Quorum Sensing Regulation of the Two hcp Alleles in Vibrio cholerae O1 Strains

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Abstract

Background: The type VI secretion system (T6SS) has emerged as a protein secretion system important to several Gram-negative bacterial species. One of the common components of the system is Hcp, initially described as a hemolysin co-regulated protein in a serotype O17 strain of Vibrio cholerae. Homologs to V. cholerae hcp genes have been found in all characterized Vibrio VI secretion systems and they are present also in the serotype O1 strains of V. cholerae that are the cause of cholera diseases but seemed to have non-functional T6SS.

Methodology/Principal Findings: The serotype O1 V. cholerae strain A1552 was shown to express detectable levels of Hcp as determined by immunoblot analyses using polyclonal anti-Hcp antiserum. We found that the expression of Hcp was growth phase dependent. The levels of Hcp in quorum sensing deficient mutants of V. cholerae were compared with the levels in wild type V. cholerae O1 strain A1552. The expression of Hcp was positively and negatively regulated by the quorum sensing regulators HapR and LuxO, respectively. In addition, we observed that expression of Hcp was dependent on the cAMP-CRP global transcriptional regulatory complex and required the RpoN sigma factor.

Conclusion/Significance: Our results show that serotype O1 strains of V. cholerae do express Hcp which is regarded as one of the important T6SS components and is one of the secreted substrates in non-O1 non-O139 V. cholerae isolates. We found that expression of Hcp was strictly regulated by the quorum sensing system in the V. cholerae O1 strain. In addition, the expression of Hcp required the alternative sigma factor RpoN and the cAMP-CRP global regulatory complex. Interestingly, the environmental isolates of V. cholerae O1 strains that showed higher levels of the HapR quorum sensing regulator in comparison with our laboratory standard serotype O1 strain A1552 where also expressing higher levels of Hcp.

Introduction

Vibrio cholerae, primarily known as the causal organism of the diarrheal disease cholera, is found as a free-living environmental organism within aquatic natural reservoirs and there are many different types distinguished by serological classification. Cholera is caused by the well studied O1 and O139 serotypes but recent discoveries include the type VI secretion system (T6SS) that is involved in virulence of V. cholerae strains but seemed to have non-functional T6SS. The type VI secretion system (T6SS) may utilize an unconventional secretion system with characteristics of both types III and IV secretion systems and indicated an involvement of this locus in the secretion of a periplasmic protein to the extracellular environment. The work defining the imp locus in R. leguminosarum and pathogenesis of this bacteria has been concomitant with work on V. cholerae classical O1 strain O395, which started with the characterization of an icmF homologue [11]. The expression of icmF in V. cholerae strain O395 was induced inside the host intestinal environment and the expression was correlated with motility of the organism. The icmF insertion mutant showed increased adherence to intestinal epithelial cells and increased conjugation frequency as a recipient [12].

It was also suggested that the icmF gene may be part of a secretion system that is capable of transferring a substrate across the outer membrane or might take part in the transport of effector molecules. The presence of genes for a phosphoprotein phosphatase and a Ser/Thr protein kinase adjacent to some of the icmF homologs also suggested the possible involvement in transport of effector molecules in signaling pathway [13]. The V. cholerae O1 icmF gene was identified as being induced in vitro in a rabbit model.
of infection [12,14]. Further studies with the O37 serogroup *V. cholerae* revealed the role of the *V. cholerae* IAHP cluster as the new T6SS [15]. The *V. cholerae* strain V52, in contrast to the O1 strain, is capable of amoeba killing when plated with *Dictyostelium discoideum* [15]. Transposon mutagenesis of strain V52 identified a series of mutants that were attenuated for their virulence on *Dictyostelium*. The transposon mapping revealed a cluster of genes called *vas* for (virulence-associated secretion). Several Vas-secreted putative effector proteins lacking a hydrophobic N-terminal signal were identified, and a double mutant lacking the Vas-secreted identical proteins Hcp (haemolysin co-regulated protein)-1 and Hcp-2 was found to be avirulent towards *D. discoideum* [15].

