H-NS Represses Biofilm Formation and c-di-GMP Synthesis in Vibrio parahaemolyticus

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Abstract

Objective This study aimed to investigate the regulation of histone-like nucleoid structuring protein (H-NS) on biofilm formation and cyclic diguanylate (c-di-GMP) synthesis in Vibrio parahaemolyticus RIMD2210633.

Methods Regulatory mechanisms were analyzed by the combined utilization of crystal violet staining, quantification of c-di-GMP, quantitative real-time polymerase chain reaction, LacZ fusion, and electrophoretic-mobility shift assay.

Results The deletion of hns enhanced the biofilm formation and intracellular c-di-GMP levels in V. parahaemolyticus RIMD2210633. H-NS can bind the upstream promoter–proximal DNA regions of scrA, scrG, VP0117, VPA0198, VPA1176, VP0699, and VP2979 to repress their transcription. These genes encode a group of proteins with GGDEF and/or EAL domains associated with c-di-GMP metabolism.

Conclusion One of the mechanisms by which H-NS represses the biofilm formation by V. parahaemolyticus RIMD2210633 may be via repression of the production of intracellular c-di-GMP.

Key words: Vibrio parahaemolyticus; Biofilm; H-NS; C-di-GMP

INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium that primarily causes seafood-associated gastrointestinal illness in humans1,2. V. parahaemolyticus produces multiple virulence determinants, such as two type-III secretion systems (T3SSs) and thermostable direct hemolysin (TDH) or TDH-related hemolysin, and forms robust biofilms on surfaces that support bacteria to survive in adverse conditions1,2. V. parahaemolyticus employs various specific structures, such as flagella, type-IV pili, and exopolysaccharide (EPS), to form mature biofilms2. EPS is the main component of the biofilm matrix3. In V. parahaemolyticus, the cpsA-J operon is responsible for the synthesis of EPS3. The deletion of cpsA-J led to smooth colonies on plates, whereas the wild-type (WT) strain formed wrinkled colonies3. Previous studies showed that the
variations between smooth and wrinkled colonies are strictly regulated by intracellular cyclic diguanylate (c-di-GMP) levels, CpsS-CpsR-CpsQ cascade, quorum sensing (QS), and histone-like nucleoid structuring protein (H-NS) in V. parahaemolyticus\textsuperscript{[4-10]}.

C-di-GMP is a ubiquitous secondary messenger involved in regulating multiple behaviors of bacteria, including biofilm formation, motility, and virulence\textsuperscript{[11]}. Increased intracellular c-di-GMP promotes biofilm formation and motility inhibition\textsuperscript{[12]}. The synthesis of c-di-GMP is catalyzed by diguanylate cyclase containing a GGDEF domain, whereas its degradation is associated with phosphodiesterases (PDE) possessing either an HD-GYP or EAL domain\textsuperscript{[12]}. In V. parahaemolyticus, two proteins, namely, ScrG and ScrC, contain both GGDEF and EAL domains and act as PDEs to degrade c-di-GMP\textsuperscript{[6,8]}. In addition, the GGDEF-type proteins ScrO, GefA, ScrJ, and ScrL and the EAL type protein LaFV are required for the regulation of motility and biofilm formation in V. parahaemolyticus\textsuperscript{[13-15]}. We recently demonstrated that a group of genes encoding the proteins with GGDEF and/or EAL domains, including scrABC and scrG, were under the direct regulation of the master QS regulator OpaR, which acts as a repressor of biofilm formation and c-di-GMP production in V. parahaemolyticus RIMD2210633\textsuperscript{[9]}.

