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Quorum Sensing and c-di-GMP-Dependent Alterations in Gene Transcripts and Virulence-Associated Phenotypes in a Clinical Isolate of *Aeromonas hydrophila*

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Abstract

Recently, we demonstrated that the LuxS-based quorum sensing (QS) system (AI-2) negatively regulated the virulence of a diarrheal isolate SSU of *Aeromonas hydrophila*, while the *ahyRI*-based (AI-1) N-acyl-homoserine lactone system was a positive regulator of bacterial virulence. Thus, these QS systems had opposing effects on modulating biofilm formation and bacterial motility *in vitro* models and *in vivo* virulence in a septicemic mouse model of infection. In this study, we linked these two QS systems with the bacterial second messenger cyclic diguanosine monophosphate (c-di-GMP) in the regulation of virulence in *A. hydrophila* SSU. To accomplish this, we examined the effect of overproducing a protein with GGDEF domain, which increases c-di-GMP levels in bacteria, on the phenotype and transcriptional profiling of genes involved in biofilm formation and bacterial motility in wild-type (WT) versus its QS null mutants. We provided evidence that c-di-GMP overproduction dramatically enhanced biofilm formation and reduced motility of the WT *A. hydrophila* SSU, which was equitable with that of the $\Delta luxS$ mutant. On the contrary, the $\Delta ahyRI$ mutant exhibited only a marginal increase in the biofilm formation with no effect on motility when c-di-GMP was overproduced. Overall, our data indicated that c-di-GMP overproduction modulated transcriptional levels of genes involved in biofilm formation and motility phenotype in *A. hydrophila* SSU in a QS-dependent manner, involving both AI-1 and AI-2 systems.

Keywords

A. hydrophila; QS; AI-1 (*ahyRI*-based) QS; AI-2 (*luxS*-based) QS; motility; biofilm; c-di-GMP

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1. INTRODUCTION

At least two modified autoinducer (AI) systems, which are known to date in other bacteria, exist in a diarrheal isolate SSU of *A. hydrophila* [1,2]. One of them is the *S*-ribosylhomocysteinase (LuxS)-based autoinducer AI-2 system [3]. LuxS plays a major role in AI-2 synthesis, and is also an integral component of the activated methyl cycle [4]. The bacterial phenotypes that are dependent on the QS systems include surface attachment and/or biofilm formation, motility, and *in vivo* virulence [5–7].

Recently, we characterized the AI-2-mediated QS in *A. hydrophila* SSU and showed that the $\Delta luxS$ mutant formed denser biofilm *in vitro*, had increased virulence in a septicemic mouse model of *A. hydrophila* infections, and exhibited decreased motility compared to that of the wild-type (WT) bacterium [1]. However, limited information was available on the AI-1 [N-acyl-homoserine lactone (AHL)-based QS (*ahyRI*)] system in the regulation of bacterial virulence. Consequently, we demonstrated that several virulence traits were altered in the $\Delta ahyRI$ mutant [2]. However, there are at least three *ahyR* homologs that exist in the genome of *A. hydrophila* SSU, and *ahyR2* is genetically linked to the type 6 secretion system (T6SS) (unpublished data). The contribution of these three *ahyR* homologs in *A. hydrophila* SSU AI-1 QS is currently unknown.

Like QS, cyclic diguanosine monophosphate (c-di-GMP), the bacterial intracellular second messenger, has also been implicated in the regulation of cell surface properties of several bacterial species [8]. The cellular level of c-di-GMP was shown to be controlled through the opposite activities of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), which contained functional GGDEF and EAL domains, respectively. Subsequently, it was found that GGDEF and EAL domain proteins were involved in c-di-GMP synthesis and degradation (hydrolysis), respectively [9,10]. Recently, it was shown that QS modulated the c-di-GMP signaling pathway to control bacterial virulence [11,12], and the role of LuxS in controlling c-di-GMP production at the transcriptional level was described in *Vibrio* species [13]. Indeed, c-di-GMP activated biofilm formation in a variety of bacteria, including *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, *Vibrio* spp., and *Yersinia pestis* [14,15] and correspondingly repressed motility of these bacteria [14,16,17].

In *V. cholerae*, an accumulation of AI-2 induced the expression of a gene encoding the master QS-regulator HapR [18]. HapR negatively affected bacterial biofilm formation and exopolysaccharide (EPS) biosynthesis [19] by lowering the intracellular level of c-di-GMP [13]. Further, HapR negatively regulated the transcription of *vpsR*, *vpsT*, and the *vps* genes, while VpsR and VpsT positively regulated the transcription of *vps* genes in *V. cholerae* [20]. The contribution of the AI-2 extracellular signal to this process is not clearly understood. Although LuxS was initially identified by its contribution to the expression of the *hapR* gene in a light production bioassay [18], deletion of the *luxS* gene in a later study did not affect HapR-dependent biofilm formation in *V. cholerae* [21].

The mechanism of coexistence and co-regulation of the above-mentioned two QS systems (AI-1 and AI-2) in *A. hydrophila* SSU is not clear. Since QS and c-di-GMP signaling regulate some of the same complex processes, like biofilm formation, motility, and virulence of bacteria, and since we observed that a GGDEF domain protein encoding gene is genetically linked to the *luxS* gene of *A. hydrophila* SSU [1], it is possible that these two signaling pathways converge in *A. hydrophila*.