The gene clusterencoded T6SS mediates the extracellular secretion of four distinct proteins (Hcp, VgrG-1, VgrG-2, and VgrG-3). Mutations in the *hcp* or the *vgrG* genes in the non-O1 non-O139 *V. cholerae* strain V52 attenuate cytotoxicity and block secretion of the other T6SS protein substrates suggesting that both of the Hcp and the VgrG proteins are T6SS machine components or involved in targeting the T6SS dependent substrates to be secreted outside the bacterial cells [2]. The functional significance of these genes was initially characterized in the non-O1 non-O139 *V. cholerae*, where the ability to perform secretion of Hcp and VgrG proteins into supernatant fluids was shown to be important as a defense against predation by *D. discoideum* [15]. Hcp assembles into hexameric ring structures that may form a regulated pore for secretion of putative effectors out of the bacterium [16]. VgrG proteins, which have homology to trimeric phage T4 tail spike proteins, are suggested to assemble into a trimmer that associates with Hcp. It is proposed that VgrG proteins thus extend away from Hcp of *V. cholerae* in Serotype O1 and O139 strains (data not shown). The Hcp was excised with HindIII restriction enzyme sites. The RpoN protein was purified using the IMPACT T7 system from New England Biolabs described by the manufacturer. Using the purified RpoN protein, polyclonal rabbit antiserum was produced by AgriSera AB, Sweden.

**Materials and Methods**

**Bacterial strains, culture conditions and plasmids**

The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were grown overnight at 37°C with shaking in Luria-Bertani (LB) broth supplemented, as appropriate, with kanamycin (50 μg/ml), or carbenicillin (100 μg/ml). The Δhup1, Δhup2, Δhup1Δhup2, ΔcqsA, Δcya, ΔluxA, ΔluxO, ΔluxK, ΔluxN, and Δvps3 mutants were constructed by making deletions of the entire reading frame using procedures that have been described previously [19,20]. Oligonucleotide primers used are listed in Table 2.

**SDS-PAGE and Immunoblot analyses**

To determine the levels of protein expression, the bacterial strains were grown in LB medium and the samples were taken at different growth phases (i.e. OD600 nm 1.0, 2.0, 3.0, and overnight) and centrifuged at 14,000 g×2 min. The bacterial cell pellets were suspended in 20 mM Tris-HCl pH 8.0 buffer and used for SDS-PAGE and immunoblot analyses. The protein samples were separated by sodium dodecyl sulfate-13% polyacrylamide gel electrophoresis (SDS-PAGE) [21]. Western blot analyses were performed as described [22] using anti-Hcp polyclonal antiserum (this study), anti-CRP polyclonal antiserum [23], anti-RpoN antiserum (this study) and anti-HapR antiserum [24]. The immunoblot detection was done by using the ECL+ chemiluminescence system (GE Healthcare, United Kingdom).

**Anti-Hcp polyclonal antiserum preparation**

Anti-Hcp polyclonal antibodies were raised in rabbits using the following method. Briefly, *V. cholerae* non-O1 non-O139 strain V5 produced a large amount of Hcp protein that was efficiently secreted into the culture supernatant. The secreted 28 kDa protein was confirmed by the Mass Spectrometric analysis as the Hcp of *V. cholerae* strains (data not shown). The Hcp was excised from the SDS gel and eluted from the gel. The eluted Hcp protein was used for immunization of the rabbits.

**Anti-RpoN polyclonal antiserum preparation**

The rpoN gene was amplified by PCR and cloned into pTYB1 using NdeI and SapI restriction enzyme sites. The RpoN protein was purified using the IMPACT T7 system from New England Biolabs described by the manufacturer. Using the purified RpoN protein, polyclonal rabbit antiserum was produced by AgriSera AB, Sweden.

**In vivo protein stability experiment**

The stability of the Hcp proteins was examined using a procedure as described earlier [25] with some modifications. Protein stability was monitored after the protein synthesis had been inhibited by the addition of 25 μg/ml chloramphenicol to bacterial cultures grown to OD600nm at 2.0 in LB medium at 37°C. Samples to be analyzed by western blotting were removed at indicated time points: 0; 15; 30; 60 min; 3 and 6 hrs after addition of chloramphenicol.