H-NS is an abundant nucleoid-associated DNA-binding protein involved in chromosome folding and gene regulation in Gram-negative bacteria\textsuperscript{[16]}. H-NS prefers to bind AT-rich DNA sequences and has been generally described as a negative regulator of gene expression\textsuperscript{[16]}. In V. parahaemolyticus, H-NS is a negative regulator of the major virulence determinants, including T3SS1, T3SS2, TDH, T6SS1, and T6SS2\textsuperscript{[17,18]}. The deletion of hns led to enhanced swarming motility and laf expression in V. parahaemolyticus\textsuperscript{[19]}. In addition, previous studies showed that H-NS acts as a positive regulator of biofilm formation by V. parahaemolyticus RIMD2210633 because the hns mutant formed smooth colonies and manifested lower expression of cpsA-J\textsuperscript{[8,20]}. However, the regulatory mechanisms remain not fully understood. The data on crystal cpsA-J\textsuperscript{[8,20]}. However, the regulatory mechanisms remain not fully understood. The data on crystal

### MATERIALS AND METHODS

#### Construction and Complementation of the hns Mutant

V. parahaemolyticus strain RIMD2210633 was used as the WT\textsuperscript{[21]}. Non-polar hns deletion mutant (Δhns) and complementary Δhns (C-Δhns) were constructed in our previous study\textsuperscript{[17]}. Control strains were also constructed by transferring the empty pBAD33 into the WT and Δhns strains to counteract the effects of arabinose and chloramphenicol on the results\textsuperscript{[17]}. Table 1 lists all the primers used in the current study.

#### Growth Conditions

The V. parahaemolyticus strains were similarly cultured as described previously\textsuperscript{[9,10]}. Briefly, V. parahaemolyticus was cultivated overnight (about 12 h) in 2.5% (w/v) Bacto heart infusion (HI) broth (BD Biosciences, USA) at 37 °C with shaking at 200 rpm. The resultant cultures were diluted 40-fold into the phosphate-buffered saline buffer (PBS, pH 7.2). Then, 150 μL of the diluted cultures were spread onto the HI plate with a diameter of 5 cm. After incubation at 37 °C for 6 h, the bacterial cells were harvested by adding 2 mL pre-cold PBS. When necessary, the media were supplemented with 100 μg/mL gentamicin, 5 μg/mL chloramphenicol, or 0.1% arabinose.

#### CV Staining

The CV staining was carried out as previously described\textsuperscript{[9,10]}. Briefly, overnight cell cultures were diluted 50-fold into 5 mL fresh HI broth and cultured at 37 °C with shaking at 200 rpm and OD\textsubscript{600} of approximately 1.4. Thereafter, the resultant cultures were 50-fold diluted into 2 mL fresh Difco marine (M) broth 2216 (BD Biosciences, USA) containing 0.1% arabinose and 5 μg/mL chloramphenicol in glass tubes and cultured at 30 °C with shaking at 150 rpm for 48 h. Media with planktonic cells were collected for the measurement of OD\textsubscript{600} values. The surface-attached cells were stained with 0.1% CV. The bound CV was dissolved with 20% ethanol, and the OD\textsubscript{570} values were measured. The relative biofilm formation was calculated with the formula: 100 × OD\textsubscript{570}/OD\textsubscript{600}.

#### Quantification of c-di-GMP

The intracellular c-di-GMP levels were measured similarly as previously described\textsuperscript{[22]}. Briefly, V. parahaemolyticus was grown on HI plates at 37 °C for 6 h, and the bacterial cells were harvested by...
adding 2 mL pre-chilled PBS. Two OD₆₀₀₀ bacterial suspensions were centrifuged at 10,000 xg for 3 min at 4 °C. The cell pellet was washed twice with ice-cold PBS, resuspended in 2 mL ice-cold PBS, and then sonicated for 30 min (power 100%, frequency 37 kHz) in an ice-water bath. After centrifugation at 10,000 xg for 10 min at 4 °C, the supernatant containing extracted c-di-GMP was collected to determine the intracellular c-di-GMP concentration with a c-di-GMP enzyme-linked immunosorbent assay kit (Mskbio, Beijing, China). Total proteins in the cell extracts were also determined by a Micro BCA Protein Assay Kit (ThermoFisher Scientific, USA), in accordance with the manufacturer’s instructions. The intracellular c-di-GMP concentration was expressed as pmol/mg of protein.

