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Impact of QseBC system in c-di-GMP-dependent quorum sensing regulatory network in a clinical isolate SSU of *Aeromonas hydrophila*

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Abstract

Our earlier studies showed that *ahyRI* (AI-1) and LuxS-based (AI-2) quorum sensing (QS) systems were positive and negative regulators of virulence, respectively, in a diarrheal isolate SSU of *A. hydrophila*. Recently, we demonstrated that deletion of the QseBC two-component signal transduction system (AI-3 QS in enterohemorrhagic *E. coli*) also led to an attenuation of *A. hydrophila* in a septicemic mouse model of infection, and that interplay exists between AI-1, AI-2, and the second messenger cyclic-di-guanosine monophosphate (c-di-GMP) in modulating bacterial virulence. To further explore a network connection between all of the three QS systems in *A. hydrophila* SSU and their cross-talk with c-di-GMP, we overproduced a protein with a GGDEF domain, which increases c-di-GMP levels in bacteria, and studied phenotypes and transcriptional profiling of genes involved in biofilm formation and motility of the wild-type (WT) *A. hydrophila* and its Δ*qseB* mutant. Over-expression of the GGDEF domain-encoding gene (*aha0701h*) resulted in a significantly reduced motility of the WT *A. hydrophila* similar to that of the Δ*qseB* mutant. While enhanced protease production was noted in WT *A. hydrophila* that had increased c-di-GMP, no enzymatic activity was detected in the Δ*qseB* mutant overexpressing the *aha0701h* gene. Likewise, denser biofilm formation was noted for WT bacteria when c-di-GMP was overproduced compared to its respective control; however, overproduction of c-di-GMP in the Δ*qseB* mutant led to reduced biofilm formation, a finding similar to that noted for the parental *A. hydrophila* strain. These effects on bacterial motility and biofilm formation in the Δ*qseB* mutant or the mutant with increased c-di-GMP were correlated with altered levels of *fleN* and *vpsT* genes. While we noted transcript levels of *qseB* and *qseC* genes to be increased in the *ahyRI* mutant, down-regulation of the *ahyR* and *ahyI* genes was observed in the Δ*qseB* mutant, which correlated with decreased protease activity. Finally, an enhanced virulence of WT *A. hydrophila* with increased c-di-GMP was noted in a mouse model when compared to findings in the parental strain with vector alone. Overall, we conclude that cross talk between AI-1- and QseBC-systems exist in *A. hydrophila* SSU, and c-di-GMP modulation on QseBC-system is dependent on the expression of the AI-1 system.

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Keywords

Aeromonas hydrophila; 2-component QseBC system; c-di-GMP; quorum sensing systems; motility; biofilm formation; gene transcription; mouse model of infection

INTRODUCTION

QseBC has recently been described as a novel, two-component-based quorum sensing (QS) system that responds to eukaryotic hormone-like signals, such as autoinducer-3 (AI-3), epinephrine, and norepinephrine [1]. In the presence of signal molecules, the inner membrane-localized QseC first undergoes autophosphorylation and then transfers this phosphate to intracellular response regulator QseB. Phosphorylated QseB binds and activates the transcription of the *flhDC* master regulator of the flagella regulon in enterohemorrhagic *E. coli* (EHEC), and it also binds to its own promoter [2]. QseC activates transcription of the genes encoding Shiga toxin, and, in addition to activating expression of the LEE (locus of enterocyte effacement)-encoded type 3 secretion system (T3SS), the majority of the genes encoding effectors and translocated through this T3SS, are also regulated by QseC [3]. It has been demonstrated that QseBC is an important virulence regulator contributing to intracellular colonization and systemic infection caused by EHEC [4], uropathogenic *E. coli* (UPEC) [5], and *Salmonella enterica* Serovar Typhimurium [6].

Recently, we demonstrated that two modified autoinducer systems exist in a diarrheal isolate SSU of *A. hydrophila.* One of them is an *N-acyl*homoserine lactone (AHL)-based QS system, designated as AI-1 [7], while the other is an *S*-ribosylhomocysteinase (LuxS)-based AI-2 system [8]. These QS systems had opposing effects on modulating biofilm formation in an *in vitro* model and virulence in a septicemic mouse model of infection [7, 8]. The regulatory network of AI-1 and AI-2 QS systems in *A. hydrophila* SSU also includes second-messenger c-di-GMP-dependent modulation of virulence genes [9].

More recently, we reported identification of a functional QseBC system in *A. hydrophila* SSU. We provided evidence that deletion of the *qseB* gene from *A. hydrophila* decreased swimming and swarming motility, increased biofilm formation, and reduced the protease and hemolytic activity associated with the cytotoxic enterotoxin (Act). Moreover, the mutant was attenuated in a septicemic mouse model of infection [10]. Since we have already demonstrated an interplay between AI-1 and AI-2 QS systems in *A. hydrophila* SSU through c-di-GMP [9], we were interested in studying the potential interaction of these two QS systems with the QseBC system found in *A. hydrophila*.

Here we present data showing an impact of the QseBC system on motility, biofilm formation, and the QS-dependent virulence regulatory network in *A. hydrophila* SSU as a function of the c-di-GMP level. We further show that transcript levels of the genes involved in AI-1- and QseBC systems are co-regulated in *A. hydrophila* SSU.

RESULTS

2.1. Identification of *qseB* and *qseC* gene transcripts in *A. hydrophila* SSU

As with the EHEC QseBC two-component system [11], the transcriptional stop codon of the *qseB* gene overlaps with the transcriptional start codon of the *qseC* gene in *A. hydrophila* [10]. However, we identified by reverse transcriptase (RT)-polymerase chain reaction (PCR) two independent transcripts, one each for the *qseB* and *qseC* gene (data not shown), in contrast to *qseBC* co-transcription which was described for EHEC [11] and *Edwarsiella tarda* [12]. Additionally, the *qseC* transcript was detected at a much reduced level, when

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compared to the results with the *qseB* transcript in *A. hydrophila,* possibly because *qseC* is a GC-rich gene (67.1%). At the same time, a 4-bp overlap at the ATGA motif, which was found for the *qseB* and q*seC* gene open reading frames (ORFs), obviously was necessary to enhance the expression of the GC-rich *qseC* gene [13].

2.2. Overproduction of GGDEF domain protein regulates motility and biofilm formation in the Δ*qseB* mutant *A. hydrophila* of SSU

Recently, we successfully used the overproduction of AHA0701h protein with a GGDEF domain, which increases c-di-GMP levels in bacteria, for gene profiling and phenotypic alterations in WT *A. hydrophila* and its *luxS* and *ahyRI* mutants [9]. The gene-encoding AHA0701h is located downstream of the *luxS* gene in the genome of *A. hydrophila* [8]. The GGDEF domain is typically found in proteins as part of modular diguanylate cyclase (DGC) enzymes, which catalyze the synthesis of the signaling molecule c-di-GMP [14]. For the present study, the gene encoding AHA0701h was cloned into the pBAD/*Myc*-HisB vector and transformed into the Δ*qseB* mutant, with the latter harboring only the vector serving as a negative control. The phenotypic alterations of these strains were studied after arabinose induction (section 4.1) and compared to the similarly transformed WT *A. hydrophila* strain.

QS functions to control population size in a biofilm and is also involved in the biosynthesis of flagella [15], which modulates swimming or swarming motility [10]. Recently, we demonstrated that over-expression of the *aha0701h* gene in the Δ*qseB* mutant resulted in no swimming motility [10]. Here, we show that overproduction of c-di-GMP in WT *A. hydrophila* significantly reduced swarming motility compared to the parental bacteria with vector alone (Fig. 1). This decrease in swarming motility of WT *A. hydrophila* with overexpression of the *aha0701h* gene was similar to that found with the Δ*qseB* mutant (Fig. 1). However, while the swimming motility of the Δ*qseB* mutant was further decreased when c-di-GMP was overproduced [10], no further reduction in the swarming motility of the Δ*qseB* mutant was observed when c-di-GMP was overproduced (Fig. 1).