In this study, we provided data showing an effect of c-di-GMP overproduction on the phenotypic and transcriptional profiling of virulence-associated genes in WT and QS mutants of *A. hydrophila* SSU. To our knowledge, our study is the first that illustrated an interplay between AI-1, AI-2, and c-di-GMP in *A. hydrophila*.

2. RESULTS

2.1. Identification of genes in *A. hydrophila* SSU which are homologs of QS-dependent c-di-GMP regulated genes in *Vibrio* and *Pseudomonas*

Although our recent studies showed the roles of AHL-mediated AI-1 QS system [2] and that of the *luxS* gene [1] in *A. hydrophila* virulence, we were interested in identifying additional genes which could be involved in the QS system of *A. hydrophila* SSU. Consequently, we took advantage of our annotation of the genome sequence of *A. hydrophila subsp. hydrophila* ATCC 7966^T [22]. By using specific primers (Table 1) and sequence analysis of the resulting polymerase chain reaction (PCR) product, we identified the presence of two more *ahyR* homologs on the genome of *A. hydrophila* SSU. The gene *ahyR2* was genetically linked to the T6SS.

We also identified in its genome the homologs of *luxQ*, *luxU* and *luxO* genes, which are involved in AI-2-dependent phosphorylation cascade [23], and exhibited 95, 96, and 92% homology with the corresponding genes found in the genome of *A. hydrophila* 7966^T strain [22]. However, we were unable to amplify the *luxP* gene which encodes an autoinducer binding protein in *V. harveyi* [24,25]. The *luxP* gene is also not present in the genome of *A. hydrophila* 7966^T strain [22].

We noted the presence of a *litR* gene in *A. hydrophila* SSU which is a homolog of *hapR* gene in *V. cholerae* [19]. The *LitR* of *A. hydrophila* SSU was highly homologous (~98%) to the corresponding gene found in the annotated genome sequences of *A. hydrophila* 7966 and *A. salmonicida subsp. salmonicida* A449 strains [22,26], and exhibited 35–40% identity with HapR protein sequences of vibrios. HapR is a DNA-binding transcription factor that initiates a program of gene expression, switching the cells from individual, low-cell-density state, to the high-cell density state [13]. HapR in *V. cholerae* regulates c-di-GMP metabolizing genes that lead to a net reduction in the level of cellular c-di-GMP, and thereby, to the negative control of biofilm formation [13]. By directly down-regulating the expression of the *vpsT* gene [13], HapR is also shown to control biofilm formation in a c-di-GMP-independent manner. VpsT is a transcriptional activator which together with VpsR is required for the expression of the *V. cholerae* polysaccharide biosynthesis operon [27,28]. We found the *vpsT* homolog, named *csgAB* (Fig. 7), in the *A. hydrophila* SSU genome which encoded a protein with 37–53% identity to VpsT of *Vibrio* species.

Interesting was also the presence of FleQ in *A. hydrophila* SSU (Fig. 7), which showed 51% identity with its homolog in *P. aeruginosa* [29], and 35% identity with VpsR in *V. cholerae* [28]. Likewise, the FleN in *A. hydrophila* SSU demonstrated 69% identity with FleN of *P. aeruginosa* [29]. FleQ, the first example of a c-di-GMP effector involved in transcription control, is a master regulator of flagellar gene expression in *P. aeruginosa* [29]. FleN, an anti-activator of FleQ, down-regulates FleQ activity through direct interactions and thereby plays a crucial role in maintaining a single flagellum in *P. aeruginosa* [30,31].

Our genome sequencing of *A. hydrophila* 7966 strain suggested the presence of 23 proteins containing GGDEF domain, 7 proteins with an EAL domain, and 15 proteins that had both GGDEF and EAL domains [22]. One of the GGDEF domain protein (AHA0701h) encoding genes was located immediately downstream of the *luxS* gene in *A. hydrophila* SSU. The AHA0701h protein exhibited 93% identity with its homolog found in *A. hydrophila* 7966 strain [22]. A homolog of *A. hydrophila* SSU AHA0701h, referred to as VCA0956 (46/68), was also found in *V. cholerae* (Fig. 1).

Therefore, *A. hydrophila* SSU QS systems have characteristics of both *Vibrio* and *Pseudomonas*, in addition to having unique signaling cascades which makes it an ideal

candidate to study QS-dependent c-di-GMP regulatory network. Such studies on *A. hydrophila* are important as the organism leads to both gastroenteritis and septicemia depending on the route of infection [2].

2.2. Overproduction of GGDEF domain protein regulates virulence-associated phenotypes of WT *A. hydrophila* SSU

The GGDEF domain and an allosteric binding site for c-di-GMP (I site) [32] are typically found as part of modular DGC enzymes, which catalyze the synthesis of the signaling molecule c-di-GMP [33]. The presence of AHA0701h with GGDEF domain, the location of its gene immediately downstream of the *luxS* gene in *A. hydrophila* SSU, together with the homology of AHA0701h with VCA0956 (Fig. 1), prompted us to test the effect of its overproduction on WT *A. hydrophila* virulence-associated phenotype(s). Indeed, overproduction of VCA0956, a cytoplasmic protein composed of a single DGC domain, was successfully used to increase c-di-GMP levels in *V. cholerae* [34,35] and *V. vulnificus* [36]. Studies have shown that the modulation of c-di-GMP level by overproducing proteins with either the DGC or the PDE domain of *V. cholerae* and *A. veronii* biovar *sobria* resulted in an altered biofilm formation and motility [35,37].