**Quantitative Real-time Polymerase Chain Reaction (qPCR)**

The qPCR assay was performed as previously described[23]. Briefly, the total RNAs were extracted from the WT and Δhns strains, and the cDNAs were generated from 1 μg total RNAs using the FastKing First-Strand cDNA Synthesis Kit (Tiangen Biotech, Beijing, China) following the manufacturer’s instructions. The relative mRNA levels of each target gene were determined using the classic 2⁻ΔΔCt method.

**LacZ Fusion and β-galactosidase Assay**

The promoter–proximal DNA region of each target gene was amplified and cloned into the

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**EMSA**

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<td>GGCCTCAGATCAGCAGCTGCTTGAGATG/CGCCGCAATTCCGAGATAGCTG</td>
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**Note.** EMSA: electrophoretic mobility shift assay.
pHRP309 vector containing a promoterless lacZ gene and a gentamicin resistance gene \[^{[24]}\]. After verification by DNA sequencing, the recombinant pHRP309 was transferred to the WT and Δhns strains, respectively. Thereafter, the transformants were cultured and lysed to measure the β-galactosidase activity in the cell extracts using the β-Galactosidase Enzyme Assay System (Promega, USA), in accordance with the manufacturer’s instructions.

The two-plasmid reporter assay in E. coli was performed as previously described \[^{[25]}\]. Briefly, the E. coli 100 λpir (Epcenter) bearing the indicated hns expression plasmid pBAD33-hns or the empty pBAD33 vector and a recombinant lacZ plasmid was cultivated overnight in Luria–Bertani (LB) broth at 37 °C with shaking at 200 rpm. The overnight cultures were diluted 1:100 into 5 mL fresh LB broth containing 0.1% arabinose and 20 μg/mL chloramphenicol and incubated at 37 °C with shaking at 200 rpm to reach an OD\(_{600}\) value of about 1.2. The E. coli cells were harvested and lysed to measure the β-galactosidase activity in the cell extracts.

Electrophoretic Mobility Shift Assay (EMSA)

The coding region of hns was amplified and cloned into the pET28a vector (Novagen, USA) and then transferred to E. coli BL21λDE3 to express 6 × His-tagged H-NS (His-H-NS). The expression and purification of His-H-NS were performed as previously described \[^{[17]}\]. The dialyzed His-H-NS was concentrated to a final concentration of 1.4 mg/mL. EMSA was performed exactly as previously described \[^{[26]}\]. The results of EMSA were detected by ethidium bromide (EB) staining, and the gel images were displayed with an ultraviolet (UV) transilluminator.

Statistical Methods

The EMSA was performed at least twice with similar results. The CV staining, c-di-GMP quantification, LacZ fusion, and qPCR were performed at least thrice with three replications each, with values expressed as the means ± standard deviation (SD). Analysis of variance with the Tukey-Kramer test was applied to calculate statistical significance, with \( P < 0.01 \) considered significant.

RESULTS

H-NS Repressed Biofilm Formation by V. parahaemolyticus

We previously reported that H-NS activated the EPS-dependent bacterial colony morphology and the transcription cpsA-J in V. parahaemolyticus RIMD2210633 \[^{[8]}\]. In this work, we investigated whether H-NS regulated biofilm formation by V. parahaemolyticus RIMD2210633 using the CV staining assay. As shown in Figure 1, the normalized CV staining by Δhns/pBAD33 significantly increased relative to that by WT/pBAD33, and C-Δhns produced a restored CV staining. This result indicated that H-NS repressed biofilm formation by V. parahaemolyticus RIMD2210633, as assessed by CV staining.

Mutation of hns Increased the Intracellular c-di-GMP Level

An increased c-di-GMP level leads to enhanced biofilm formation in bacteria \[^{[2]}\]. Thus, the increased CV staining by Δhns prompted us to investigate whether H-NS affects the production of c-di-GMP in V. parahaemolyticus RIMD2210633. As shown in Figure 2, the intracellular c-di-GMP level was significantly enhanced in Δhns relative to that in WT, indicating that H-NS inhibited the synthesis of c-di-GMP in V. parahaemolyticus RIMD2210633.