Biofilm formation is critical not only for environmental survival but also to cause successful infection in the host by numerous pathogenic bacteria. To measure solid surface-associated biofilm formation, we performed a crystal violet (CV) staining assay and also examined biofilm formation on thermanox cover slips by scanning electron microscopy (SEM). The biofilm formation in polystyrene tubes was observed after 24 h of growth of WT *A. hydrophila* and its Δ*qseB* mutant as well as of the Δ*qseB* mutant with overproduction of GGDEF domain protein after induction of the latter strain with 0.2% arabinose in the Luria-Bertani (LB) medium. Recently, we demonstrated that the Δ*qseB* mutant formed significantly increased solid-surface-associated biofilms in polystyrene tubes, with a more than 2-fold increase in CV staining when compared to that of the WT *A. hydrophila* harboring the pBAD/*Myc*-HisB vector [10]. While an increase in c-di-GMP levels in WT *A. hydrophila* led to a significant increase in biofilm formation (Fig. 2), similar to that with the Δ*qseB* mutant, overproduction of c-di-GMP in the Δ*qseB* mutant resulted in decreased biofilm formation; the latter finding was similar to that noted for the WT *A. hydrophila* with vector alone (Fig. 2).

We then used SEM to investigate in detail the surface attachment and architecture of bacterial cell aggregation in biofilms formed by the WT *A. hydrophila* and its *qseB* mutant, both harboring the pBAD/*Myc*-HisB vector alone, as well as when c-di-GMP overproduced, after 48 h of cultivation on thermanox cover slips. As shown in Fig. 3A, biofilms of WT *A. hydrophila* with pBAD/*Myc*-HisB vector alone demonstrated compact and three-dimensional cell aggregation with exopolysaccharide (EPS) that covered and extended over the cells similar to that found in WT *A. hydrophila* without the plasmid vector [8, 10]. On the contrary, SEM of WT *A. hyrophila,* when c-di-GMP was overproduced, showed

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biofilms with greater number of cells forming highly compact three-dimensional structure and EPS appeared as pellets of condensed material around aggregated sessile cells (Fig. 3B). Aggregated cells of the *qseB* mutant harboring the pBAD/*Myc*-HisB vector alone formed a dense biofilm with intercellular filament connections and a very little EPS (Fig. 3C) that appeared similar to the biofilms in the *qseB* mutant [10]. Overproduction of c-di-GMP in the *qseB* mutant resulted in irregular shape of the biofilms with sparser cell aggregation (Fig. 3Da). Importantly, several biofilm regions of the *qseB* mutant with c-di-GMP overproduction included extensive EPS surrounding the mutant cells (Fig. 3Db).

2.3. Overproduction of the GGDEF domain protein further ablates protease activity of the Δ*qseB* mutant *A. hydrophila* of SSU

We examined the protease activity in the culture supernatants of WT *A. hydrophila* and its *qseB* and *ahyRI* mutants with and without the overproduction of c-di-GMP. While as we showed earlier, deletion of the *ahyRI* and *qseB* genes resulted in decreased protease production [7, 10], increase of c-di-GMP levels in the Δ*qseB* mutant further ablated protease production, with no further change in protease activity in the *ahyRI* mutant with increased levels of c-di-GMP.

On the contrary, the protease level was increased in the WT *A. hydrophila* when c-di-GMP was overproduced, compared to the parental strain with the pBAD/*Myc*-HisB empty vector (Fig. 4). These results were different from our earlier published data [10] in which we showed decreased protease level when c-di-GMP was overproduced from WT *A. hydrophila* compared to the parental strain with vector alone. We believe these differences could be attributed to bacterial growth conditions used. For example, in our earlier study [10], the bacteria were directly grown from −80°C stocks in the liquid culture for measuring enzymatic and other biological activities. We followed this method based on a reported study of Waters et al. [16] which indicated that levels of c-di-GMP were much higher in *Vibrio cholerae* when the bacteria were directly grown from −80°C stocks compared to when the bacteria were first grown in a liquid culture overnight and then subcultures were made. This was possibly related to decrease in c-di-GMP levels over time in the stationary phase of the bacterial growth.

However, in the current paper, we grew the cultures overnight in the liquid medium from −80°C stocks and then the subcultures were made. As a result, we noticed increase in protease activity in the WT *A. hydrophila* with increased c-di-GMP when compared to the parental bacteria with vector alone. We also noted that this effect on c-di-GMP levels in bacteria in terms of how we grew them modulated only some biological effects and only in some mutants of *A. hydrophila.*

2.4. Addition of aspartate does not restore the altered phenotypes (motility and biofilm formation) in the *luxS* mutant of *A. hydrophila* SSU

Mutations in the *qseB* and *luxS* genes of *A. hydrophila* resulted in decreased motility [8, 10]. The *luxS* mutation seems to alter cellular metabolism leading to decreased AI-3 production, possibly by reducing tyrosine levels in *E. coli,* while AI-3 signaling had little effect on bacterial metabolism [17]. To examine whether the decreased motility noted in the Δ*qseB* mutant(Fig. 1) could be interrelated with the LuxS-dependent metabolism of *A. hydrophila*, we assessed the restoration of QS-dependent phenotypes by complementing the defects in the *luxS* mutant at the level of oxaloacetate-homocysteine pathway. The addition of aspartate to the growth medium could change the nitrogen and carbon levels in the *luxS* mutant [17]. However, the addition of L-aspartate to the *luxS* mutant was not able to restore either the *luxS* mutant's motility or biofilm formation (data not shown).

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2.5. Alteration in the transcription of genes that encode three QS systems in the *qseB*, *luxS*, and *ahyRI* mutants of *A. hydrophila* SSU

The existence in *A. hydrophila* of three QS systems (Fig. 5) prompted us to study the possibility of interconnections between them. Indeed, we found transcriptional interplay between LuxS-dependent and AhyRI-based QS systems [9]. We did not find alterations in the transcript levels of *qseB* and *qseC* genes in the *luxS* mutant of *A. hydrophila* when compared to that in the WT bacteria (Supplemental data, Table I). However, the transcription levels of *qseB* and *qseC* genes were increased (by over 2 fold) in the *ahyRI* mutant (Fig. 6 [yellow bars], Supplemental data, Table I). The mutation in the *qseB* gene did not alter the *luxS* transcript level (Supplemental data, Table 1), which was in contrast to the *ahyRI* deletion that caused the *luxS* gene transcript to be up-regulated [9]. The *ahyR* and *ahyI* genes were down-regulated (by 2 fold) in the Δ*qseB* mutant compared to that in the WT *A. hydrophila* (Fig. 6 [cyan bars], Supplemental data, Table I).

2.6. Alteration in the transcription of genes involved in biofilm formation and motility in the Δ*qseB* mutant *A. hydrophila* of SSU

In our recent study, we examined the transcription of five major genes involved in biofilm formation and motility of the WT *A. hydrophila* and its *ΔahyRI* and *luxS* mutants [9]. We analyzed transcript levels of the same genes in the Δ*qseB* mutant, and, in contrast to findings in the *luxS* and *ahyRI* mutants, the transcript levels of only *vpsT* and *fleN* genes (Fig. 7, Supplemental data, Table 1 were altered in the Δ*qseB* mutant. As noted, the transcript level of *fleN* was increased and that of *vpsT* decreased in the Δ*qseB* mutant compared to that of the WT bacteria.

2.7. Effect of overproduction of the GGDEF domain protein on *qseB* and *qseC* gene expression in WT *A. hydrophila* SSU and its Δ*ahyRI* mutant

The *qseB* and *qseC* gene transcript levels were increased (approximately 2–3 fold) when c-di-GMP was overproduced in the WT *A. hydrophila* compared to those of the parental bacteria with pBAD/*Myc*-HisB vector alone (Fig. 6 [green bars], Supplemental data, Table I). The level of expression of the *qseB* and *qseC* genes in the *ahyRI* mutant, when c-di-GMP was overproduced was comparable to the level seen in WT bacteria which harbored only the pBAD/*Myc*-HisB vector (Supplemental data, Table I). This was in contrast to increased expression levels of these genes (*qseB* and *qseC*) in the *ahyRI* mutant with the pBAD/*Myc*-HisB vector alone (Fig. 6 [yellow bars], Supplemental data, Table I). Thus, increased production of c-di-GMP in the Δ*ahyRI* mutant normalized the transcriptional response of the *qseB* and *qseC* genes and made their levels comparable to those seen in the WT *A. hydrophila*.