**Results**

**Growth phase dependent levels of intracellular Hcp in *V. cholerae* O1 strain A1552**

Earlier it was reported that *V. cholerae* O1 and O139 strains also carry *hcp* and *vas* genes in their genomes but e.g. the O1 strain N16961 was unable to secrete Hcp into the culture supernatant indicating that the T6SS was not expressed or was non-functional in the O1 serotype [15]. As an initial study we tested the effect of bacterial growth phase on the expression of Hcp in *V. cholerae* O1 strain A1552. The bacteria were grown in a standard LB medium and the level of Hcp was measured at different growth phases by...
immunoblot analyses. We detected expression of Hcp at OD<sub>600</sub> 1.0 and the highest expression was observed at OD<sub>600</sub> 2.0 (Fig. 1). Interestingly, the Hcp band was not detectable in whole cell samples of overnight grown bacteria. Similar to what was reported for the O1 strain N16961 [15] there was no detectable secreted Hcp in culture supernatants from <i>V. cholerae</i> O1 strain A1552 at any of the different growth phases or in the late stationary phase after overnight incubation (data not shown).

The apparent lack of Hcp in bacteria harvested from the stationary phase after overnight incubation might be due to an intracellular proteolytic degradation of the Hcp in late stationary phase and/or that the expression was totally shut off in the late

Table 1. Bacterial strains and plasmids.

| Strains/Plasmids | Relevant Genotype/Phenotype | Reference/Source |
|------------------|-----------------------------|-----------------|
| **Bacteria**     |                             |                 |
| *E. coli* DH5α   | F<sup>−</sup>,araD139ΔZAM15, Δ[lacZYA-argF]U169, deoR, recA1, endA1, hsdR17(k<sup>R</sup>, mk<sup>+</sup>), phoA, supE44, λ<sup>−</sup>, thi-1, gyrA96, relA1 | [34] |
| *E. coli* SM10::pir | thi thr leu tonA lacY supE recA R6K<sup>R</sup> RP4-2 Tc::Mu Km λpir | [35] |
| *V. cholerae* A1552 | O1 El Tor, Inaba, Rif<sup>+</sup> | [36] |
| *V. cholerae* A1552::hcp1 | .hcp1 derivative of A1552 | This study |
| *V. cholerae* A1552::hcp2 | .hcp2 derivative of A1552 | This study |
| *V. cholerae* A1552::hcp1,2 | .hcp1,2 derivative of A1552 | This study |
| *V. cholerae* A1552::hapR | .hapR derivative of A1552 | This study |
| *V. cholerae* A1552::rpoS | .rpoS derivative of A1552 | This study |
| *V. cholerae* A1552::rpoN | .rpoN derivative of A1552 | This study |
| *V. cholerae* A1552::lcrp | .lcrp derivative of A1552 | This study |
| *V. cholerae* 1552::lcrp/pHA7 | complementation of A1552::lcrp | This study |
| *V. cholerae* 1552::lcrp/pBR322 | A1552::lcrp with vector control | This study |
| *V. cholerae* A1552::lcrp | .lcrp derivative of A1552 | This study |
| *V. cholerae* A1552::luxO | .luxO derivative of A1552 | This study |
| *V. cholerae* A1552::luxS | .luxS derivative of A1552 | This study |
| *V. cholerae* A1552::lcrpA | .lcrpA derivative of A1552 | This study |
| *V. cholerae* A1552::hfpA | .hfpA derivative of A1552 | [37] |
| *V. cholerae* V5/04 | non-O1 non-O139 clinical isolate (2004) | Swedish Institute of Infectious Diseases |
| *V. cholerae* C6706 | O1 El Tor, Inaba, Str<sup>R</sup> | [38] |
| *V. cholerae* AJ3 | O1 Environmental isolate (1981) | Ryukyu University |
| *V. cholerae* AJ5 | O1 Environmental isolate (1981) | Ryukyu University |
| *V. cholerae* CB4 | O1 Classical Inaba (1982) | Ryukyu University |
| *V. cholerae* 86B1 | O1 Classical Ogawa (1986) | Ryukyu University |
| **Plasmids**     |                             |                 |
| pBR322           | Cb<sup>R</sup> cloning vector plasmid | [39] |
| pH7              | pBR322-based crp expression plasmid | [40] |
| pGEM-TEasy       | Cb<sup>R</sup> TA-cloning vector plasmid | Promega<sup>®</sup> |
| pCVD442          | Cb<sup>R</sup> positive selection suicide vector plasmid | [41] |
| pKAS32           | Cb<sup>R</sup> positive selection suicide vector plasmid | [42] |
| pTY18            | Cb<sup>R</sup>, expression vector plasmid | New England Biolabs<sup>®</sup> |
| p.Thcp1          | pCVD442-based suicide plasmid for generating .Tcp1 | This study |
| p.Thcp2          | pCVD442-based suicide plasmid for generating .Tcp2 | This study |
| p.TrpoS          | pCVD442-based suicide plasmid for generating .TrpoS | [43] |
| p.TrpoN          | pCVD442-based suicide plasmid for generating .TrpoN | This study |
| p.ThapR          | pKAS32-based suicide plasmid for generating .ThapR | [19] |
| p.Tscp            | pKAS32-based suicide plasmid for generating .Tscp | This study |
| p.Tcya           | pKAS32-based suicide plasmid for generating .Tcya | This study |
| p.TluxO          | pKAS32-based suicide plasmid for generating .TluxO | [20] |
| p.TluxS          | pKAS32-based suicide plasmid for generating .TluxS | [20] |
| p.TcqsA          | pKAS32-based suicide plasmid for generating .TcqsA | [20] |

