strain | ATCC |
| SSU-R | $Rif^r$ strain of *A. hydrophila* SSU | Laboratory stock |
| Δ*qseB* | *qseB* gene deletion mutant of *A. hydrophila* SSU-R strain $Rif^r$ $Km^r$ | This study |
| Δ*ahyRI* | *ahyRI* gene deletion mutant of *A. hydrophila* SSU-R strain $Rif^r$ $Sm^r$ $Sp^r$ | Khajanchi *et al.* (2009) |
| Δ*luxS* | *luxS* mutant of *A. hydrophila* SSU-R strain $Rif^r$ $Km^r$ | Kozlova *et al.* (2008) |
| Δ*qseB*/pBR322-*qseBC* | *qseB* mutant complemented with the *qseBC* genes (ATCC) via pBR322 $Rif^r$ $Km^r$ $Ap^r$ | This study |
| ***E. coli*** | | |
| DH5α | Production of recombinant plasmids. *recA gyrA* | Life Technologies |
| SM10 | $Km^r$, *λpir* | Edwards *et al.* (1998) |
| **Plasmids** | | |
| pCR2.1 | TA cloning vector $Ap^r$ $Km^r$ | Invitrogen |
| pCR2.1/*qseB* | TA cloning vector carrying *qseB* gene $Ap^r$ $Km^r$ | This study |
| pUC-4K | Contains a 1.2 kb kanamycin$^r$ gene cassette | Amersham |
| pCR2.1/*qseB*-Km | TA cloning vector harbouring the *qseB* gene disrupted by Km cassette, $Ap^r$ $Km^r$ | This study |
| pDMS197 | Suicide vector; R6K *ori*, *sacB*, $Tc^r$ | Edwards *et al.* (1998) |
| pDMS197/*qseB*-Km | Suicide vector containing *qseB* gene with Km cassette, $Tc^r$ $Km^r$ | This study |
| pBR322 | $Ap^r$ $Tc^r$ | Amersham |
| pBR322-*qseBC* | Contains the *qseBC* genes (ATCC), $Ap^r$ | This study |
| pBAD/*Myc*-HisB | Vector, *ara*BAD promoter $Ap^r$ | Invitrogen |
| pBAD::*ggdef* | GGDEF-domain-encoded gene of *A. hydrophila* cloned into pBAD/*Myc*-HisB *ara* $Km^r$ $Ap^r$ | Laboratory stock |

upstream DNA sequence containing the potential promoter region of the *qseBC* genes for complementation studies. This DNA fragment (2648 bp) was cloned in pBR322 vector ($Tc^r$ $Ap^r$) at the *Hin*dIII–*Nhe*I sites and transformed into the *E. coli* DH5α strain (Table 1). The pBR322/*qseBC* ($Tc^s$ $Ap^r$) recombinant plasmid was isolated from the *E. coli* strain and electroporated into an *A. hydrophila* SSU Δ*qseB* mutant (Sha *et al.*, 2002) (Table 1).

We complemented the *qseB* mutant with both the *qseBC* genes, as they exist in an operon, and we suspected that deletion of the *qseB* gene would have impacted the transcript level of the *qseC* gene.

**Swimming and swarming motility assay.** LB medium with 0.3 % Difco Bacto-agar was used to characterize the swimming motility, while Difco nutrient broth with 0.5 % Eiken agar (Eiken Chemical) was employed for measuring the swarming motility of WT *A. hydrophila* SSU, its Δ*qseB* mutant, and the complemented strain, as described in our previous studies (Khajanchi *et al.*, 2009; Kozlova *et al.*, 2008). For both the swimming and swarming motility assay, media supplemented with ampicillin (450 μg $ml^{-1}$) were used for the complemented strain. Briefly, the overnight cultures grown in LB medium in the presence of the respective antibiotics were adjusted to the same optical density, and equal numbers of WT *A. hydrophila*, its Δ*qseB* mutant bacteria or the complemented strain ($1 \times 10^8$ c.f.u.) were stabbed into agar plates. The swimming and swarming agar plates were incubated at 37 and 30 °C, respectively, for 16–18 h and then motilities were assessed by examining migration of bacteria through the agar from the centre towards the periphery of the plate.

**Measurement of the haemolytic activity.** For measuring the haemolytic activity associated with Act of WT *A. hydrophila* SSU, its Δ*qseB* mutant or the complemented strain, the culture filtrates from bacteria grown for 18 h in LB medium at 37 °C with shaking (180 r.p.m.) were first treated with trypsin [final concentration 0.05 % to activate Act (Sha *et al.*, 2004)] at 37 °C for 1 h and then subjected to a haemolytic assay by using rabbit red blood cells (RBCs), as described previously (Sha *et al.*, 2002). Briefly, 100 μl Dulbecco's PBS (DPBS) was added to each well of a 96-well microtitre plate. Aliquots (100 μl) of culture filtrates were added to the first well, followed by a serial twofold dilution, with subsequent addition of 100 μl 2.5 % rabbit erythrocytes (Colorado Serum). The plate was incubated at 37 °C for 1 h and observed for the lysis of RBCs. The supernatant was taken from those wells that showed a partial lysis of rabbit erythrocytes, and the release of haemoglobin was then evaluated by measuring absorbance at 540 nm. The haemolytic activity titres were calculated as the absorbance value of the haemoglobin release multiplied by the dilution of the culture filtrates. The haemolytic units were reported per ml cell filtrate per $1 \times 10^8$ c.f.u.

For the neutralization assays, culture filtrates of WT and Δ*qseB* mutant strains were mixed with either pre-immune (control) or hyper-immune rabbit sera (laboratory stock, 1 : 10 dilution) containing antibodies to Act (Erova *et al.*, 2007; Khajanchi *et al.*, 2009) before we measured the haemolytic activity.