2.8. Effect of overproduction of GGDEF domain protein on QS and QS-dependent gene transcripts in the Δ*qseB* mutant *A. hydrophila* of SSU

We found that, when compared to the WT *A. hydrophila* with the pBAD/*Myc*-HisB vector alone, *ahyR* and *ahyI* gene transcripts were even more down-regulated by approximately 3 fold [red bars] when c-di-GMP was overproduced in the Δ*qseB* mutant, compared to its already decreased level of expression [cyan bars] caused by *qseB* deletion (Fig. 6, Supplemental data, Table I). This finding was similar to the decreased levels of expression of these genes (*ahyR* and *ahyI*) in the *luxS* mutant, again when compared to the WT bacteria with the pBAD/*Myc*-HisB vector alone (Fig. 6 [blue bars], Supplemental data, Table I). When c-di-GMP was overproduced in the Δ*qseB* mutant, the expression of the *fleN* gene was down-regulated, and the transcription level of *vpsT* was restored to the level found in the WT bacteria (Fig. 7, Supplemental data, Table I). Thus, overall our results showed that the increased production of c-di-GMP in the Δ*qseB* mutant resulted in differential

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transcriptional responses of studied genes (motility and biofilm formation) when compared to that seen in the WT and the *ahyRI* mutant of *A. hydrophila.*

2.9. Effect of overproduction of the GGDEF domain protein on WT *A. hydrophila* SSU virulence in an animal model

To study the effect of overproduction of c-di-GMP in WT *A. hydrophila*, we injected mice via the intraperitoneal (i.p.) route with either the WT *A. hydrophila* harboring the pBAD/*Myc*-HisB vector alone or the parental bacteria having increased c-di-GMP levels. As noted in Fig. 8, 56% of the animals infected with the WT *A. hydrophila* (pBAD/*Myc*-HisB) died within 2 days at a dose of 1×10^7 colony forming units (cfu), and when GGDEF domain protein was overproduced in the WT bacteria, an 89% mortality rate was noted at the same dose. These data indicated that increased production of c-di-GMP enhanced the virulence of the WT *A. hydrophila*.

DISCUSSION

In this study, we described the impact of the QseBC system on QS and that of c-di-GMP network regulation in *A. hydrophila* SSU. Our earlier report demonstrated that the AI-1 and AI-2 QS systems were interconnected, at least at the transcriptional level in *A. hydrophila* [9], and both of them were involved in the regulation of biofilm formation, albeit in an opposite way. Interestingly, however, the LuxS-dependent AI-2 QS system regulated bacterial motility [8], while the AhyRI-based AI-1 QS system did not regulate either the swimming or swarming motility of *A. hydrophila* [7].

The deletion of the *qseB* gene in *A. hydrophila* SSU resulted in decreased swarming and swimming motility, and an increase in c-di-GMP levels in the Δ*qseB* mutant further ablated the swimming motility [10]. Importantly, our current results showed contrasting data in terms of the swimming and the swarming motility associated with the Δ*qseB* mutant when c-di-GMP was overproduced (Fig. 1). While increased c-di-GMP levels led to a further decrease in the swimming motility [10], no further ablation of the swarming motility of the Δ*qseB* mutant was noted. Clarke *et al.* reported that QseBC regulated flagella and motility through the flagellar master regulator FlhDC in *E. coli* [4, 11]. They also demonstrated that in order to control motility, QseB directly bound to the *flhDC* promoter, both at the low- and high-affinity binding sites [4]. However, the *flhDC* genes do not exist in the *A. hydrophila* genome [18]. Recently, QseB was demonstrated as a negative regulator of bacterial motility in UPEC [5] and *S. enterica* serovar Typhimurium [19]. It was shown the *qseB* gene deletion did not influence the flagellar gene expression; however, the expression of the *qseB* gene was upregulated in the absence of QseC and that resulted in the downregulation of flagella, pili and curli genes in UPEC [5]. In contrast, the Δ*qseB* and Δ*qseC* mutants displayed significantly impaired motility, and this defect was rescued by complementation of the respective mutants with either the intact *qseB* or the *qseC* gene in *E. tarda* [12]. We showed that the deletion of the *qseB* gene in *A. hydrophila* SSU resulted in a dramatic decrease in the *qseC* transcript; at least we could not detect this transcript by RT-PCR. However, detectable levels of *qseB* and *qseC* gene transcripts were seen in the WT *A. hydrophila* strain.

Interestingly, over-expression of the *aha0701h* gene encoding the GGDEF domain protein in WT *A. hydrophila* SSU resulted in decreasing both swimming [9] and swarming motility (Fig. 1). These results demonstrated that high levels of c-di-GMP regulate the polar and lateral flagella of WT bacteria; however, this effect is restricted to polar flagellar regulation when the *qseB* gene was deleted, as polar and lateral flagella are involved in swimming and swarming motility, respectively [20]. Since we also showed that an increase in c-di-GMP levels in WT *A. hydrophila* led to enhanced transcripts for *qseB* and *qseC* genes (Fig. 6),

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this could lead to down-regulation of the flagellar genes and, hence, bacterial motility, as shown for UPEC [5]. In the future, it will be intriguing to delete the *qseC* gene and show the effect of this deletion on the expression of the *qseB* gene in WT *A. hydrophila*.

Biofilm formation represents a virulence mechanism in both Gram-positive [21, 22] and Gram-negative bacteria [23, 24]. Further, c-di-GMP has been shown to induce biofilm formation in *V. cholerae* [25, 26]. Although increased c-di-GMP levels resulted in enhanced biofilm formation in *V. vulnificus*, the degree of induction and the final level of biofilm formed were strain specific [27]. Our recent results showed that the overproduction of AHA0701h protein resulted in a dramatic increase in biofilm formation, as measured by CV staining and SEM, in WT *A. hydrophila* SSU (Figs. 2 and 3B) compared to that in bacteria harboring only the vector (Figs. 2 and 3A). However, increased production of c-di-GMP in the *ahyRI* mutant did not cause any quantitative alteration in biofilm formation (data not shown). Surprisingly, overproduction of c-di-GMP in the Δ*qseB* mutant decreased biofilm formation when compared to that in the Δ*qseB* mutant with vector alone (Figs. 2, 3C, 3Da and 3Db), and it was similar to that noted for the WT *A. hydrophila* SSU as measured by CV staining (Fig. 2). However, the distribution of *qseB* mutant cells with overproduction of GGDEF domain protein that formed a biofilm on the thermanox cover slip, was different, when compared to that of WT bacteria with increased c-di-GMP levels (Fig. 3B), and could be described as cells attached to islands including extensive EPS surrounding the mutant cells uniformly distributed all over the cover-slip surface (Fig. 3D). The differential biofilm-forming capacities of the strains could be due to differences in the expression or activity of downstream effector proteins, such as the proteins that bind c-di-GMP and transduce the signal to systems that regulate adhesion factor(s) [28].

Although many bacteria produce metalloproteases that have a zinc (II) ion in the catalytic site, other types of proteases, such as serine proteases, are also produced by pathogenic bacteria [29]. The role of proteases in the virulence of *A. hydrophila* has been well established [30]. The contribution of the AI-2 system, but not of the AI-1 system, to protease production in *V. vulnificus* was suggested [31]. However, this regulation was temperature dependent [32]. It appears that *V. vulnificus* produces protease via the QS system only in the tissues of the limbs, as the temperature there is lower than that in the bloodstream [29]. However, in human serum, *V. vulnificus* protease production increased at 37°C without an increase in the expression of the *luxS* gene [33]. Thus, *V. vulnificus* protease production in the host serum may be regulated by a system other than the AI-2-dependent QS system [29]. In our study, we found that protease activity in WT *A. hydrophila* SSU was modulated by the QseBC and AI-1 systems in a c-di-GMP-dependent manner (Fig. 4). In addition, our observation in a mouse model of the increased virulence of WT *A. hydrophila* with GGDEF domain protein over-production, compared to that of its parental strain with vector alone, demonstrated the role of c-di-GMP in the regulation of *A. hydrophila* virulence.