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stationary phase. Nothing was known about the possible degradation or turn over of Hcp in the bacterium. We therefore performed an experiment to monitor the stability of Hcp in V. cholerae wild type strain A1552 after inhibiting the total protein synthesis by addition of chloramphenicol into the culture medium as described in Materials and Methods (Fig. 2A&B). The Hcp level appeared stable during at least one hour after the total protein synthesis was inhibited and at least half of the protein remained after three hours suggesting that there was not much degradation or turn over of Hcp at that growth stage (Fig. 2B). In the control culture the level of Hcp was almost the same throughout the late exponential phase of bacterial growth. However, the Hcp level was reduced about 10-fold when the bacteria entered into the early stationary phase. In the late stationary phase (6 hr samples) the Hcp level was totally abolished in the control culture. The results suggested that the absence of Hcp in the bacteria of an overnight

Table 2. Primers used in this study.

| Primer  | Sequence | Source   |
|---------|----------|----------|
| HCP1-A  | 5’CGCTCTAGAGGGTGCTGCTCGATAT3’ | This study |
| HCP1-B  | 5’CCCATCCACATAAACACTACAGCCGTAAGCTCGAAGATA3’ | This study |
| HCP1-C  | 5’TGTTAGTTATAGTGATGGGCTGTAATTTACGCTGCAGAA3’ | This study |
| HCP1-D  | 5’GCCTCTAGACCCATCTCCTGCAGCAAGATA3’ | This study |
| HCP2-A  | 5’CGCTCTAGACCCATCTCCTGCAGCAAGATA3’ | This study |
| HCP2-B  | 5’CCCATCCACATAAACACTACAGCCGTAAGCTCGAAGATA3’ | This study |
| HCP2-C  | 5’TGTTAGTTATAGTGATGGGCTGTAATTTACGCTGCAGAA3’ | This study |
| HCP2-D  | 5’GCCTCTAGACCCATCTCCTGCAGCAAGATA3’ | This study |
| RpoN-A  | 5’CGCTCTAGACCTCGGTTAGAAGACACATC3’ | This study |
| RpoN-B  | 5’CCCATCCACATAAACACTACAGCCGTAAGCTCGAAGATA3’ | This study |
| RpoN-C  | 5’TGTTAGTTATAGTGATGGGCTGTAATTTACGCTGCAGAA3’ | This study |
| RpoN-D  | 5’CGCTCTAGACCCATCTCCTGCAGCAAGATA3’ | This study |
| CRP-A   | 5’CCCATCCACATAAACACTACAGCCGTAAGCTCGAAGATA3’ | This study |
| CRP-B   | 5’CGCTCTAGACCCATCTCCTGCAGCAAGATA3’ | This study |
| CRP-C   | 5’TGTTAGTTATAGTGATGGGCTGTAATTTACGCTGCAGAA3’ | This study |
| CRP-D   | 5’CCCATCCACATAAACACTACAGCCGTAAGCTCGAAGATA3’ | This study |
| Cya-A   | 5’TGTTAGTTATAGTGATGGGCTGTAATTTACGCTGCAGAA3’ | This study |
| Cya-B   | 5’CCCATCCACATAAACACTACAGCCGTAAGCTCGAAGATA3’ | This study |
| Cya-C   | 5’TGTTAGTTATAGTGATGGGCTGTAATTTACGCTGCAGAA3’ | This study |
| Cya-D   | 5’AATAATCGCCACCGATTAGGA3’ | This study |
| Cya-E   | 5’CCCATCCACATAAACACTACAGCCGTAAGCTCGAAGATA3’ | This study |
| Cya-F   | 5’TGTTAGTTATAGTGATGGGCTGTAATTTACGCTGCAGAA3’ | This study |