**Measurement of the protease activity.** Protease activity was measured in culture filtrates of overnight-grown WT *A. hydrophila*, its Δ*qseB* mutant or the complemented strain, as described earlier (Erova *et al.*, 2006; Khajanchi *et al.*, 2010). The protease activity was calculated per ml cell filtrate per $1 \times 10^8$ c.f.u. The hide azure powder substrate (Calbiochem) was used for measuring protease activity because of the sensitivity and rapidity of the assay. Further, this substrate could detect both metallo and serine proteases, which are the two major classes of this enzyme produced by *Aeromonas* species (Swift *et al.*, 1999). The substrate incubated with DPBS alone served as a negative control.

**Crystal violet (CV) biofilm assay.** The WT *A. hydrophila* SSU, its Δ*qseB* mutant or the complemented strain were grown in 3 ml LB broth contained in polystyrene tubes at 37 °C for 24 h with shaking.

Biofilm formation was quantified according to the procedure described elsewhere (Khajanchi *et al.*, 2009; O'Toole & Kolter, 1998). The biofilm formation results were normalized to $1 \times 10^9$ c.f.u. to account for any minor differences in the growth rate of various bacterial strains used. The experiment was repeated independently three times.

**Scanning electron microscopy (SEM) of biofilms.** SEM of biofilm formation of *A. hydrophila* SSU and its Δ*qseB* mutant was performed by using 13 mm diameter thermanox plastic coverslips. After 48 h incubation, unattached cells were removed, and then 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) by using the procedure described in our previous studies (Khajanchi *et al.*, 2009; Kozlova *et al.*, 2008).

**Western blot analysis.** Overnight cultures of WT and Δ*qseB* mutant strains were diluted 1 : 20 in fresh LB medium and grown for 2 h ($OD_{600}$~0.8) and/or 4 h ($OD_{600}$~1.4) at 37 °C with shaking at 180 r.p.m. Western blot analysis was performed to measure production and secretion of T6SS effector Hcp in the LB medium (Khajanchi *et al.*, 2009). We also examined production of T3SS effector AexU in the bacterial whole-cell lysates as well as in the insoluble fraction collected after co-culturing of WT and Δ*qseB* mutant of *A. hydrophila* SSU with HeLa cells in the Dulbecco's modified Eagle medium (DMEM) (Khajanchi *et al.*, 2009; Sha *et al.*, 2007).

**Animal experiments.** Groups of 10 Swiss Webster mice (Taconic Farms) were infected by the intraperitoneal route with $5 \times 10^7$ c.f.u. (WT or its Δ*qseB* mutant) in accordance with the approved animal care protocol. One group of mice was inoculated with DPBS ($n$=10) and served as a control. Deaths were recorded for 16 days post-infection. The animal experiments were repeated three times.

**Statistical analysis.** All of the experiments were performed in triplicate, and wherever appropriate, the data were analysed by using the Student's $t$ test, and a $P$-value of $\leqslant 0.05$ was considered significant. The data were presented as an arithmetic mean ± SD. The animal data were analysed by using the Kaplan–Meier's survival estimates.

## RESULTS

### Characterization of the two-component QseBC QS system from *A. hydrophila* SSU

By analysing the protein sequences of QseB and QseC in the NCBI conserved domains database, a Pfam protein sequence search (http://pfam.sanger.ac.uk/) and SMART analysis (http://smart.embl-heidelberg.de/), it was revealed that QseB possesses two domains: a receiver domain (REC) and a transcriptional regulatory protein/C-terminal helix–turn–helix (HTH) domain (Supplementary Fig. S1a, available with the online version of this paper). QseC, on the other hand, harbours three domains: a HAMP/transmembrane domain, a His-kinase (HisKA) and a ATPase domain (Supplementary Fig. S1b).

### Deletion of the *qseB* gene from *A. hydrophila* SSU diminishes both swimming and swarming motility

To characterize the role of the QseBC system in the regulation of virulence in *A. hydrophila* SSU, we deleted the *qseB* gene by double crossover homologous recombination and subsequently generated a complemented strain with both of the *qseBC* genes that were supplied *in trans*. Motility is considered to be an important virulence factor in the pathogenesis of *Aeromonas*-associated infections, as it facilitates pathogens to adhere and invade the host cells (Kirov *et al.*, 2002; Kirov, 2003; Kirov *et al.*, 2004). *Aeromonas* species possess polar flagella for swimming motility and lateral flagella for swarming motility (Kirov *et al.*, 2002; Kirov, 2003; Kirov *et al.*, 2004). To cause infection by avoiding the host defence, the motility of the pathogens must be tightly regulated. In this study, we examined whether the two-component QseBC system involved in QS regulates motility in *A. hydrophila*. The results of the motility assay revealed that the Δ*qseB* mutant had significantly reduced migration in the swimming and the swarming agar plates, compared with that of the WT *A. hydrophila* SSU (Fig. 1a, b). The latter finding indicated that both the swimming and swarming motility in *A. hydrophila* SSU were regulated by the QseBC QS system. Further, the swimming and the swarming motility were restored in the *qseBC*-complemented strain (Fig. 1a, b). Importantly, when the GGDEF-domain-containing protein (AHA0701h) was overproduced in the Δ*qseB* mutant (Kozlova *et al.*, 2011), the swimming motility of this strain was further reduced compared with the Δ*qseB* mutant harbouring the pBAD vector alone (Fig. 1c).