Based on our observation of LuxS-dependent and AhyRI-based QS system interplay (Fig. 5) [9], we studied the possible cross talk between three QS systems which exist in *A. hydrophila* SSU. A study on the QseBC system of *E. coli* showed that a mutation in the *qseC* gene did not have any effect on the transcription of other genes and phenotype regulation of the AI-2 QS mechanism [34]. However, the *luxS* gene mutation led to decreased levels of AI-3 in *E. coli,* which was dependent on the metabolic defect present in the Δ*luxS* mutant [17]. No phenotypic alterations were noted when L-aspartate was used as an alternative source of the homocysteine pathway during the growth of the *A. hydrophila* SSU Δ*luxS* mutant. In agreement with this, we did not find an alteration in the transcript levels of the *qseB* and *qseC* genes in the Δ*luxS* mutant of *A. hydrophila* (Supplemental data, Table I). However, the transcript levels of *qseB* and *qseC* genes were increased in the *ahyRI* mutant (Fig. 6, Supplemental data, Table I).

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Mutation in the *qseB* gene did not alter the *luxS* transcript level, while the *ahyR* and *ahyI* genes were down-regulated in the Δ*qseB* mutant (Fig. 6). Of note is that only one common phenotype in addition to *in vivo* virulence attenuation, when the *qseB* and *ahyRI* genes were deleted, was the reduction in protease activity (Fig. 4). The observation of decreased *ahyRI* transcript levels in the Δ*qseB* mutant is in agreement with earlier studies [7, 35, 36]. Overall, mutations in either the *qseB* gene or the *ahyRI* genes caused an alteration in gene transcripts of the remaining functional QS system of *A. hydrophila;* these data lead us to suggest that the QseBC system interplays with the AI-1 QS system.

Recently, we observed that mutations in either the *luxS* gene or the *ahyRI* genes caused an alteration in the expression of the major genes involved in biofilm formation and motility of the WT *A. hydrophila*. [9]. In contrast to the *luxS* and *ahyRI* mutants, the transcription levels of *vpsT* and *fleN* genes only were altered in the Δ*qseB* mutant (Figs. 5 and 7). VpsT is a transcriptional activator involved in biofilm formation in *Vibrio* spp. [37]. Because we already demonstrated that *vpsT* gene expression is dependent on the existence of *ahyRI*, *luxS* and c-di-GMP levels in *A. hydrophila* SSU [9], we were interested in studying the effects of AHA0701h (GGDEF) over-production in the Δ*qseB* mutant.

Consequently, we showed that over-expression of one of the genes encoding a protein with a GGDEF domain in the Δ*qseB* mutant *A. hydrophila* of SSU resulted in no swimming motility, had no effect on swarming motility, exhibited an altered biofilm phenotype, and demonstrated no detectable protease activity. Interestingly, the *qseB* and *qseC* gene transcript levels were increased when c-di-GMP was overproduced in the WT *A. hydrophila* (Fig. 6). However, the level of expression of the *qseB* and *qseC* genes returned to that of the WT bacteria when c-di-GMP was overproduced in the *ahyRI* mutant (Supplemental data, Table I), while the Δ*ahyRI* mutant showed an increase in these transcripts (Fig. 6).

We demonstrated that the QseBC system, in addition to c-di-GMP-dependent AI-1 system regulation, is involved in the modulation of expression of two major regulators of biofilm and motility, such as VpsT and FleN. An over-expression of the GGDEF domain protein-encoding gene in the Δ*qseB* mutant down-regulated the *fleN* gene transcript level (Fig. 7) in a manner similar to that seen in the *ahyRI* mutant [9]. However, the effect of overproduction of c-di-GMP on the expression of the *vpsT* gene was different in those two (*qseB* and *ahyRI*) mutants. For example, in contrast to the down-regulation of the *vpsT* gene expression by high levels of c-di-GMP in the *ahyRI* mutant [9], overproduction of the GGDEF domain protein in the Δ*qseB* mutant resulted in normalization of the *vpsT* gene expression to the level of WT *A. hydrophila* SSU (Fig. 7, Supplemental data, Table I).

Interestingly, interruption/mutation of either the QseBC or the AhyRI QS system abolished positive regulation of c-di-GMP overproduction on protease activity and, consequently, the mortality rate in mice, which was noted in animals infected with the WT *A. hydrophila* (pBAD::*aha0701h*). In contrast, Wang et al. [38] showed that c-di-GMP negatively regulated the production of HA/protease in *V. cholerae.* The authors proposed a model showing an interplay between c-di-GMP, HapR and RpoS in this regulation. Based on our observations on the gene transcript levels for *qseB*, *qseC*, *ahyR*, and *ahyI* in the *qseB*, *ahyRI* and *luxS* mutants, we believe that QseB regulates protease production indirectly through the AI-1 QS system in *A. hydrophila*. In addition, an interplay which alters transcript levels of *vpsT* and *vpsR* genes in the *qseB*, *ahyRI* and *luxS* mutants after c-di-GMP overproduction suggested that a downstream regulatory system, e.g., O-antigen lipopolysaccharide biosynthesis or flagellin glycosylation [39], could be involved in QS-dependent regulatory network and impact the virulence of *A. hydrophila*. Moreover, some of these genes could be regulated by local c-di-GMP, independent of the global alteration in c-di-GMP levels [40].

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Overall, our data indicated an impact of the QseBC system on the c-di-GMP-dependent, quorum-sensing virulence regulatory network in a clinical isolate SSU of *A. hydrophila*, and demonstrated the interplay between three existing QS systems.

4. MATERIALS AND METHODS

4.1. Bacterial strains and plasmids

The sources of *A. hydrophila* strains, as well as the plasmids used in this study, are listed in Table 1. The antibiotics ampicillin (Ap), kanamycin (Km), streptomycin (Sp), and spectinomycin (Sm) were used at concentrations of 50–500, 50–100, 50–100 and 50–100 μg/ml, respectively, in Luria-Bertani (LB) medium or agar plates. Rifampicin (Rif) was utilized at a concentration of 100 μg/ml for bacterial growth and 300 μg/ml during transformation experiments. All of the antibiotics used were obtained from Sigma (St. Louis, MO). Aspartate at a concentration of 0.5 mM was added to the growth medium for the Δ*luxS* mutant cultivation. The Advantage cDNA PCR Kit was purchased from Clontech (Palo Alto, CA). The digested plasmid DNA or DNA fragments from agarose gels were purified by using a QIApreps Miniprep Kit (Qiagen, Inc., Valencia, CA). The medium was supplemented with L-arabinose (0.2%) when the *ggdef* gene was expressed from the pBAD/Myc-HisB::*aha0701h* plasmid (Table 1) under the control of an arabinose-inducible *araC* promoter in the pBAD vector.

4.2. Motility assay

LB medium with 0.3% Difco Bacto-agar (Difco Laboratories, Detroit, MI) was used to characterize the swimming motility, while Difco nutrient broth with 0.5% Eiken agar (Eiken Chemical Co., Ltd., Tokyo, Japan) was employed for measuring the swarming motility of WT *A. hydrophila* SSU and its Δ*qseB* mutant strain, as described in our previous study [10]. The overnight cultures grown in the presence of the antibiotics used were adjusted to the same optical density, and equal numbers of cfu (10^6) were stabbed onto 0.35% LB agar plates. Plates were incubated at 37°C overnight, and the motility was assayed by examining the migration of bacteria through the agar from the center towards the periphery of the plate.

4.3. Crystal violet (CV) biofilm assay

The WT *A. hydrophila* SSU and its Δ*qseB* mutant were grown in 3 ml of LB broth contained in polystyrene tubes at 37°C for 24 h with shaking. Biofilm formation was quantified according to the procedure described elsewhere [10]. The biofilm formation results were normalized to 1×10^9 cfu to account for any minor differences in the growth rate of the various bacterial strains used. The experiment was repeated independently three times.