Figure 1. Hcp levels in V. cholerae O1 wild type strain A1552 at different growth phases. Bacteria were grown as described in Materials and Methods and the whole cell lysate samples were taken at different optical density. Immunoblot analysis was performed with an anti-Hcp antiserum that also contained antibodies recognizing the OmpA major outer membrane protein as confirmed by analysis of ompA mutant V. cholerae (data not shown).

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culture would be due to both loss of expression and to an increased turnover by proteolytic degradation presumably by some stationary phase expressed protease(s).

Figure 2. Analysis of Hcp stability in *V. cholerae* O1 wild type strain A1552. (A) The growth curve and time of sampling for Hcp analyses of *V. cholerae* wild type strain A1552 with and without chloramphenicol (Cm) treatment. The bacterial cells were grown to OD 2.0 and 25 μg/ml Cm was added. Arrows indicate the time points of sampling for immunoblot analysis after the addition of Cm. (B) Immunoblot analysis of the stability of Hcp in *V. cholerae* wild type strain A1552. The samples were taken at different time points after the addition of Cm (for the test sample). For the control experiment, the samples were taken at different time points during normal growth of bacteria. The vertical arrows show the time points when the samples were taken.

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Figure 3. The effect of ΔhapR and ΔluxO mutations on Hcp levels in *V. cholerae* strain A1552. The whole cell lysate samples were taken at OD 1.0 and OD 2.0 and immunoblot analysis was performed using anti-Hcp polyclonal antiserum.

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The *V. cholerae* O1 strain A1552 has two *hcp* genes separately located in the two *V. cholerae* chromosomes. Mutant derivatives with deletion of either of the two loci still expressed Hcp in a
growth phase dependent manner from the intact locus as confirmed by immunoblot analysis (data not shown). The complete disappearance of Hcp in the stationary phase as judged by the results from the immunoblot analyses (Fig. 1 and 2) showed that there was loss of expression from both hcp loci and it appeared that expression was quite coordinated.

Quorum sensing regulators HapR and LuxO influence the expression of Hcp in V. cholerae O1 strain A1552

In order to analyze further how the expression of Hcp might be regulated in the growth phase dependent manner, we compared the level of Hcp in wild type V. cholerae strain A1552 with those of different global regulator mutant strains. We first tested the levels of Hcp expression in quorum sensing (QS) regulatory mutants since the QS is the main growth phase mediated regulatory system in V. cholerae. As shown in Fig. 3, the expression of Hcp was strongly reduced in the hapR mutant. Furthermore, the level of Hcp was clearly increased at an earlier growth phase (OD 1.0) in the luxO mutant V. cholerae derivative when compared to the wild type strain A1552. Our findings suggested that the expression of Hcp in the V. cholerae O1 strain was positively regulated by the quorum sensing regulator HapR and negatively regulated by the regulator LuxO.