### Haemolytic activity of T2SS-associated Act is significantly reduced in the Δ*qseB* mutant

We earlier showed that Act is one of the most potent virulence factors that contributes to the pathogenesis of *A. hydrophila* SSU (Chopra *et al.*, 2000; Galindo *et al.*, 2004; Sha *et al.*, 2002). To examine whether the QseBC-system regulates Act production, we measured the haemolytic activity associated with Act in the culture supernatant of WT *A. hydrophila* SSU, the Δ*qseB* mutant and the *qseBC*-complemented strain. Interestingly, we found that the haemoglobin release from rabbit RBCs was significantly reduced when culture filtrate from the Δ*qseB* mutant was used, compared with culture filtrate from the WT strain, and the haemoglobin release was restored in the *qseBC* complemented strain (Fig. 2).

To demonstrate that the majority of this haemolytic activity was associated with Act, we neutralized the toxin by using specific antibodies. Indeed we noted much reduced and similar residual haemolytic activity in the culture supernatants of both WT and Δ*qseB* mutant. This residual activity is contributed by another haemolysin that we characterized in isolate SSU of *A. hydrophila* (Erova *et al.*, 2007). These data indicated that the haemolytic activity associated with this haemolysin gene was not altered by deletion of the *qseB* gene and that the QseBC system specifically controlled the haemolytic activity of Act in *A. hydrophila* SSU.

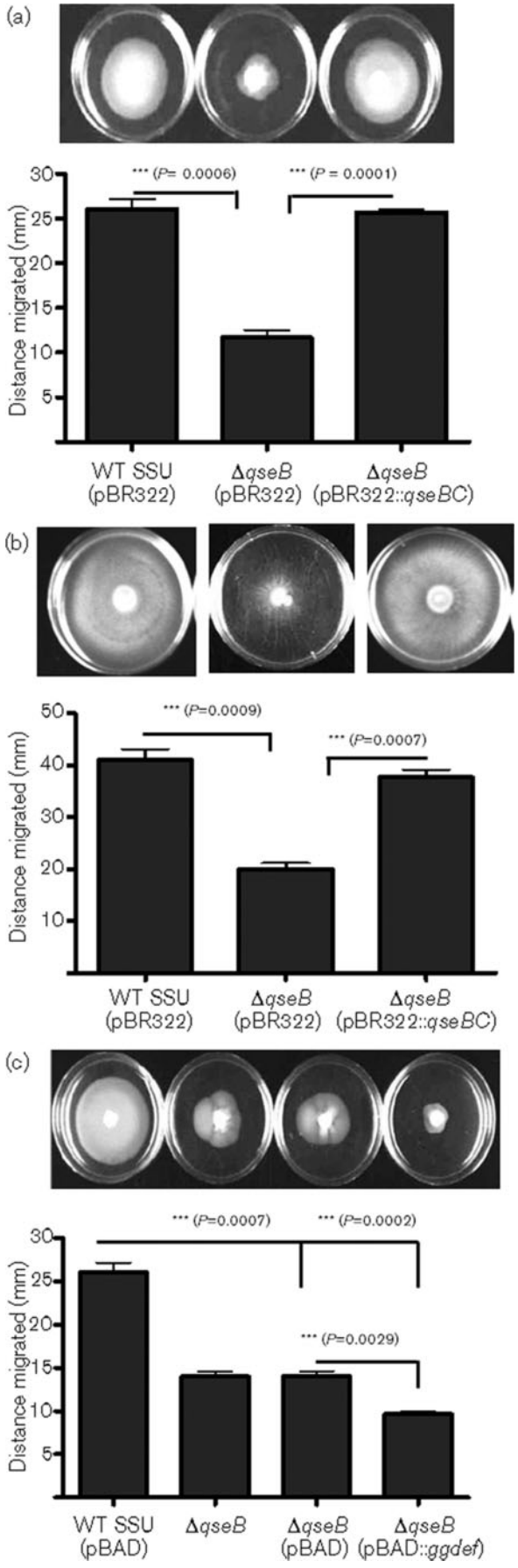


**Fig. 1.** Swimming and swarming motility of WT *A. hydrophila* SSU, the Δ*qseB* mutant and its *qseBC*-complemented strain. (a) LB medium with 0.3 % Difco Bacto-agar (supplemented with ampicillin for the complemented strain) was used to characterize the swimming motility. The Δ*qseB* mutant showed significantly decreased migration compared with WT *A. hydrophila* SSU. The *qseBC*-complemented strain migrated in a manner similar to that of the WT strain. Asterisks (***) indicate a significant difference in migration between WT *A. hydrophila* SSU and its Δ*qseB* mutant ($P$=0.0006), and between the Δ*qseB* mutant and the *qseBC*-complemented strain ($P$=0.0001). (b) Difco nutrient broth with 0.5 % Eiken agar was used to determine swarming motility. A strong swarming response was observed for the WT *A. hydrophila* SSU and the *qseBC*-complemented strain, while reduced swarming motility was noted for the Δ*qseB* mutant. Asterisks (***) indicate a significant difference in migration between the WT *A. hydrophila* SSU and the Δ*qseB* mutant ($P$=0.0009), and between the Δ*qseB* mutant and the *qseBC*-complemented strain ($P$=0.0007). (c) Influence of *ggdef* encoding gene overexpression in the Δ*qseB* mutant on swimming motility. The Δ*qseB* mutant with *ggdef* overexpression did not show any migration but grew at the inoculation site. Asterisks (***) indicate statistically significant differences between the WT and *qseB* mutant ($P$=0.0007), and the *qseB* mutant and the *qseB* mutant with an overexpressed *ggdef*-encoding gene ($P$=0.0029). Three independent experiments were performed, and the arithmetic means ± SD are plotted. Statistical analyses between the WT and the mutant and complemented strains were performed by using Student's *t* test.