4.4. *In vitro* growth of the biofilm

Briefly, 6-well, sterile polystyrene microtiter plates were filled with 2.5 ml of LB broth supplemented with the appropriate antibiotics. Next, a sterile, 13-mm-diameter thermanox plastic cover slip was laid in each of the wells. Medium was inoculated with 10^6 cfu of WT *A. hydrophila* or its mutants, and the plates were incubated at 37°C. After 12, 24 or 48 h of incubation with gentle shaking (65 rpm), those bacterial cells that were not sufficiently adherent, were removed along with the planktonic cells, by 3 washes of water. Glass or Thermanox plastic cover slips containing the attached biofilms were removed, rinsed with water, and viewed under light microscopy.

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4.5 Scanning electron microscopy (SEM) of biofilms

SEM on biofilm formation of *A. hydrophila* SSU and its mutants was performed using 13-mm-diameter thermanox plastic cover slips. After 48 h of incubation, unattached cells were removed by washing with distillate water, the cover slips were fixed, 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 studies [7, 8].

4.6. Primers and PCR assays

The primers used for various experiments are indicated in Table 2 and were synthesized by Sigma-Aldrich Biotechnology LP (The Woodlands, TX). The PCR assays were performed with 100 ng of genomic DNA (gDNA) and 50 ng of plasmid DNA.

4.7. Reverse transcription–polymerase chain reaction (RT-PCR)

Standard conditions for the isolation of total RNA from cells grown overnight in LB medium, cDNA generation, reverse-transcription PCR procedure, and an estimation of gene transcript levels were performed as described [9]. Briefly, RT-PCR was performed by using SuperScript™ III Platinum (Invitrogen, Carlsbad, CA). Equal amounts of DNase-treated total RNA (500 ng) were used to generate cDNA with random hexamer primers according to the manufacturer's protocol. Sequences of primers used in RT-PCR are shown in Table 2. The relative levels of the cDNAs of RT-PCR were determined by densitometric analyses with AlphaEasyFC software (AlphaInnotech, San Leandro, CA) by using 16S rRNA genes as references. Each RT-PCR reaction which was reported in our earlier paper [9] was rerun as control in the present study. The results were very reproducible and demonstrated exactly the same fold increases compared to a similar supplemental data table presented in our earlier study [9].

4.8. Measurement of the protease activity

Protease activity was measured in culture filtrates of overnight-grown WT *A. hydrophila* SSU and its Δ*qseB* and *ahyRI* mutants as described earlier [41]. The cultures were grown overnight in a liquid medium from −80°C stocks and then the subcultures were made. The protease activity was calculated per ml of the culture filtrate per 10^8 cfu. The hide azure powder substrate (Calbiochem, La Jolla, CA) was used for measuring protease activity because of the sensitivity and rapidity of the assay. The substrate incubated with Dulbecco's phosphate-buffered saline (DPBS) alone served as a negative control.

4.9. Animal experiments

Groups (n=9) of Swiss Webster female mice (Taconic Farms, CA) were infected via the intraperitoneal (i.p.) route with the WT *A. hydrophila* (pBAD/*Myc*-HisB) and the WT *A. hydrophila* in which the GGDEF domain protein was overproduced, in accordance with an approved Institutional Animal Care and Use Committee protocol. The animals were infected at doses of 1×10^7 cfu of the above-mentioned strains. Deaths were recorded for 10 days post-infection. The data were statistically analyzed by using Kaplan-Meier survival estimates. * represents statistical significance at $p < 0.05$.

4.10. Statistical analysis

All of the experiments were performed in triplicate, and, wherever appropriate, the data were analyzed by using the Student's *t* test, with a *p* value of ≤ 0.05 considered significant. The data were presented as an arithmetic mean ± standard deviation.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Connection of 3 QS systems through c-di-GMP was established in *A. hydrophila*.
- Increase of c-di-GMP in WT *A. hydrophila* regulated bacterial virulence.
- c-di-GMP levels in the *qseB* mutant modulated *A. hydrophila* virulence phenotypes.
- c-di-GMP-dependent transcription of *fleN* and *vpsT* was noted in the *qseB* mutant.
- Interplay between QseBC and AhyRI systems at the transcript level was established.

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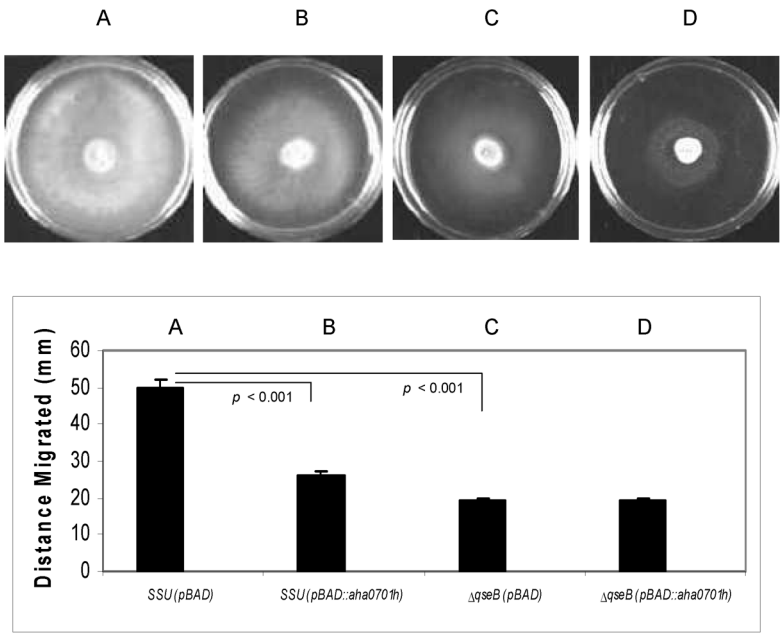

Fig. 1.

Swarming motility of the WT *A. hydrophila* SSU and its Δ*qseB* mutant with either the empty pBAD/*Myc*-HisB vector or the pBAD::*aha0701h* plasmid under arabinose induction. The WT *A. hydrophila* with vector alone **(A)** migrated in the swarming Difco nutrient agar plate with 0.5% Eiken agar, whereas the WT bacteria with overproduction of c-di-GMP **(B)** showed a decrease in motility ($p < 0.001$).The *ggdef*-overexpression in the Δ*qseB* mutant **(D)** did not alter its migration compared to the Δ*qseB* mutant with vector alone **(C)**, but the swarming motility of the latter was decreased statistically when compared to WT *A. hydrophila* harboring the pBAD/*Myc*-HisB vector alone **(A)** ($p < 0.001$). Three independent experiments were performed, and the arithmetic means ± standard deviations were plotted.

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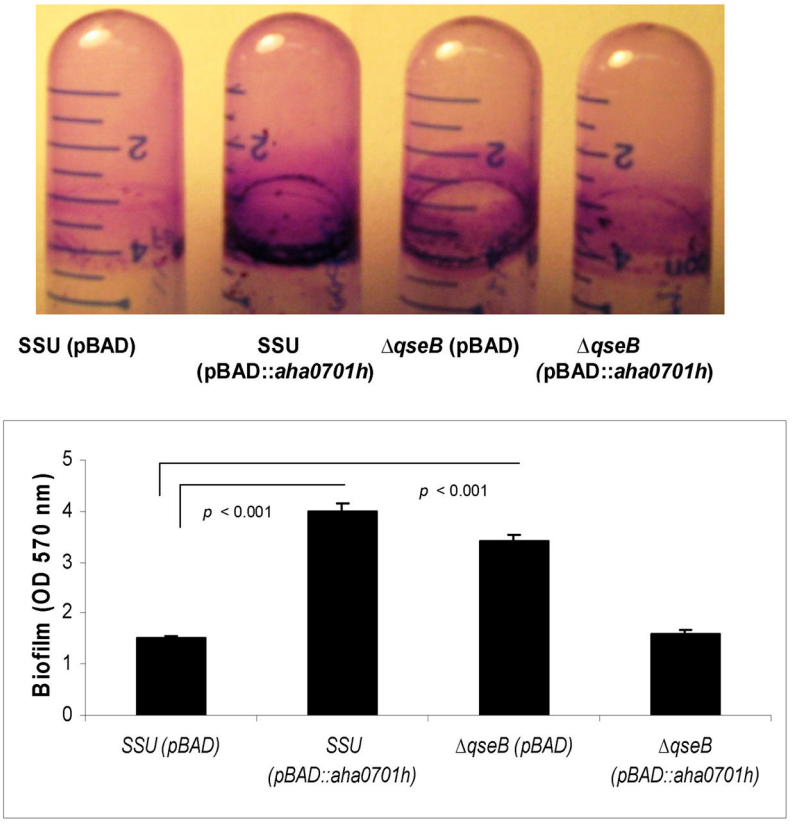

Fig 2.