For these studies, we cloned the gene encoding AHA0701h into the pBAD/Myc-HisB vector. Subsequently, the empty vector and pBAD/Myc-His::*aha0701h* (Table 2) were transformed into WT *A. hydrophila* SSU and its Δ *ahyRI* mutant, and the phenotype of these strains was studied after arabinose induction.

Overproduction of AHA0701h resulted in a dramatically increase in biofilm formation, as measured by quantitative crystal violet (CV) staining, in WT *A. hydrophila* SSU compared to that of bacteria harboring only the vector (Fig. 2A & B). The biofilm formed by WT *A. hydrophila* with vector alone, visualized by light microscopy, appeared as uniform sparse cell attachment on the surface of Thermanox plastic cover slips after 12 h of incubation, with a greater number of bacterial cells adherent at the line of the edge of the cover slips after 24 h of incubation (Fig. 3A). In contrast, biofilm formed by the WT *A. hydrophila* with pBAD/Myc-His B::*aha0701h* plasmid appeared as a mature, dense three-dimensional structure after 24 h of incubation, uniformly distributed all along the cover slip's surface (Fig. 3B). Thus, overexpression of the gene encoding AHA0701h in WT *A. hydrophila* dramatically increased the number of attached cells and accelerated the dynamics of three-dimensional structured biofilm formation.

The number of biofilm forming cell-units of WT *A. hydrophila* SSU with overproduced GGDEF protein (AHA0701h) was greater compared to that of the Δ *luxS* mutant (Fig. 2B & C). Further, the Δ *luxS* mutant formed a matured biofilm after 48 h of incubation [1], while a mature biofilm of WT *A. hydrophila* SSU with overproduced GGDEF protein (AHA0701h) appeared after 24 h (Fig. 3B).

While over-expression of the gene encoding AHA0701h in WT *A. hydrophila* SSU positively regulated biofilm formation, motility of the bacteria was decreased compared to that of the WT bacteria (Fig. 4A-3 versus A-1). Additionally, the WT *A. hydrophila*, which overproduced GGDEF protein (AHA0701h), exhibited minimal motility similar to that of the *A. hydrophila* Δ *luxS* mutant (Fig. 4A-3 versus B-3).

Thus, over-expression of the gene encoding AHA0701h in WT *A. hydrophila* led to enhanced biofilm formation and decreased motility, and these data correlated with the observations of Rahman *et al.* (2007) who showed that overproduction of the GGDEF domain protein AdrA increased c-di-GMP concentration and biofilm formation and reduced motility in *A. veronii* biovar *sobria* [37].

2.3. The effect of over-production of GGDEF domain protein on biofilm formation and motility in the Δ ahyRI mutant of *A. hydrophila* SSU

We transformed empty vector and pBAD/Myc-HisB::*aha0701h* plasmid in the Δ ahyRI mutant of *A. hydrophila*. Recently, we demonstrated that the *ahyRI* deletion resulted in decreased biofilm formation but the motility of the mutant was not affected compared to that of the parental bacteria [2]. In contrast to WT *A. hydrophila* with overproduced GGDEF protein (AHA0701h), which resulted in increased biofilm formation (Figs. 2B & 3B), biofilm formed by the Δ ahyRI mutant with over-production of GGDEF protein was marginally increased after 48 h of incubation (data not shown).

Importantly, both WT *A. hydrophila* and its Δ ahyRI mutant had similar swarming and swimming motility [2] (also Fig. 4 A-2 versus B-1). However, overproduction of the GGDEF protein (AHA0701h) in the Δ ahyRI mutant did not alter its motility compared to that of the parental mutant strain (Fig. 4 B-1 versus B-2). This was in contrast to decrease in motility that was observed with the WT *A. hydrophila* overproducing the GGDEF protein (AHA0701h) (Fig. 4 B-2 versus A-3). Thus, overproduction of GGDEF protein in WT versus its Δ ahyRI mutant exhibited contrasting phenotypic alterations.

These data prompted us to study alteration in the transcription of major genes involved in biofilm formation and motility of the WT *A. hydrophila* and its Δ ahyRI mutant after overproduction of GGDEF protein (AHA0701h), as compared to the parental background strains and the *luxS* mutant. Such studies were performed to evaluate a possible correlation between the presence and expression of QS system-encoding genes and c-di-GMP overproduction in *A. hydrophila*.

2.4. Alteration in the transcription of genes involved in biofilm formation and motility in *luxS* and *ahyRI* mutants of *A. hydrophila* SSU

The analysis of transcripts by reverse-transcription PCR demonstrated increases in the levels for *litR* and *fleQ* (*vpsR*), and decreases in the levels of *csgAB* (*vpsT*) and *fleN* in the Δ luxS mutant compared to that of WT *A. hydrophila* (Fig. 5A; Supplemental data, Table I). On the contrary, the Δ ahyRI mutation in WT *A. hydrophila* led to down-regulation of the *litR* (Figs. 5B and 6A) and an up-regulation of the *fleN* gene transcript (Fig. 5B), but the levels of expression for the *csgAB* (*vpsT*) (Fig. 6B) and *fleQ* (*vpsR*) genes were not altered compared to that of the WT *A. hydrophila* (Supplemental data, Table I). The Δ ahyRI mutation also caused an increase in the *luxS* gene transcript as compared to that of the WT *A. hydrophila* (Fig. 5B). The expression level of the *aha0701h* gene in the Δ ahyRI mutant was comparable to that in the WT bacteria (Supplemental data, Fig. I, lanes 1 & 3) in contrast to an over-expression of this gene which we noticed in the Δ luxS mutant (Supplemental data, Fig. I, lanes 1 & 2).