## The Δ*qseB* mutant produces lower amounts of protease

Earlier studies have shown that the pathogenic and virulence characteristics of *A. hydrophila* were associated in part with the production of proteases (Leung & Stevenson, 1988; Sakai, 1985). Consequently, we measured protease production and found that the Δ*qseB* mutant exhibited a significantly reduced level of protease production when compared with the WT *A. hydrophila* SSU, and this enzyme activity was restored in the *qseBC*-complemented strain (Fig. 3), possibly meaning that the QseBC system also controlled protease production in *A. hydrophila*. Further, to investigate the influence of overproduction of GGDEF protein on protease level, we examined protease activity in WT *A. hydrophila* SSU and the Δ*qseB* mutant with overproduced GGDEF protein. Interestingly, our data showed that the protease level was decreased when GGDEF protein was overproduced in WT *A. hydrophila* SSU, when compared with the parental strain with the empty pBAD vector. In addition, the Δ*qseB* mutant with overproduced GGDEF protein further reduced protease activity compared to that in the Δ*qseB* mutant with the pBAD vector alone (Fig. 4). These data clearly indicate a link between the QseBC QS system with *A. hydrophila* virulence (motility, haemolytic activity associated with Act and protease production) and c-di-GMP.

To examine whether the regulation of protease production by QseBC is dependent on cell density, we measured the

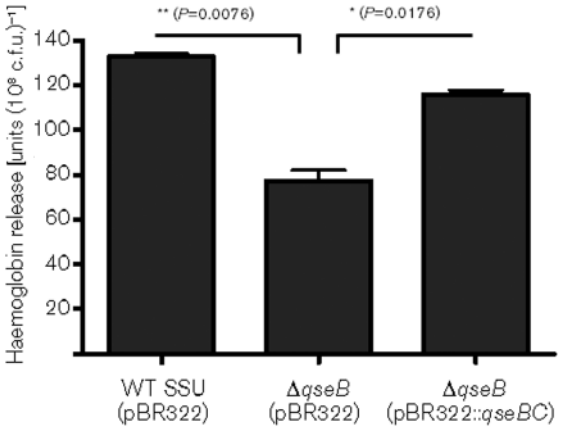


**Fig. 2.** Measurement of the haemolytic activity associated with Act of WT *A. hydrophila* SSU, Δ*qseB* mutant and its *qseBC*-complemented strain by using rabbit RBCs. Haemoglobin release was quantified by measuring absorbance at 540 nm, and the haemolytic titres were calculated as the absorbance value of the haemoglobin release multiplied by the dilution of the culture filtrate. The data were normalized to $1\times10^8$ c.f.u. to account for any minor differences in the growth rates between the various bacterial strains. Three independent experiments were performed, and the arithmetic means ± SD are plotted. Asterisks indicate statistically significant difference in haemoglobin release between the WT and its Δ*qseB* mutant (***P*=0.0076) as well as between the Δ*qseB* mutant and the *qseBC*-complemented strain (**P*=0.0176) by Student's *t* test.

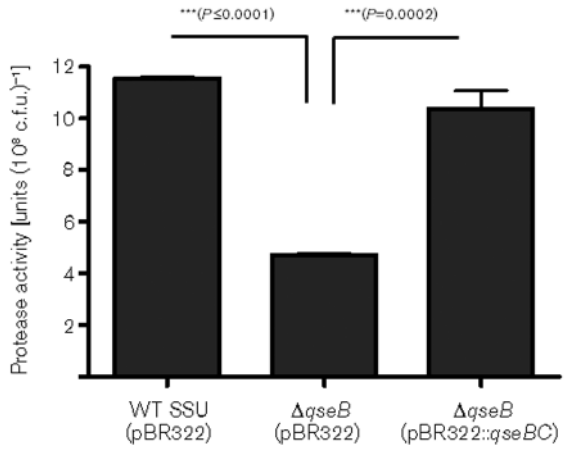


**Fig. 3.** Protease activity in the culture supernatants of WT *A. hydrophila* SSU, the Δ*qseB* mutant and its *qseBC*-complemented strain. The data were normalized to $1\times10^8$ c.f.u. to account for any minor differences in the growth rates between the various bacterial strains. Three independent experiments were performed, and the arithmetic means ± SD are plotted. Asterisks (***) indicate statistically significant differences in protease activity between the WT and its Δ*qseB* mutant ($P\leqslant0.0001$), as well as between the Δ*qseB* mutant and the *qseBC*-complemented strain ($P=0.0002$) by Student's *t* test.

protease activity of WT *A. hydrophila* SSU and its Δ*qseB* mutant at different bacterial growth phases (exponential, mid-exponential, late-exponential, early stationary and late stationary). Although a slight reduction in protease activity was noted at the mid- and late-exponential phases in the Δ*qseB* mutant compared with the WT strain, protease production by the Δ*qseB* mutant was significantly reduced in late stationary phase compared with that produced by the WT strain, indicating that QseBC regulates protease production in *A. hydrophila* SSU at high cell density (data not shown).