The role of up-stream quorum sensing regulators in expression of the Hcp

We next tested the levels of Hcp in autoinducer synthesizer mutants such as ΔcqsA and ΔluxS and in a mutant defective in one of the master regulatory components of the quorum sensing system, the hff gene. As shown in Fig. 4, in the Δhff mutant the expression of Hcp was increased at the earlier growth phase to the same extent as in the case of the ΔluxO mutant where the level of HapR also was expected to be up-regulated. In contrast, the level of Hcp was reduced in the ΔcqsA and ΔluxS mutants where the level of the hofR expression was expected to be reduced. The results were all consistent with our suggestion that expression of a functional hapR gene was essential for the expression of the hcp genes in the V. cholerae O1 strain.

As it was reported that the carbon catabolite repressor, CRP (cAMP-receptor protein) is required for the biosynthesis of cholera autoinducer 1 (CAI-1) and affects the expression of multiple HapR-regulated genes [26], we analyzed whether the expression of the Hcp would also be regulated by CRP and cAMP. We compared the levels of the Hcp expression in wild type V. cholerae strain A1552 and its crp mutant by immunoblot analyses. The Hcp protein was not detectable in the Δcrp mutant and Hcp expression was fully complemented when we tested the strain containing a crp+ allele in trans (Fig. 5). As the adenylate cyclase enzyme encoded by the cya gene catalyzes formation of cAMP, which is indispensable for the function of CRP, we also constructed a cya deficient mutant and analyzed the level of Hcp. As expected, the expression of Hcp was abolished in the Δcya mutant (Fig. 5). These results indicate that in addition to the quorum-sensing system regulation, the metabolic regulators may also affect the expression level of Hcp.

Furthermore, a quorum sensing and HapR-mediated regulation of Hcp was also suggested by results obtained with other V. cholerae

Figure 4. Hcp levels in different quorum sensing regulatory system of V. cholerae strain A1552. The samples were taken at OD 1.0 and OD 2.0 from wild type V. cholerae O1 strain A1552 and its quorum sensing regulator mutants and immunoblot analyses were done using anti-Hcp antiserum. doi:10.1371/journal.pone.0006734.g004

Figure 5. Hcp levels in Δcrp and Δcya mutants of V. cholerae strain 1552. Immunoblot analysis of Hcp levels in whole cell lysates of V. cholerae O1 wild type strain A1552, Δcrp, Δcya, Δcrp/pcrp, and Δcrp/vector control strains. The whole cell lysates were taken at OD 1.0 and OD 2.0 and immunoblot was done using anti-Hcp antiserum. doi:10.1371/journal.pone.0006734.g005
O1 strains. As shown in Fig. 6A and B, the expression of Hcp was correlating to the HapR status of the different V. cholerae O1 isolates (A1552 & C6706; O1 El Tor, AJ3 & AJ5; O1 environmental, CB4 & 86B1; O1 Classical). Interestingly, the AJ3 and AJ5 strains, the environmental isolates of serotype O1 V. cholerae strains, showed even higher levels of Hcp expression than our standard strain A1552.

RpoN (σ54) is essential for the expression of Hcp in the V. cholerae O1 strain A1552

Next we considered whether Hcp expression might also be regulated by common alternative sigma factors of Gram-negative bacteria. The most likely positions of the hcp1 and hcp2 promoters in strain A1552, as determined by sequence analysis, included the (-12/-24) σ54 consensus sequences in both cases (Fig. 7A). On the basis of the sequence data analysis, we performed genetic experiments to directly test the prediction that the two hcp promoters would be σ54 dependent. We constructed in-frame deletion mutations in the rpoN and rpoS alternative sigma factor...
genes of *V. cholerae* O1 strain A1551 and monitored the expression of Hcp in these mutants by immunoblot analyses. As shown in Fig. 7B, the expression of Hcp was completely abolished in the *rpoN* mutant whereas there was no significant change in the *rpoS* mutant (Fig. 7B; panel a). The results indicated that the RpoN (σ^34^) sigma factor would be essential for the expression of Hcp from either of the two loci in strain A1552. Our finding are also giving support to the previous prediction of a potential, but unconfirmed, σ^34^ binding sequence in the promoter regions of the *hcp* genes in *V. cholerae* non-O1 strains [17].