It is known that the elevation of c-di-GMP in the low-cell-density state of *V. cholerae* induces the transcriptional activator *vpsT* involved in HapR/LitR-dependent regulation of biofilm formation [27]. Further, regulation by VpsR has also been linked to signal transduction by bacterial c-di-GMP [38,39]. These studies prompted us to overexpress GGDEF protein (AHA0701h) encoding gene for studying the effect of high level of intracellular c-di-GMP on the transcriptional expression of selected genes mentioned above in WT *A. hydrophila* and its Δ ahyRI mutant.

2.5. The effect of overproduction of GGDEF domain protein on genes involved in biofilm formation and regulation of motility in WT and Δ ahyRI mutant of *A. hydrophila* SSU

We found that *luxS*, *litR*, *csgAB* (*vpsT*), *fleQ*, and *fleN* gene transcripts were significantly increased by AHA0701h overproduction in WT bacteria (Fig. 5C; Supplemental data, Table

I). It should be noted that the expression levels of these genes in WT and the WT with vector alone were similar. When GGDEF protein was overproduced in the $\Delta ahyRI$ mutant, the *litR* gene transcript was increased compared to its level noted in the $\Delta ahyRI$ mutant with vector alone (Fig. 6A). At the same time, this level of *litR* gene transcript in the $\Delta ahyRI$ mutant with overproduced GGDEF protein was comparable to that in the WT bacteria with vector alone (Fig. 6A; Supplemental data, Table I). Most likely, AHA0701h overproduction normalized the transcriptional level of *litR* gene in the $\Delta ahyRI$ mutant, making it similar to that in the WT bacteria.

The same trend of transcript level normalization was also noted for the *luxS* gene in the $\Delta ahyRI$ mutant with AHA0701h over-production (Supplemental data, Table I) but occurred as a result of down-regulation of this gene (Supplemental data, Table I). While the transcripts for *csgAB* (*vpsT*) and *fleQ* (*vpsR*) were not altered in the $\Delta ahyRI$ mutant compared to that of WT bacteria (Supplemental data, Table I), the AHA0701h overproduction led to decreased levels of *csgAB* (*vpsT*) (Figs. 5D and 6B) and the increased levels of *fleQ* (*vpsR*) (Fig. 5D) in the $\Delta ahyRI$ mutant.

Thus, the *ahyRI* deletion affected on the differentiation in the transcriptional responses of selected genes under AHA0701h over-production conditions compared to that in the WT *A. hydrophila*.

2.6. Co-modulation of QS gene transcripts and the effects of GGDEF domain protein overproduction on the expression of gene transcript levels in *A. hydrophila* SSU

Our data showed that the *luxS* gene transcript modulation was dependent on the presence and expression of AI-1 genes and on the c-di-GMP levels (Figs. 5B, C & D). This prompted us to study the effects of *luxS* mutation and c-di-GMP level modulation on the expression of *ahyR*, *ahyI*, and two additional *ahyR* (*ahyR2* and *ahyR3*) homologs which exist in *A. hydrophila* SSU. The *ahyR* and *ahyI* gene transcripts were down-regulated in the $\Delta luxS$ mutant, while the *ahyR2* and *ahyR3* gene transcripts were over-expressed compared to that in the WT bacteria (Fig. 5A, Supplemental data, Table I). The GGDEF protein when overproduced in WT *A. hydrophila* resulted in the up-regulation of *luxS*, *ahyI* and all of the three homologs of *ahyR* genes as compared to that in the WT bacteria with vector alone (Fig. 5C, Supplemental data, Table I).

In the *ahyRI* mutant, the expression of *ahyR2* and *ahyR3*, together with the *luxS* gene was increased as compared to that in the WT *A. hydrophila* (Fig. 5B, Supplemental data, Table I). However, overproduction of GGDEF protein in the *ahyRI* mutant resulted in down-regulation of *luxS*, *ahyR2*, and *ahyR3* gene transcripts compared to that in the $\Delta ahyRI$ mutant with vector alone. The level of expression of these genes returned to the level of WT bacteria (Supplemental data, Table I). Thus, our results demonstrated that AI-1 and AI-2 QS systems were linked at least at the transcriptional level in *A. hydrophila* SSU. The GGDEF domain protein overproduction modulated the transcriptional levels of genes involved in AI-1 and AI-2, and this modulation was dependent on the presence of both of them in the cells.

3. DISCUSSION

In this study, we identified additional components of LuxS- and AhyRI- dependent QS and c-di-GMP networks in *A. hydrophila* SSU. Majority of reports on the $\Delta luxS$ mutant indicated that the lack of *luxS* gene in bacteria led to attenuation in biofilm formation and virulence, or retention of virulence but to a decrease in biofilm formation [40–43]. However, Ali and Benitez [44] recently showed that in *V. cholerae* $\Delta luxS$ mutant, there occurred an increase in biofilm formation similar to our findings in *A. hydrophila* SSU [1]. This finding

subsequently was correlated with the model of QS regulation and biofilm formation in *V. cholerae* through the modulation of intracellular c-di-GMP level [13].