## CV staining and light microscopic observations on biofilm formation demonstrate a more efficient attachment of the Δ*qseB* mutant to the polystyrene tubes and Thermonox coverslips

To measure the solid surface-associated biofilm formation, we performed a CV staining assay and examined biofilm formation on Thermonox coverslips by using light microscopy. The biofilm formation was observed after 24 h growth of WT, Δ*qseB* mutant and its *qseBC* complemented strain in LB medium. The Δ*qseB* mutant formed a significantly increased, solid-surface-associated biofilm in polystyrene tubes, with a more than twofold increase in the CV staining when compared with that of the

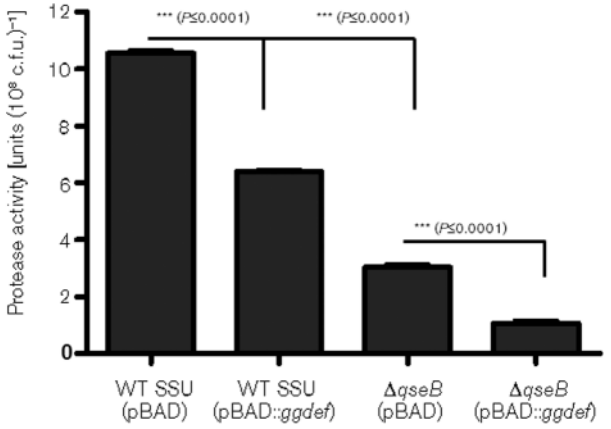


**Fig. 4.** Influence of *ggdef*-encoding gene overexpression in the WT and the Δ*qseB* mutant on protease production of *A. hydrophila* SSU. The data were normalized to $1\times10^8$ c.f.u. to account for any minor differences in the growth rates between the various bacterial strains. The results were reproduced in three independent experiments, and the error bars represent SD. Asterisks (***) indicate statistically significant differences in protease activity between the WT and WT overexpressing the *ggdef*-encoding gene ($P\leqslant0.0001$), WT and the Δ*qseB* mutant ($P\leqslant0.0001$), and the Δ*qseB* mutant and the Δ*qseB* mutant overexpressing the *ggdef*-encoding gene ($P\leqslant0.0001$) by Student's *t* test.

WT *A. hydrophila* SSU strain (Fig. 5). Likewise, the Δ*qseB* mutant also produced more aggregated biofilms which were uniformly distributed all over the coverslip (Supplementary Fig. S2b, available with the online version of this paper) compared with the biofilms of the WT strain which produced less aggregation (Supplementary Fig. S2a). In addition, the *qseBC*-complemented strain produced biofilms that were similar to those from the WT strain when examined by CV staining on polystyrene tubes (Fig. 5), as well as on the Thermonox coverslips when examined by using light microscopy (Supplementary Fig. S2c). We used Δ*ahyRI* (biofilm-deficient) *and* Δ*luxS* (enhances biofilm) mutants as controls to compare biofilm formation by different QS mutant strains (Supplementary Fig. S3). These data suggested to us that, similar to the Δ*luxS* mutant, the QseBC QS system also negatively controlled biofilm formation in *A. hydrophila* SSU.

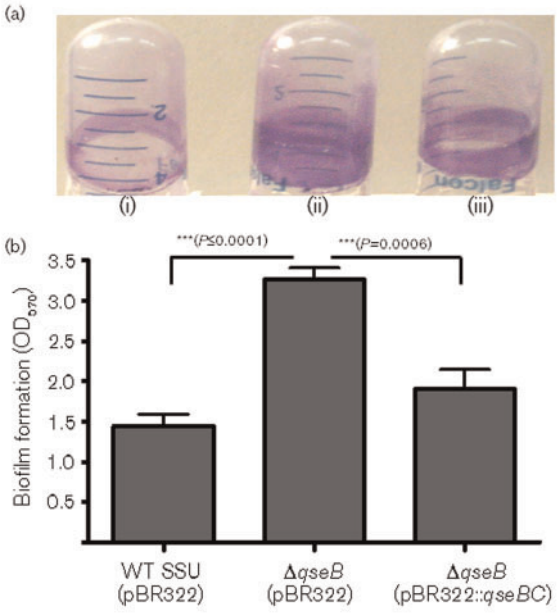


**Fig. 5.** Measurement of biofilm mass by CV staining of biofilms formed on polystyrene by the WT *A. hydrophila* SSU (i), Δ*qseB* mutant (ii) and its *qseBC*-complemented strain (iii). Biofilms were quantified after 24 h incubation at 37 °C. (a) Adherent bacteria were stained with 1 % CV and washed with distilled water; the extracted colour (with 95 % ethanol) was measured at $A_{570}$. (b) The data were normalized to $1\times10^9$ c.f.u. to account for any minor differences in the growth rates of various bacterial strains used. The results were reproduced in three independent experiments and the error bars represent SD. Asterisks (***) represent statistically significant differences in biofilm formation between the WT and its Δ*qseB* mutant ($P\leqslant0.0001$), and between the Δ*qseB* mutant and the *qseBC*-complemented strain ($P=0.0006$) by Student's *t* test.

## A 3D structured and/or denser biofilm is observed in the Δ*qseB* mutant when examined by SEM

To investigate in detail the surface architecture of bacterial cells aggregated in biofilms formed by the WT and its Δ*qseB* mutant, we performed SEM. We used ruthenium red, which binds strongly to negatively charged polysaccharides, and represents an excellent method for visualization of the surface properties of bacteria. We observed that the Δ*qseB* mutant produced very thick intercellular filaments and bundles of aggregated cells that formed a dense biofilm (Fig. 6b). In addition, at a higher magnification, the Δ*qseB* mutant was found to have formed a 3D structure of biofilm covered with thick exopolysaccharides (Fig. 6d), when we compared these findings with the flattened biofilms formed by the WT bacterium which were less aggregated and connected with fewer filaments (Fig. 6a, c). The SEM observations further confirmed CV staining results that the Δ*qseB* mutant produced denser biofilms than those produced by the WT, indicating that the QseBC QS system functioned as a negative regulator of biofilm formation in *A. hydrophila* SSU.