Measurement of biofilm mass by CV staining formed on polystyrene plastic by the WT *A. hydrophila* SSU and its Δ*qseB* mutant. Biofilms were quantified after 24 h of incubation at 37°C. The results were reproduced in three independent experiments, and the error bars represent standard deviations. Overproduction of AHA0701h in the WT *A. hydrophila* statistically increased biofilm formation compared to the biofilm formed by the WT bacteria with empty vector ($p < 0.001$). The Δ*qseB* mutant with pBAD/*Myc*-HisB vector alone formed biofilms comparable to that seen with the WT *A. hydrophila* with GGDEF domain protein overproduction. When AHA0701h was overproduced in the Δ*qseB* mutant, the biofilm mass was similar to that seen in the WT *A. hydrophila* with the pBAD/*Myc*-HisB vector alone.

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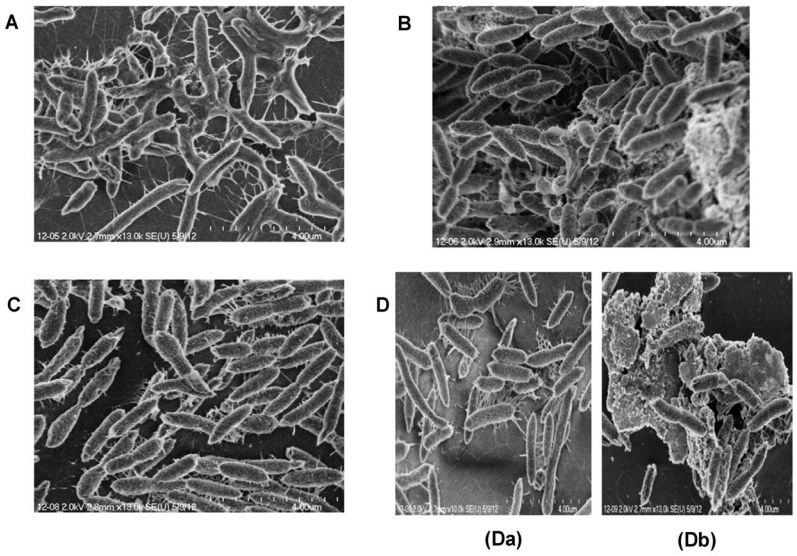

Fig. 3.

Representative SEM images of biofilm formation by WT *A. hydrophila* SSU and its Δ*qseB* mutant after 48 h of cultivation at on37°C thermanox cover slips: **A**. WT *A. hydrophila* with the pBAD/*Myc*-HisB vector alone; **B**. WT *A. hydrophila* when c-di-GMP was overproduced; **C.** Δ*qseB* mutant with the pBAD//*Myc*-HisB vector alone; **D (a, b)** Δ*qseB* mutant with overproduction of c-di-GMP.

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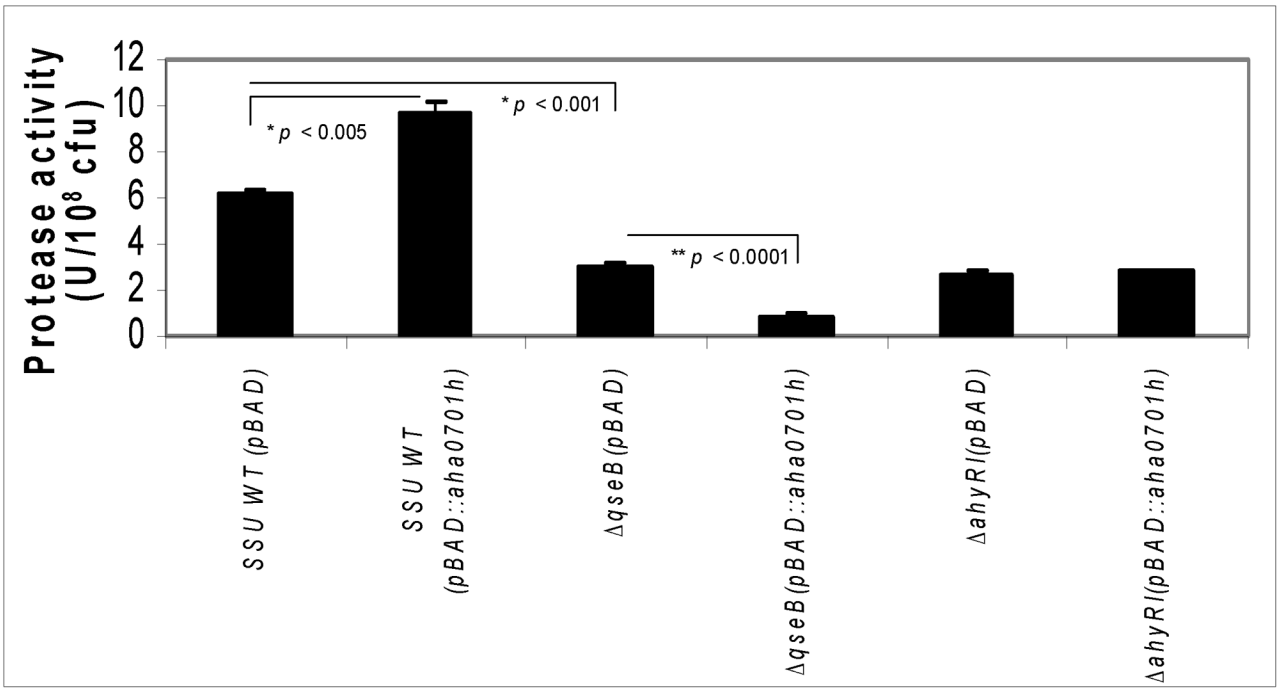

Fig. 4.

Protease activity in the culture supernatants of WT *A. hydrophila* SSU and its Δ*qseB* and *ahyRI* mutants with pBAD/*Myc*-HisB vector alone, compared to the WT and mutants with overproduction of GGDEF domain protein. Overproduction of c-di-GMP in the WT *A. hydrophila* resulted in statistically significant increases of protease activity compared to that seen in the WT bacteria with the pBAD/*Myc*-HisB vector ($p < 0.005$). No protease activity was measured when AHA0701h-encoding gene was over-expressed in the Δ*qseB* mutant. No statistically significant differences in protease activity were found when the GGDEF domain protein was overproduced in the *ahyRI* mutant as compared to the mutant with the empty pBAD/*Myc*-HisB vector. The data were normalized to 1×10^8 cfu to account for any minor differences in the growth rates between the WT bacteria and mutant strains. All of the experiments were performed in triplicate and the data presented as arithmetic means ± standard deviations. * denotes statistically significant increase (WT SSU versus SSU with overproduction of c-di-GMP) and decrease (WT SSU versus Δ*qseB* mutant), respectively. ** denotes statistically significant decrease between the Δ*qseB* mutant with and without the overproduction of c-di-GMP ($p < 0.0001$).