In *Vibrio* species, sensing of extracellular AI-2 involves a regulation cascade consisting of a binding protein LuxP and the response regulator LuxQ (Fig. 7) [24,19,47]. The VarS/A-CsrA/BCD system is an additional signaling mechanism that acts in parallel with the two QS systems to input information into LuxO [48] (Fig. 7). The global regulators CsrA and HapR both largely contribute to the control of c-di-GMP levels by modulating selected panels of GGDEF and EAL domain-encoding genes in response to internal signals [13,48] (Fig. 7). In addition to QS-dependent HapR regulating cascade, we also identified the VarS/VarA (LetS/LetA)-CsrA regulatory system in the *A. hydrophila* SSU genome (unpublished data) by examining gene homologs that exist in a variety of gram-negative bacteria [48]. However, subtle differences were noted, for example, between the HapR-dependent QS systems of *V. cholerae* and *A. hydrophila* SSU. Further, how these two regulatory systems co-function in *A. hydrophila* is not known yet.

To date, two types of AI-2 receptors have been identified: LuxP, present in *Vibrio* species and LsrB, first identified in *S. Typhimurium* [24,49] (Fig. 7). However, we could not identify any homolog of such receptors in *A. hydrophila* SSU. Moreover, no information about sRNAs involved in *A. hydrophila* SSU QS regulation is available from the genome sequence of *A. hydrophila* ATCC 7966 [22].

We did identify homologs of *luxQ*, *luxU*, *luxO*, and *litR/hapR* genes in *A. hydrophila* SSU genome similar to that found in *Vibrio* [58]. LuxR/HapR is the only TetR family member that appears to act as both activator and repressor [50–53]. While the mechanism by which LuxR (HapR) activates *qrr* (encoding sRNA) gene expression in *V. harveyi* is yet to be determined, it is known that the expression of the *qrr* gene requires LuxO~P acting in conjunction with the alternative sigma factor sigma-54 [23,54]. Recently, Waters *et al.* [13] demonstrated that HapR altered the expression of 14 genes encoding proteins with GGDEF and/or EAL domains. The QS control of these genes resulted in increased intracellular levels of c-di-GMP at low cell density and decreased c-di-GMP levels at high cell density. The major consequence of increased c-di-GMP in the low-cell-density state is the induction of biofilm transcriptional activator *vpsT* (Fig. 7) [27]. We identified the homolog of *vpsT* in WT *A. hydrophila* SSU genome which is designated as *csgAB*. VpsT is a member of the FixJ, LuxR, and CsgD family of prokaryotic response regulators [55–57].

We did not find any candidate protein which was closely related to VpsR of *V. cholerae*, a key regulator of biofilm formation [28,58], in the *A. hydrophila* genome, but FleQ (found in *P. aeruginosa*) [29], a distant VpsR homolog, encoding gene was detected (Fig. 7). In *V. parahaemolyticus*, the role of VpsR is reported to be strain dependent [59] while in *V. fischeri*, VpsR might function as a repressor of an unknown polysaccharide that could contribute to mucoidal versus non-mucoidal bacterial colonies, and together with a positive regulator of cellulose biosynthesis, might involve in the complexity of the biofilm formation [60].

VpsR and FleQ are transcriptional regulators containing an AAA-type ATPase domain and a DNA-binding domain, and belong to Fis family of prokaryotic response regulators. FleQ was identified as a c-di-GMP responsive transcriptional regulator that controls the transcription of exopolysaccharide biosynthesis genes in *P. aeruginosa* (Fig. 7) [29]. However, FleQ also activates expression of the two component regulatory genes *fleSR* (Fig. 7) as well as genes that are necessary for the assembly of the flagellar export apparatus [29,61–64]. We demonstrated the presence of *fleQ*, *fleS* and *fleR* gene homologs in *A. hydrophila* SSU genome (unpublished data) in contrast to *A. hydrophila* AH-3 strain, where

no master regulatory genes encoding homologs of *P. aeruginosa* FleQ, FleS, and FleR were found [65]. We also identified FleN which is an anti-activator of FleQ in *P. aeruginosa* [29–31,38]. We believe that FleQ of *A. hydrophila* SSU is a significant player in biofilm formation and bacterial motility.

While searching for genes encoding c-di-GMP metabolic proteins in *A. hydrophila* SSU genome in addition to *aha0701h*, we identified the presence of *aha4220* and *aha1018* gene homologs. These genes encode DGC and PDE, respectively, but we could not identify, for instance, a homolog of *A. hydrophila* ATCC 7966 *aha0996* gene that encodes DGC (unpublished data). Thus, QS-dependent c-di-GMP regulatory network in *A. hydrophila* SSU includes the elements of cascade related to that in *Vibrio* and *Pseudomonas*, thus making this system unique.

To better understand the role of LuxS and AhyRI in QS-dependent c-di-GMP regulatory network in *A. hydrophila* SSU, we monitored transcript levels of the selected genes involved in biofilm and motility regulation and the effects on them by overproduction of GGDEF protein.