## Production of AexU, a T3SS effector, and Hcp, a T6SS effector, is not affected in the Δ*qseB* mutant of *A. hydrophila* SSU

To demonstrate any regulation of the QseBC QS system on the T3SS, we examined the expression and production of AexU in bacterial cell pellets of WT *A. hydrophila* SSU and its Δ*qseB* mutant grown in LB medium. We also infected HeLa cells with WT *A. hydrophila* and its Δ*qseB* mutant in DMEM and monitored the production of AexU in bacterial cells by Western blot analysis. We found that the Δ*qseB* mutant had similar levels of AexU to the WT when grown in LB medium (Supplementary Fig. S4a) and also during co-culture with HeLa cells (Supplementary Fig. S4b). These data indicated that the QseBC QS system had no effect on the production of the T3SS effector, AexU.

Similarly, the QseBC system did not regulate the production and secretion of the T6SS effector, Hcp, as the Western blot analysis data showed that both the WT and the Δ*qseB* mutant produced and secreted Hcp at similar levels (Supplementary Fig. S5).

## The Δ*qseB* mutant shows a marginally decreased virulence in an animal model

By using *in vitro* experiments, we demonstrated that the QseBC QS system positively regulated swimming and swarming motility, protease and Act production, and negatively modulated biofilm formation. To further examine whether these changes in virulence factors regulated by the QseBC QS system had any influence on the virulence of *A. hydrophila* SSU *in vivo*, we injected mice via the intraperitoneal route with the Δ*qseB* mutant and the WT strain of *A. hydrophila* at a lethal dose of $5\times10^7$ c.f.u.

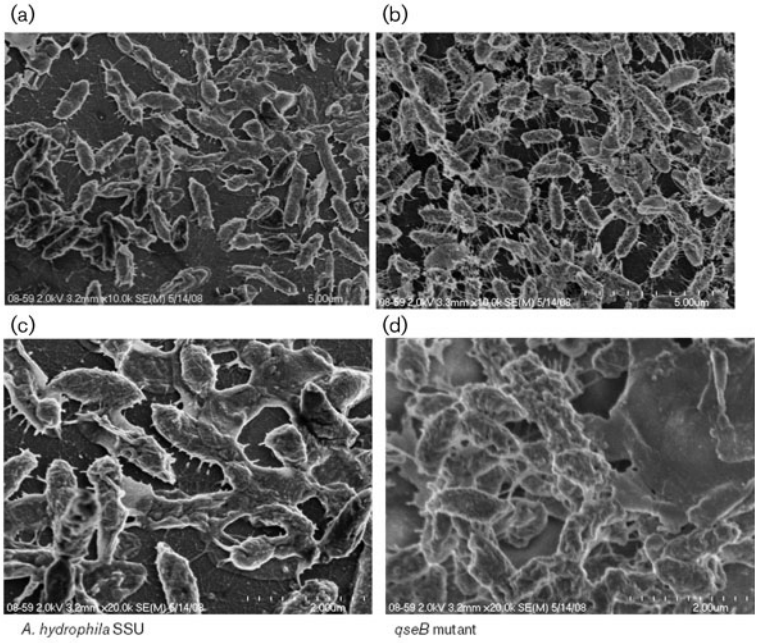


**Fig. 6.** Representative SEM images of biofilm formation by WT *A. hydrophila* SSU and its Δ*qseB* mutant after 48 h culture at 37 °C on Thermonox coverslips stained with ruthenium red. Compact aggregated cells were well connected with filaments, and denser 3D biofilms were formed by the Δ*qseB* mutant (b, d) compared with the less aggregated cells connected with fewer filaments and flattened biofims produced by WT bacteria (a, c). SEM images (a) and (b) are at low magnification (×10 000) and (c) and (d) are at higher magnification (×20 000).

(Fig. 7). We noted that 100 % of the animals infected with WT *A. hydrophila* SSU died within 2–3 days. However, mice infected with the Δ*qseB* mutant strain exhibited 30 % lower mortality over a test period of 16 days, suggesting that a marginal but statistically significant ($P$=0.0058) bacterial attenuation occurred when we deleted the *qseB* gene from *A. hydrophila* (Fig. 7).

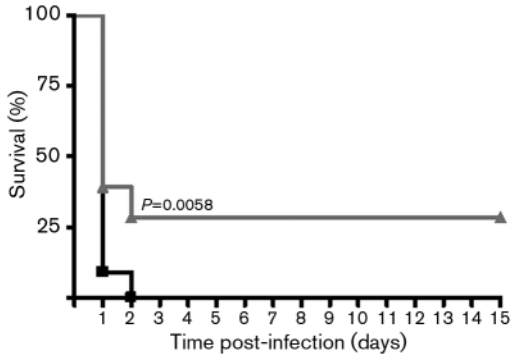


**Fig. 7.** Marginal decrease in *in vivo* virulence of the Δ*qseB* mutant in a septicaemic mouse model of *A. hydrophila* infection. Swiss Webster mice were injected via the intraperitoneal route with $5\times10^7$ c.f.u. of WT *A. hydrophila* SSU ($n$=22; ■). The same dose was used to infect mice with the Δ*qseB* mutant ($n$=27; ▲), and both groups were monitored for death over a 16 day period. The percentage of surviving mice over time combined from three independent experiments is shown. The data were analysed by using Kaplan–Meier's survival estimates showing statistically significant differences in animal survival between the Δ*qseB* mutant and the WT *A. hydrophila* SSU ($P$=0.0058).

## DISCUSSION

In this study, we demonstrated the role of the QseBC two-component system in controlling virulence of *A. hydrophila* SSU, which possesses all three functional QS circuits and might establish a complex QS network to exert its regulatory role during pathogenesis (Khajanchi *et al.*, 2009; Kozlova *et al.*, 2008). Therefore, *A. hydrophila* SSU represents an excellent model organism to study the role of different QS networks in bacterial pathogenesis.