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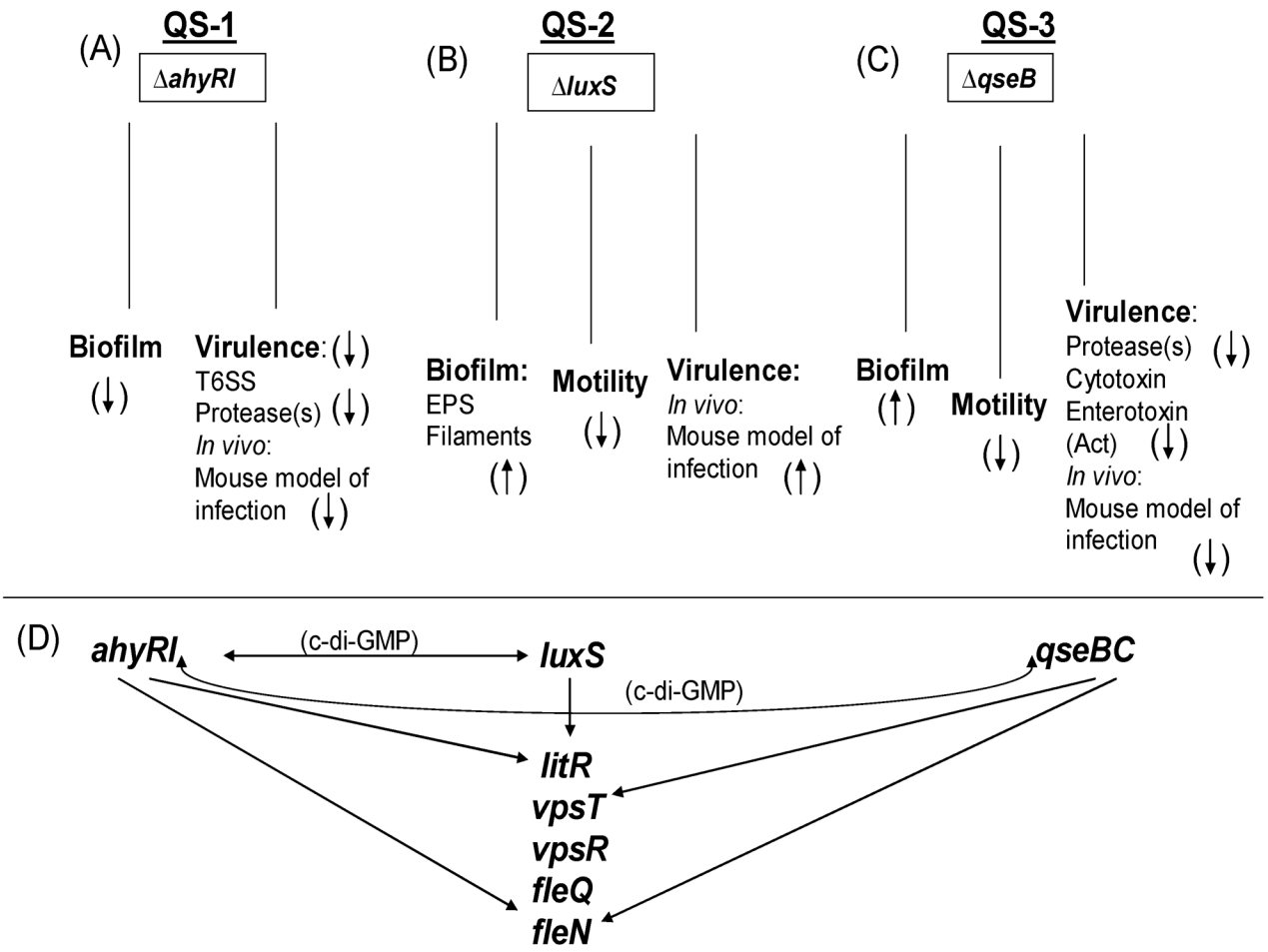

Fig. 5.

Integrated schematic illustrating QS-dependent regulatory network in *A. hydrophila* SSU. Three modified autoinducer (AI) systems, which are known to date in other bacteria, exist in *A. hydrophila.* **(A)** While the *ahyRI*-based (AI-1) QS system positively regulates bacterial virulence [7], the LuxS-based QS system (AI-2) negatively regulates *A. hydrophila* virulence [8] **(B). (C)** QseBC has both positive and negative regulation on various virulence factors/mechanisms of *A. hydrophila* [10] that play an important role in fine tuning the expression of virulence genes (Current study). **(D)** Cross talk between AI-1- and QseBC-systems was identified (Current study) in addition to interplay between AI-1 and AI-2 systems [10]. An alteration in the transcription of major genes involved in biofilm formation and motility of the WT *A. hydrophila* is dependent on the existence of AI-1, AI-2, and QseBC systems, and they are co-regulated via di-GMP. (↑) denotes an increase and (↓) shows a decrease in biofilm formation, motility, and virulence in Δ*ahyRI*, Δ*luxS*, and Δ*qseB* mutants of *A. hydrophila*.

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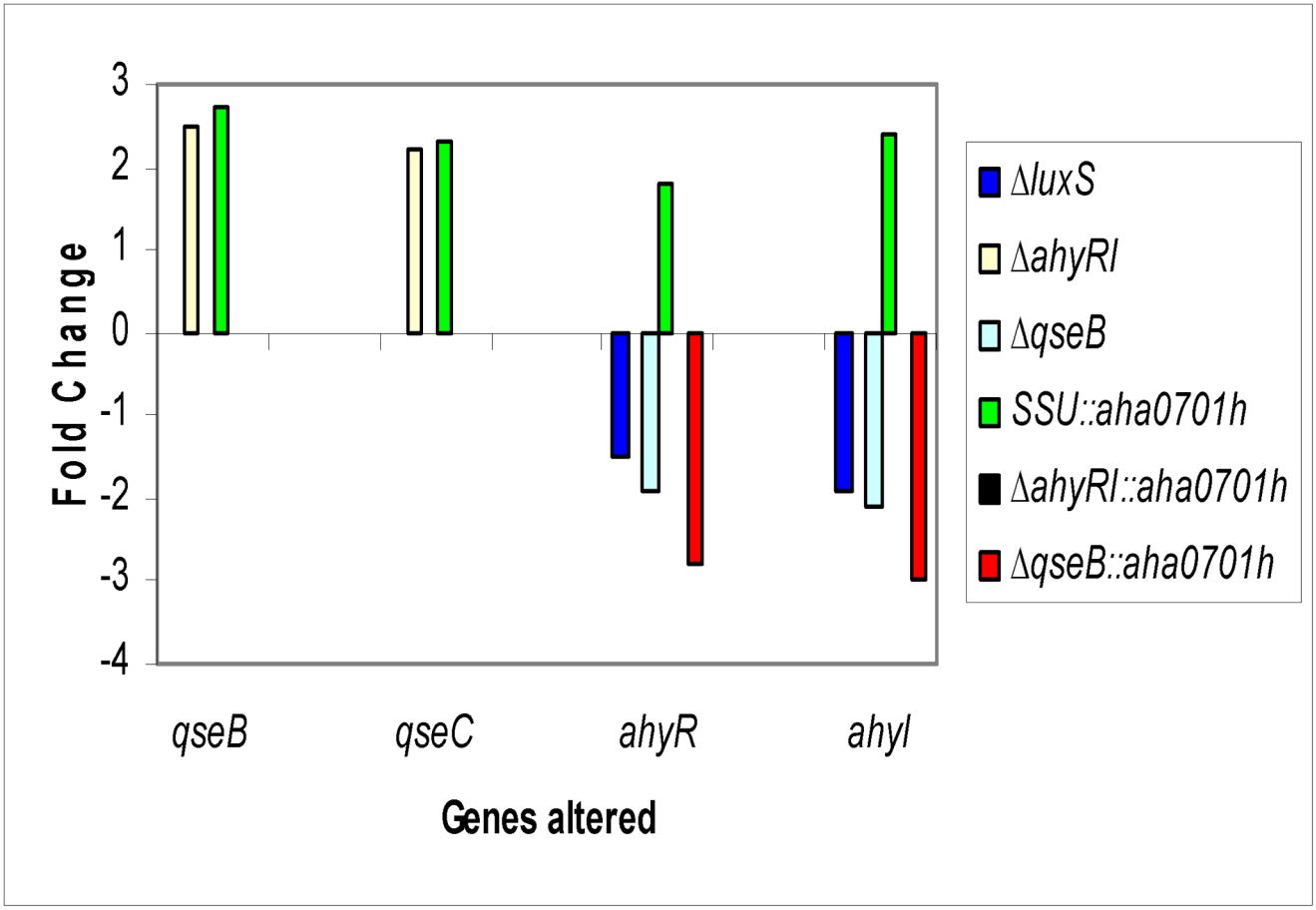

Fig. 6.