Interestingly, the fact that the *litR* gene was up-regulated in the $\Delta luxS$ mutant of *A. hydrophila* SSU was surprising in itself because it is totally distinct compared to the QS-dependent *hapR* regulation which exists in *Vibrio* [23] (Fig. 7). Together with the absence of major components of the QS-dependent regulating cascade, such as the LuxP and the genes encoded cascade involving sRNA, suggest a different mechanism of LuxS-dependent QS gene regulation in *V. cholerae* [48,58] and *A. hydrophila*.

Biofilm formation in *V. cholerae* is primarily controlled by two transcriptional activators VpsT and VpsR, which are required for the expression of *Vibrio* polysaccharide biosynthesis operon *vps* [58]. HapR represses *vpsT* expression both by reducing the cellular concentration of c-di-GMP via altering transcription of genes encoding GGDEF and EAL proteins and by directly binding to the *vpsT* promoter [13]. In addition, recently, Krasteva *et al.* [15] showed that the transcriptional regulator VpsT from *V. cholerae* directly senses c-di-GMP to inversely control biofilm and motility.

Our study showed that the $\Delta luxS$ mutant of *A. hydrophila* SSU formed denser biofilm and exhibited decreased motility, however, the *csgAB* (*vpsT*) transcript was down-regulated, while the *fleQ* (*vpsR*) was increased. Importantly, VpsR in *A. hydrophila* SSU was more closely related to FleQ in *P. aeruginosa* [29] thus making LitR-VpsT-FleQ system of *A. hydrophila* SSU unique.

Importantly, in contrast to the *luxS* mutant, the transcript levels for the *vpsT* and *vpsR* genes in the *ahyRI* mutant of *A. hydrophila* SSU were similar to that in the WT bacteria in spite of *litR* gene down-regulation, and no alteration in biofilm formation and motility was noted. However, we evidenced that *ahyRI* deletion sufficiently altered transcriptional response and phenotype changes when GGDEF protein was overproduced in the *ahyRI* mutant compared to that in the WT *A. hydrophila* SSU.

Notably, overproduction of GGDEF protein in WT *A. hydrophila* SSU resulted in increase of all selected genes compared to that in the WT bacteria (Fig. 5C). An increase in the *luxS* and *litR* transcripts could provide a decrease in c-di-GMP level that correlated with the common scheme of LuxS-dependent HapR c-di-GMP regulation in *Vibrio* [66] (Fig. 7). However, because GGDEF protein is overproduced, the c-di-GMP level continued to be very high that consequently could stimulate *csgAB* (*vpsT*) and *fleQ* (*vpsR*) expression levels as noted in Fig. 5C. The phenotypic alterations noted with the overproduction of GGDEF protein in the WT *A. hydrophila* SSU were not surprising, and corroborated with the effects

of high level of c-di-GMP on biofilm formation and motility in *A. veronii* [37] and *V. vulnificus* [36]. Interestingly, these alterations were similar to that found in the $\Delta luxS$ mutant, however, the transcriptional level of expression of the selected genes involved in the biofilm formation and motility regulation were distinct.

In contrast to the up-regulation of all selected genes which were revealed in the WT *A. hydrophila* SSU when GGDEF protein was overproduced, in the $\Delta ahyRI$ mutant, the overproduction of AHA0701h mostly resulted in adjustment of the selected gene expression levels to the level of these genes in the WT bacteria. The exceptions to that were *vpsT* (*csgAB*) and *fleN*, which were down-regulated, and *vpsR* (*fleQ*), which was up-regulated when compared to the same gene transcripts in the WT *A. hydrophila* SSU. These gene transcript level alterations were not essentially affecting motility and biofilm formation in the $\Delta ahyRI$ mutant. More likely, the deletion of the *ahyRI* genes abrogated the effect of GGDEF protein overproduction on gene expression in the $\Delta ahyRI$ mutant compared to that in the WT *A. hydrophila* SSU when GGDEF protein was overproduced.

In addition, we observed for the first time to our knowledge, that mutation in either the *luxS* gene or the *ahyRI* genes caused an alteration in gene transcripts of the remaining functional QS system of *A. hydrophila*. Interestingly, together with the *luxS* gene up-regulation, the expression levels of two additional copies of the *ahyR* gene were increased in the *ahyRI* mutant. Moreover, the overproduction of GGDEF protein brought down these transcripts to the level comparable to that in WT *A. hydrophila* SSU.

In conclusion, the transcript level of genes involved in AI-1 and AI-2 QS systems were co-regulated in *A. hydrophila* SSU. A balance of transcriptional level of gene expression between *luxS* and *ahyR* was regulated by modulation of c-di-GMP level. The *luxS* and *luxR*-type (*ahyR*) regulator of AI-1 mutually compensated each other at least at the transcriptional level. The effects of c-di-GMP modulation on a number of common genes involved in motility and biofilm formation regulation were dependent on the expression AI-1 and AI-2 systems.

4. MATERIALS AND METHODS

4.1. Bacterial strains and plasmids

The sources of *A. hydrophila* and *E. coli* strains, as well as the plasmids used in this study, are listed in Table 2. 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 50 µg/ml for bacterial growth and 300 µg/ml during transformation experiments. All of the antibiotics used were obtained from Sigma (St. Louis, MO). Restriction endonucleases and T4 DNA ligase were obtained from Promega (Madison, WI). The Advantages cDNA PCR Kit was purchased from Clontech (Palo Alto, CA). The digested plasmid DNA or DNA fragments from agarose gels were purified using a QIAprep 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::*ggdef* plasmid (Table 1) under the control of an arabinose-inducible pBAD promoter.