Flagella are not only important for bacterial movement but also contribute to pathogenesis by aiding in an organism's adherence to the target host cells and biofilm formation (Kirov *et al.*, 2002; Kirov, 2003; Kirov *et al.*, 2004). *Aeromonas* species possess two distinct flagellar systems: a polar flagellum for swimming motility and several lateral flagella for swarming motility over surfaces (Kirov *et al.*, 2004). We observed that both the swimming and swarming motility were diminished in *A. hydrophila* SSU when the *qseB* gene was deleted, suggesting to us that *qseB* is a regulator of both polar and lateral flagella. In accordance with our data, recent studies also showed that QseBC regulated the swimming motility of several pathogens such as *E. coli* (Sperandio *et al.*, 2002), *S.* Typhimurium (Bearson & Bearson, 2008; Moreira *et al.*, 2010) and *Edwardsiella tarda* (Wang *et al.*, 2011). However, the investigators in these reports only examined the swimming and not the swarming motility.

In the present study, we noted that in addition to swimming motility, QseB also regulated the swarming motility of *A. hydrophila* SSU. Further, Clarke & Sperandio (2005a) reported that QseBC regulated flagella and motility through the flagellar master regulator FlhDC. They also

demonstrated that in order to control motility, QseB directly bound to the *flhDC* promoter at both low and high affinity binding sites (Clarke & Sperandio, 2005a). Our future studies will examine the specific mechanism(s) of how QseBC regulates motility by controlling both the polar and lateral flagella systems in *A. hydrophila* SSU.

Interestingly, the *ahyRI*-mediated (AI-1) QS did not regulate either swimming or swarming motilities (Khajanchi *et al.*, 2009), but deletion of the *luxS* gene (AI-2 QS) reduced the motility of *A. hydrophila* SSU (Kozlova *et al.*, 2008).

In contrast with our study in *A. hydrophila*, it was noted that the *qseB* single mutant and the *qseBC* double mutant showed motility phenotypes similar to that of the WT *S.* Typhimurium (Bearson *et al.*, 2010). However, in the latter study, the authors reported a decreased motility in the *qseC* mutant compared to that of the WT *S.* Typhimurium strain. Based on these observations, they concluded that the decreased motility in the *qseC* mutant was due to the negative regulation of QseB in the absence of QseC (Bearson *et al.*, 2010).

Studies in *E. coli* have shown that QseC is an adrenergic receptor for AI-3 and host hormones epinephrine and/or norepinephrine (Clarke *et al.*, 2006). Indeed, addition of epinephrine and/or AI-3 in the growth medium increased expression of many virulence genes, including that of the flagellar regulon genes in *E. coli* (Kendall *et al.*, 2007). Likewise, addition of norepinephrine in the soft agar increased the motility of *S.* Typhimurium (Bearson & Bearson, 2008; Bearson *et al.*, 2010). However, since supplementing the medium with norepinephrine also increased the motility of the QseC mutant, the authors questioned the role of QseBC in the motility of *S.* Typhimurium in response to norepinephrine (Bearson *et al.*, 2010).

Our initial data indicated that addition of epinephrine and/or norepinephrine to the soft agar did not influence the motility of *A. hydrophila*. However, further study is necessary to demonstrate the effect of AI-3, epinephrine and norepinephrine on the QseBC-mediated regulation of virulence mechanisms in *A. hydrophila*.

In this report, we showed that QseB positively regulated protease production in *A. hydrophila* SSU. Likewise, in our recent study, we observed that AI-1 QS positively modulated protease activity, particularly that of metalloprotease (Khajanchi *et al.*, 2009). It will be very intriguing to delineate the interaction of these three different QS systems to control various virulence mechanisms in *A. hydrophila* SSU, as our data indeed suggested the possibility of cross-talk between the AI-1 and AI-2 QS systems of *A. hydrophila* (Kozlova *et al.*, 2011). In this context, we observed that the gene transcript levels for *qseB* and *qseC* were increased in the *ahyRI* mutant, while the *ahyR* and *ahyI* genes were downregulated in the *qseB* mutant (unpublished data). Based on these observations and the reduction of protease activity in both the *qseB* and the *ahyRI* mutant, we speculate that QseB regulates protease production indirectly through the AI-1 QS system. Thus, these data also suggested to us that there could be cross-talk between AI-1 and the QseBC QS systems in *A. hydrophila*, which will be explored further in our future studies.

Act is one of the most potent virulence factors of *A. hydrophila* SSU, which possesses several biological activities (Chopra *et al.*, 2000; Galindo *et al.*, 2004). Further, the Act mutant of *A. hydrophila* SSU is significantly attenuated in an animal model, indicating that Act contributes to *in vivo* virulence of this pathogen (Xu *et al.*, 1998). In this study, we further showed that QseB regulated Act production, which could be very important for *A. hydrophila* as it is establishing an infection in the host. In a similar fashion in EHEC, QseC also regulated Shiga toxin by controlling the transcription of another two-component system QseEF (Hughes *et al.*, 2009). It is interesting that we also detected a homologue of a QseEF two-component system in *A. hydrophila* SSU (unpublished data). In the future, we intend to study cross-talk between the QseBC and QseEF systems in the pathogenesis of *A. hydrophila* and to delineate specific mechanisms that regulate the biological activity of Act. Importantly, we observed that AI-1- and AI-2-mediated QS had no influence on the haemolytic activity of Act, which may mean that different QS systems in *A. hydrophila* SSU regulate different sets of virulence factors (Khajanchi *et al.*, 2009; Kozlova *et al.*, 2008).