Comparison by RT-PCR of the expression of *qseB*, *qseC, ahyR, and ahyI* genes in different genetic backgrounds of *A. hydrophila* SSU. The transcript levels of the *qseB* and *qseC* genes were increased in the *ahyRI* mutant compared to those of the WT *A. hydrophila* (yellow bars). The *qseB* and *qseC* gene transcript levels were increased when c-di-GMP was overproduced in the WT *A. hydrophila* compared to those of the parental strain with pBAD/*Myc*-HisB vector (green bars). The level of expression of the *ahyR* and *ahyI* genes was down-regulated in the Δ*luxS* mutant. The transcript levels *ahyR* of the and *ahyI* genes were down-regulated in the Δ*qseB* mutant (cyan bars). The increased levels of c-di-GMP further down-regulated the expression levels of *ahyR* and *ahyI* genes in the Δ*qseB* mutant (red bars). Finally, the expression *ahyR* of the and *ahyI* genes was increased in the WT *A. hydrophila* with increased c-di-GMP levels (green bars). The data used to generate fold changes (arithmatic means ± standard deviations) with statistical analysis are shown in Supplemental data, Table I.

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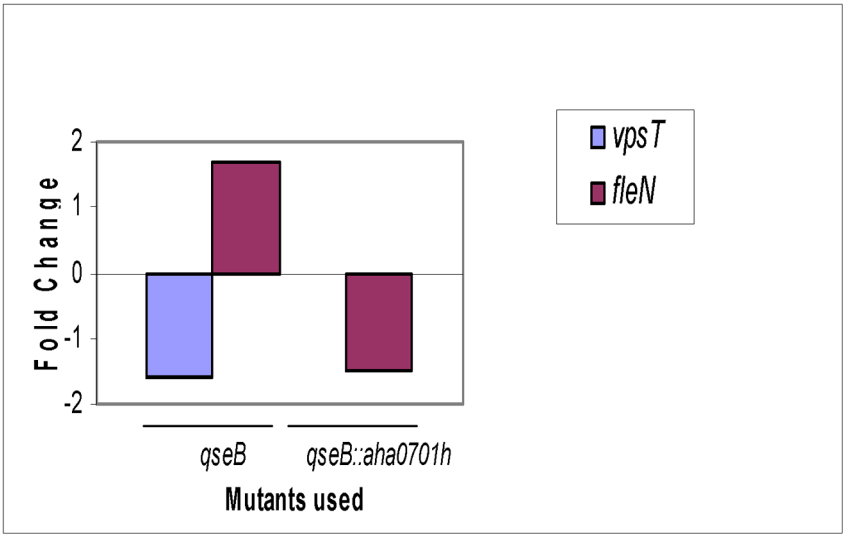

Fig. 7.

Alteration in the transcription of genes involved in biofilm formation and motility in the Δ*qseB* mutant *A.* of *hydrophila* SSU. The analysis of transcripts by RT-PCR demonstrated increases in the levels of the *fleN* gene and decreases in the levels of *vpsT* gene, compared to that of WT *A. hydrophila.* The GGDEF domain protein overproduction in the Δ*qseB* mutant returned the transcript level of *vpsT* the gene to the level found in the WT bacteria with the pBAD/*Myc*-HisB vector alone, while the expression of the *fleN* gene was down-regulated. The data used to generate fold changes (arithmatic means ± standard deviations) with statistical analysis are shown in the Supplemental data, Table I.

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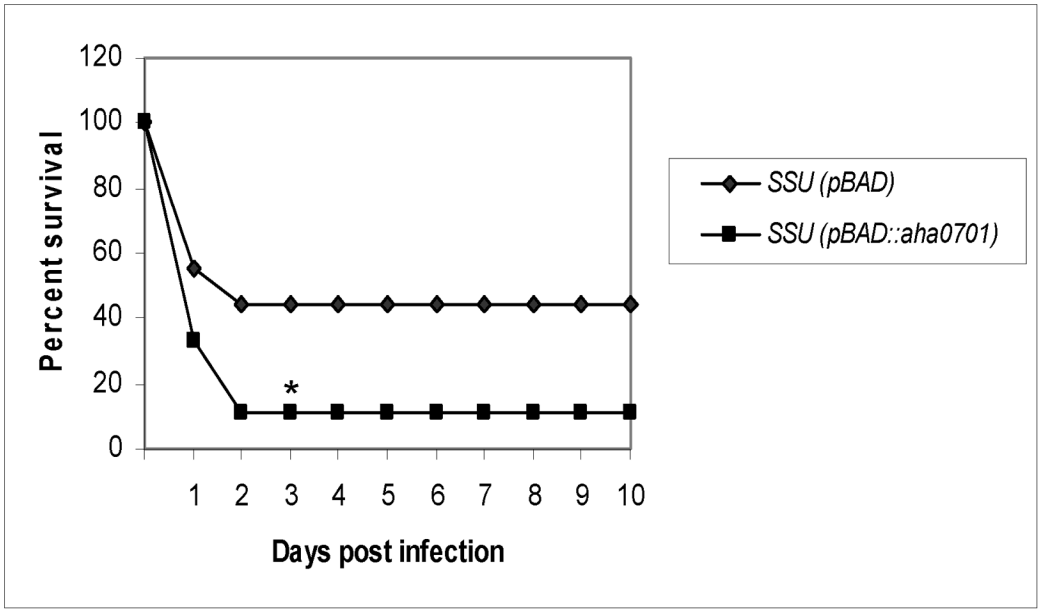

Fig. 8.

The virulence of the WT *A. hydrophila* SSU and the parental bacteria with increased production of c-di-GMP in a mouse model. Swiss-Webster mice (n=9/group) were infected at doses of 1×10^7 cfu of the above-mentioned strains by the i.p. route. The animals were observed for mortality over a period of 10 days. The data were statistically analyzed by using a Kaplan-Meier survival estimate. * represents statistical significance at a p value of < 0.05.

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Table 1

Strains and plasmids used in this study

Strain or Plasmid	Relevant characteristic (s)	Source or reference
A. hydrophila SSU		CDC[a]
SSU-Rif^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	[10]
Δ*ahyRI*	*ahyRI* gene deletion mutant of *A. hydrophila* SSU-R strain Rif^r Sm^r Sp^r	[7]
Δ*luxS*	*luxS* mutant of *A. hydrophila* SSU-R strain Rif^r Km^r	[8]
SSU (pBAD::*aha0701h*)	WT *A. hydrophila* SSU with GGDEF domain protein overproduced	[9]
Δ*ahyRI* (pBAD::*aha0701h*)	*ahyRI* gene deletion mutant of *A. hydrophila* with GGDEF domain protein overproduced	[9]
Δ*qseB* (pBAD::*aha0701h*)	*qseB* gene deletion mutant of *A. hydrophila* SSU with GGDEF domain protein overproduced	This study
Plasmids		
pBAD/*Myc*-HisB	vector, *ara* BAD promoter Ap^r	Invitrogen
pBAD:: *aha0701h*	GGDEF domain encoded gene of *A. hydrophila* cloned into pBAD/*Myc*-HisB *ara* Km^r Ap^r	[9]

Abbreviations: Rif, rifampin; Km, kanamycin, Sm, streptomycin, Sp, spectinomycin,

[a]Centers for Disease Control and Prevention

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Table 2

Oligonucleotides used and RT-PCR products

Primers	Oligonucleotide sequences	Amplification of the genes	RT-PCR product (bp)
qseBF2	TGCTCAAGAGCGAGGAGTTTG	*qseB*	551
qseBR2	CTTCTTGCGCAGGTGGTGAAT		
qseCF2	ATGGAGGAGCTGTTCGATGCC	*qseC*	793
qseCR2	AGCAGCATCTTCTGCAGGGAGT		
vpsTF1	TCAGAGATACTCCTTGGCCCA	*vpsT (csgAB)*	645
vpsTR1	CGCTTCATGATCACCCCATA		
fleNF1	TGGTCTGCGCAAAATGCGT	*fleN*	856
fleNR1	TTATTCACGGGAACCTTCCTG		
luxSF2	ACCTCCAAGTGGGATGCGTAT	*luxS*	971
luxSR2	CGGGCCATCGAAAAAATGT		
ahyRF1	TATTGCATCAGCTTGGGGAA	*ahyR*	781
ahyRR1	TGAAACAAGACCAACTGCTTG		