4.2. Primers and PCR assays

The primers used for various experiments are indicated in Table 1 and were synthesized by Sigma-Aldrich Biotechnology LP. PCR assays were performed with 100 ng of gDNA and 50 ng of plasmid DNA.

4.3. Cloning of the *aha0701h* gene into pBAD/Myc-HisB

The *aha0701h* gene was amplified with primers ggdeF1 and ggdefR1 from *A. hydrophila* SSU genomic DNA. The PCR product was digested with the indicated enzymes and cloned into the XhoI and XbaI sites of the conjugative expression vector pBAD/Myc-HisB. Correct clones were verified by restriction digestion and sequencing. Expression of the *aha0701h* gene was induced with arabinose.

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

Standard conditions for the isolation of total RNA from cells grown overnight in LB medium were used (Ambion, Austin, TX). RNA concentration was determined by optical density at 260 nm. Triple measurements were performed for each RNA sample and an average value was obtained as a final concentration. RT-PCR was performed 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.

The resultant cDNA was diluted eight times with Nuclease-free water and concentration was determined by optical density at 260 nm. The diluted cDNA was subsequently used in 50 µl of RT-PCR reaction. Sequences of primers used in RT-PCR are shown in Table 1. The 16S rRNA was used as the internal control gene to confirm that equal amounts of total RNA was used in each reaction [67,68]. As a negative control and a control to detect DNA contamination, water and DNase treated RNA were used in place of cDNA template, respectively. For RT-PCR analysis, 30–35 cycles of PCR reaction was used. Standard techniques were employed to prevent and detect *in vitro* contamination of the PCR reactions. Amplified products were identified by size, by using agarose-gel electrophoresis and ethidium-bromide staining. Specificity of the amplified products was demonstrated by DNA sequence analysis. The relative level of the cDNAs of RT-PCR was determined by densitometric analyses using AlphaEasyFC software (AlphaInnotech, San Leandro, CA) using 16S rRNA genes as references.

4.5. *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 10⁶ colony forming units (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 by light microscopy.

4.6. Motility assay

LB medium with 0.35% agar was used to characterize the motility phenotype of WT *A. hydrophila* SSU and its mutants strain. The overnight cultures grown in the presence of respective antibiotic were adjusted to the same optical density, and equal numbers of CFU (10⁶) were stabbed into 0.35% LB agar plates. Plates were incubated at 37°C overnight, and the motility was assayed by examining migration of bacteria through the agar from the center towards the periphery of the plate [69].

4.7. Crystal violet (CV) biofilm assay

The WT *A. hydrophila* SSU and its mutants 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 [70,71]. The biofilm formation results were normalized to 1×10^9 cfu to account for any differences in the growth rate of various bacterial strains used. The experiment was repeated independently three times.

4.8. 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 $\pm$ standard deviation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
A homology of *A. hydrophila* AHA0701 protein with its VCA0956 homolog found in *V. cholerae*.

Fig. 2. Measurement of biofilm mass formed on polystyrene plastic after crystal violet staining: **A** – WT *A. hydrophila* SSU with pBAD/Myc-HisB vector alone; **B** – WT *A. hydrophila* SSU with pBAD/Myc-His::aha0701h; **C** – $\Delta luxS$ mutant.

Fig. 3.

Light microscopy images of biofilm formation on Thermanox cover slips by WT *A. hydrophila* with uniform sparse cell attachment on the surface of plastic cover slip (**A**), and WT *A. hydrophila* with overproduction of GGDEF domain protein (AHA0701h), forming much thicker and denser biofilm (**B**), after 24 h of cultivation.

Fig. 4.

Effect of the overproduction of AHA0701h on the motility of *A. hydrophila* inoculated onto the surface of 0.35% LB agar plates containing 0.2 % arabinose: **A 1** – WT *A. hydrophila* SSU; **A 2** – WT *A. hydrophila* SSU with pBAD/Myc-HisB vector alone; **A 3** – WT *A. hydrophila* SSU with pBAD/Myc-His::aha0701h; **B 1** – Δ ahyRI mutant with pBAD/Myc-HisB vector alone; **B 2** – Δ ahyRI mutant with pBAD/Myc-His::aha0701h; **B 3** – Δ luxS mutant with pBAD/Myc-HisB vector alone; **C** - the bar graph shows distances in millimeters by which these strains migrated from the point of inoculation in soft agar plates. Data from three independent experiments were plotted with standard deviations. Statistical significance

by Student's *t* test ($p \leq 0.05$) was found for A3 and B3 compared to other genetic background strains.

Fig. 5. Schematic representation of fold-changes in the expression of selected genes in various genetic backgrounds of *A. hydrophila* SSU: the *luxS* mutant (**A**); the *ahyRI* mutant (**B**); WT with AHA0701h over-production (**C**); the *ahyRI* mutant with AHA0701h over-production (**D**). The data used to generate fold changes (arithmetic mean $\pm$ SD) with statistical analysis is shown in Table I; Supplemental data.

Fig. 6. Reverse-transcription PCR analysis of the *litR* (A) and *vpsT* (B) gene expression in different genetic backgrounds of *A. hydrophila* SSU.

Fig. 7.