Most bacteria in nature are not present as free-floating, isolated cells; rather they prefer to form surface-associated communities known as biofilms (Costerton *et al.*, 1987; Costerton *et al.*, 1999). Bacteria present in biofilms are more robust in nature in that they have the ability to withstand chemical and physical stresses and are more resistant to host defences than when they are free-living or in a planktonic state (Costerton *et al.*, 1999; Singh *et al.*, 2006). Therefore, biofilm formation is considered one of the most important virulence mechanisms that contribute to human disease transmission and pathogenesis (Huq *et al.*, 2008; Oggioni *et al.*, 2006). Several studies also pointed out that QS plays a crucial role in controlling biofilm development and establishing efficient infections in both Gram-positive (Oggioni *et al.*, 2006; Petersen *et al.*, 2006) and Gram-negative bacteria (de Kievit, 2009; Zhu & Mekalanos, 2003).

Previously, we demonstrated that AhyRI (AI-1) QS positively regulated biofilm formation in *A. hydrophila* SSU (Khajanchi *et al.*, 2009), while this was negatively regulated by LuxS/AI-2-based QS (Kozlova *et al.*, 2008). In the present study, we further showed that QseBC, in a manner similar to the *luxS* system, also negatively regulated biofilm formation in *A. hydrophila*. In agreement with our study, Moreira *et al.* (2006) showed that deletion of the *qseA* gene (encoding LysR-type regulator), which is also involved in AI-3 mediated QS circuits

(Kaper & Sperandio, 2005) from enteropathogenic *E. coli* (EPEC), enhanced biofilm formation. However, we observed differences in solid surface biofilm formation by using CV staining and light microscopy at 24 h, as well as noticeable differences in the architecture of biofilm by using SEM at 48 and 72 h between the WT and the *qseB* mutant of *A. hydrophila*. On the other hand, Moreira *et al.* (2006) noticed that the *qseA* mutant formed denser/more compact biofilms than those seen with WT EPEC at earlier time points, such as at 6 and 12 h, following light microscopy examination and measurement of the biofilm biomass (c.f.u. $cm^{-2}$).

In contrast with our findings, it was shown that inactivation of the *qseC* gene in *Aggregatibacter actinomycetemcomitans* reduced biofilm growth (Novak *et al.*, 2010). These data indicate that regulation of biofilm formation by the QseBC system is distinct in different bacterial pathogens. Further, it was also observed that alterations in the biofilm formation ability of *Aggregatibacter actinomycetemcomitans* through the QseBC system was dependent on the AI-2/LuxS-based QS system (Novak *et al.*, 2010). The production of AI-3 could be altered by cellular metabolism when the *luxS* gene is mutated in EHEC (Walters *et al.*, 2006). Therefore, it is worth examining whether these AI-2 and QseBC QS systems in *A. hydrophila* SSU are linked in controlling biofilm formation.

In addition, we also showed that overproduction of c-di-GMP, the bacterial intracellular second messenger, altered biofilm formation and motility in *A. hydrophila* SSU in a QS-dependent manner involving both AI-1 and AI-2 systems (Kozlova *et al.*, 2011). The loss in the motility and reduction of protease activity by GGDEF overproduction in the *qseB* mutant, which we described here, may mean that the QseBC QS system is involved in the c-di-GMP-dependent regulatory network in *A. hydrophila*. Because we argued that a balance of the transcriptional level of gene expression between *luxS* and *ahyR* was regulated by the modulation of c-di-GMP (Kozlova *et al.*, 2011), we are interested in further studying a network connection between all three QS systems and c-di-GMP signalling in *A. hydrophila* SSU.

We also demonstrated that AI-1- and AI-2-mediated QS systems had opposite effects on the virulence of *A. hydrophila* in a septicaemic mouse model (Khajanchi *et al.*, 2009; Kozlova *et al.*, 2008). While deletion of the *ahyRI* QS genes attenuated the bacterium, the *luxS* mutant of *A. hydrophila* SSU had increased virulence (Khajanchi *et al.*, 2009; Kozlova *et al.*, 2008). Similar to the *luxS* mutant, deletion of the *qseB* gene from *A. hydrophila* enhanced *in vitro* biofilm formation; however, the *qseB* mutant was less virulent compared with the WT *A. hydrophila* in an *in vivo* model. In an animal model, we expected a marginal decrease in the virulence of the *qseB* mutant compared with that of the WT bacterium. This was based on *in vitro* experiments, where we noted that in addition to serving as a negative regulator of biofilm formation, QseBC also positively regulated some of the important virulence factors, such as Act and protease, and the latter (reduced Act and protease production) could possibly balance overall the pathogenicity of *A. hydrophila*. In agreement with our study, other investigators also showed that the *qseC* mutant and/or interference of QseC signalling in different bacteria resulted in attenuation of bacteria in different animal models tested (Bearson & Bearson, 2008; Kostakioti *et al.*, 2009; Novak *et al.*, 2010; Rasko *et al.*, 2008). Although the present study has provided some evidence that the QseBC system might influence the virulence of *A. hydrophila* in an animal model, future *in vivo* studies are needed to elucidate the roles of AI-3 and the QseBC system in the virulence of *A. hydrophila*.

In conclusion, we characterized the role of the QseBC two-component system in order to better understand how different QS systems regulate the virulence of *A. hydrophila*. We have shown that QseBC both positively and negatively regulates various virulence factors/mechanisms of *A. hydrophila* SSU that could play an important role in fine-tuning the expression of virulence genes at an appropriate time to facilitate the establishment of infection by the pathogen in a highly efficient manner.

## ACKNOWLEDGEMENTS

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