We also investigated the expression levels of the CRP, HapR and RpoN proteins in the Δ*apoV* and Δ*apoS* derivatives by immunoblot analyses using the same samples that were used for the detection of Hcp (Fig. 7). The HapR levels were increased in the Δ*apoV* mutant at both OD 1.0 and 2.0 (Fig. 7C), in agreement with the previous reports that the *hapR* gene expression is negatively regulated by the RpoN sigma factor through four small RNAs, Qrrs, and Hfq in the quorum-sensing regulatory pathway of *V. cholerae* [27,28]. Furthermore, this result supports our conclusion that RpoN would be directly required for *hcp* gene expression since in the Δ*apoV* mutant the expression of Hcp was totally abolished despite the fact that in this mutant the level of HapR was increased.

**Discussion**

In this study we obtained evidence that there was expression of the T6SS substrate Hcp in *V. cholerae* O1 strain A1552 and we found that expression was strictly controlled by the quorum sensing regulatory system, the cAMP-CRP global metabolic regulator complex, and the alternative sigma factor RpoN as illustrated in Fig. 8. We monitored the expression of Hcp at different growth phases and observed that it was growth phase dependent and the maximum expression level was observed at OD 2.0. The Hcp was not detectable after the prolonged incubation in over-night growth cultures. However, as was pointed out earlier, the O1 serotype *V. cholerae* did not appear able to secrete Hcp and it was kept intracellular in the bacteria. It remains to be clarified when and how the complete T6SS determinant might be induced and functioning for protein secretion in the *V. cholerae* O1 strains. Our findings that the two *hcp* loci are expressed in the serotype O1 strains in a highly coordinated and regulated fashion suggest that these genes are functional and that Hcp may play a role in the bacterial growth and maintenance.

Bacterial pathogens use various protein secretion systems to deliver virulence effectors into hosts to cause diseases. The type III secretion system islets [29] and the recently identified type VI secretion system [15] are also virulence factors that are present in clinical and environmental isolates of different *V. cholerae* serotypes. Most secreted products of such gene clusters are associated with toxicity for eukaryotic cells and therefore may play a role in human disease or other pathobiological interactions with host or predatory environmental organisms. Protein secretion systems are generally precisely regulated by various global regulators to respond to various environmental changes or stresses. Regulation of the T6SS in *P. aeruginosa* is complex; the system is stringently regulated post-translationally by the Gac/Rsm pathway and post-translationally by threonine phosphorylation [3,16,30]. There is also some precedence for quorum sensing control of T6SS in bacteria with an active secretion system. The VgrG ortholog ECA3427 is secreted by *Pectobacterium atrosepticum* [31] and expression of five VgrG orthologs and various other T6SS components was recently shown to be regulated by quorum sensing in plant host tissues or after exposure to host extracts [31,32,33]. The expression level of four *P. atrosepticum* hcp homologs was clearly upregulated when plant extract was added to the medium and suggested that the expression of Hcp was under the influence of environmental signals [31]. The regulatory features of T6SS suggest that an optimal timing of T6SS gene expression is necessary to ensure correct function. Our characterization of the Hcp expression in serotype O1 *V. cholerae* should aid and prompt further studies of how Hcp in these human pathogenic strains may play some role in the bacterial life style. In particular it will be of interest to find out if there are conditions where Hcp would be actively secreted by the T6SS of the serotype O1 *V. cholerae* strains.

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**Author Contributions**

Conceived and designed the experiments: TI SNW. Performed the experiments: TI PKR BL. Analyzed the data: TI PKR BL DLM SNW. Contributed reagents/materials/analysis tools: DLM SNW. Wrote the manuscript. This work was supported by grants from the Swedish Research Council, Carl Tryggers Stiftelse, Clinical Hematology Oncology Study Group (CHOTSG) Scholarship (Japan), the Swedish Foundation for International Cooperation in Research and Higher Education (STINT), the Faculty of Medicine, Umeå University and was performed within the Umeå Centre for Microbial Research (UCMR) Linnaeus Program.

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