Integrated schematic based on the published information illustrating QS-dependent regulatory network in *Vibrio*, *Pseudomonas*, and *A. hydrophila*. LuxR/HapR/LitR represents a major regulator of QS target genes [23,50–53] involved in the AI-2 phosphorylation cascade [19,45–47,72]. HapR negatively affects biofilm formation and exopolysaccharide biosynthesis [19] by lowering the intracellular level of c-di-GMP [13] and repressing the positive regulator VpsT [73]. VpsT from *V. cholerae*, a master regulator for biofilm formation [15], directly senses c-di-GMP to inversely control extracellular matrix production and motility. VpsR and FleQ are the transcriptional regulators containing an AAA-type ATP-ase domain and a DNA-binding domain [29]. The orthologous systems CsrA LetA/LetS exist in many gram-negative bacteria, such as *S. enterica* (BarA/SirA), *Erwinia carotovora* (ExpA/ExpS), *V. cholerae* (VarA/VarS), *Pseudomonas* spp. (GacA/GacS), *Photobacterium luminescens* (UvrY/BarA) and *E. coli* (UvrY/BarA) [74]. The central regulator CsrA is antagonized by sRNA and CsrC, whose expression is positively regulated by the BarA-UvrY two-component system.[48,75]. An unconventional GG-DEF-EAL protein CsrD regulates sRNAs at the level of RNA stability [76–78]. The global regulator CsrA contributes to the control of c-di-GMP levels by modulating selected panels of GGDEF and EAL genes in response to internal signals [79]. Also CsrA, a post-transcriptional regulator, can activate LuxO, possibly through an additional regulatory factor (denoted X) [80].

Table 1

Oligonucleotides used and PCR products

Primer	Oligonucleotide sequence	Use	PCR product (bp)
ggdefF1 ggdefR1	CCGCTCGAGATGATCAAGTATCAAATCCCCA GCTCTAGATCAGGATGGCAGCGGCATC	GGDEF domain encoded gene of <i>A. hydrophila</i> cloned into pBAD/ <i>Myc</i> -HisB <i>ara</i> Km ^r Ap ^r	966
litRF1 litRR1	GAACCACGCGCAGAAATAAG GGGCCGTCACGTACTTGA	<i>litR</i>	595
vpsT (csgAB)F1 vpsT (csgAB)R1	TCAGAGATACTCCTTGGCCCA CGCTTCATGATCACCCCAT	<i>vpsT</i> (<i>csgAB</i>)	645
vpsR(fleQ)F1 vpsR(fleQ)R1	TGCTTCCTTGCTCATCCCGTA TGTTGATTGTTGACGCCGAT	<i>vpsR</i> (<i>fleQ</i>)	1403
fleN F1 fleN R1	TGGTCTGCGCAAAATGCGT TTATTACGGGAACCTTCCTG	<i>fleN</i>	856
luxSF2 luxSR2	ACCTCCAAGTGGGATGCGTAT CGGGCCATCGAAAAATGT	<i>luxS</i>	971
ahyRF1 ahyRR1	TATTGCATCAGCTTGGGGAA TGAAACAAGACCAACTGCTTG	<i>ahyR</i>	781
ahyIF1 ahyIR1	AGGAAAATTAAGAAGACACCC TTATTCTGTGACCAGTTCGC	<i>ahyI</i>	610
ahyR2F2 ahyR2R2	TCCTCATACCCCGGAAATGA TCACTGCTCCTCATCCACAT	<i>ahyR2</i>	790
ahyR2F1 ahyR2R1	ATGTTCAACGATGCGATCTG TTATGACAGCCGAGGTTGGT	<i>ahyR3</i>	612
aha4220F1 aha4220R1	CAACCTTACCCATCAAGGCTA TCTATACCCACGCCTCATTCA	<i>aha4220</i> homolog	1101
aha1018F1 aha1018R1	AGAGAGGGGGGAGGGCAT GCATTTCAAGAAGAGTACCCA	<i>aha1018</i> homolog	720
aha0145F aha0145R	AGAGTTTGATCATGGCTCAG GTATTACCGCGCTGCTG	<i>aha0145</i> homolog	531

Table 2

Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>A. hydrophila</i> SSU		CDC _a , Atlanta
SSU-Rif ^r	Rifampin resistance (Rif ^r)	Laboratory stock
SSU Δ <i>luxS</i>	<i>luxS</i> mutant of <i>A. hydrophila</i> SSU-R; Rif ^r Km ^r	This study
SSU Δ <i>luxS</i> /pBR322:: <i>luxS</i>	<i>luxS</i> mutant complemented Rif ^r Km ^r Ap ^r	This study
Δ <i>ahyRI</i>	Δ <i>ahyRI</i> deletion mutant of <i>A. hydrophila</i> SSU-R; Rif ^r Sm ^r Sp ^r	This study
Δ <i>ahyRI</i> /pBR322:: <i>ahyRI</i>	Δ <i>ahyRI</i> mutant complemented Rif ^r Sm ^r Sp ^r Ap ^r	
<i>E. coli</i>	TOP10	Invitrogen
Plasmids		
pBAD/Myc-HisB	vector, <i>ara</i> BAD promoter Ap ^r	Laboratory stock
pBAD:: <i>ggdef</i>	GGDEF domain encoded gene of <i>A. hydrophila</i> cloned into pBAD/Myc-HisB <i>ara</i> Km ^r Ap ^r	Laboratory stock