H-NS Repressed the Transcription of a Set of Genes Associated with c-di-GMP Metabolism

The transcription of a group of genes encoding proteins with GGDEF and/or EAL domains, namely, scrABC, scrG, VP0117, VPA0198, VPA1176, VP0699, and VP2979, were directly regulated by the QS master regulator OpaR \[^{[9]}\]. H-NS is also a transcriptional repressor of scrABC \[^{[8]}\], but whether H-NS can directly bind scrABC promoters is still unknown. In this study, we investigated the
regulatory mechanisms of H-NS on the transcription of these seven genes. The results of qPCR demonstrated that the mRNA levels of all of the seven genes significantly increased in Δhns relative to those in WT (Figure 3A), suggesting that their transcription was repressed by H-NS. The upstream DNA region of each of the seven genes was cloned into the pHRP309 plasmid containing a gentamicin resistance gene and a promoterless lacZ gene and then transferred into Δhns and WT to investigate the β-galactosidase activities in the cell extracts. As shown in Figure 3B, the promoter activities of all the seven genes were considerably higher in Δhns than those in WT, suggesting that the expressions of these genes were under the negative control of H-NS. Altogether, H-NS acted as a repressor of scrABC, scrG, VP0117, VPA0198, VPA1176, VP0699, and VP2979 in V. parahaemolyticus RIMD2210633.

**H-NS Repressed the Expression of Target Genes in a Heterologous Host**

To test whether H-NS can control the expressions of the seven target genes in a heterologous host, we expressed H-NS from the pBAD33 vector in E. coli 100 λpir, which also contained a lacZ fusion reporter plasmid[23]. As shown in Figure 4, after induction by the addition of 0.2% arabinose in the culture media, the promoter activities for all of the seven target genes in the E. coli 100λpir containing the empty pBAD33-Δhns were significantly lower than those in E. coli 100λpir containing the recombinant pBAD33 vector. These results suggested that H-NS can bind the promoter DNA region of each of the seven target genes to repress their expression.

![Figure 2](image_url) Intracellular c-di-GMP levels in V. parahaemolyticus. The data are expressed as the mean ± SD of at least three independent experiments. WT: wild-type.

**Binding Activities of His-H-NS to the Promoter DNA Regions of Target Genes**

The EMSA was employed to detect whether His-H-NS had binding activities to the regulatory DNA regions of the seven target genes in vitro. As shown in Figure 5, His-H-NS can bind the regulatory DNA regions of all of the seven target genes in dose-dependent manners, but it cannot bind the DNA fragment of 16S rDNA, which was used as the negative control. These results further suggested that H-NS directly inhibited the transcription of scrABC, scrG, VP0117, VPA0198, VPA1176, VP0699, and VP2979 in V. parahaemolyticus RIMD2210633.

**DISCUSSION**

H-NS has been shown to be a positive regulator of biofilm formation by V. parahaemolyticus BB22 because the deletion of hns forms defective submerged biofilms relative to the WT strain[20]. The hns mutant of V. parahaemolyticus RIMD2210633 formed smooth colonies and expressed low cpsA-J, indicating that H-NS may also act as a positive regulator of biofilm formation by V. parahaemolyticus RIMD2210633[8]. However, the data on CV staining presented here showed that H-NS repressed biofilm formation by V. parahaemolyticus RIMD2210633 (Figure 1). This result is not only contrary to the previous observations on V. parahaemolyticus BB22 but also V. parahaemolyticus RIMD2210633[8,20]. The genetic information contained in the genomes of BB22 and RIMD2210633 are different, and RIMD2210633 contains many unique genes relative to BB22[27]. Thus, the discrepancy between the data presented here and the previous conclusion regarding BB22 may be explained by these genetic differences. However, why the results of CV staining and colony morphology are contrary to each other in RIMD2210633 is difficult to understand. Colony morphology is mainly attributed to the EPS content, which is associated with the expression of cpsA-J[3]. However, a previous study showed that the biofilm formation by the V. parahaemolyticus trh positive strain ATCC17802 was strongly correlated with extracellular DNA and protein content in the extracellular polymeric substances but not with the EPS content[28]. Thus, similar observations may be obtained with V. parahaemolyticus RIMD2210633. Such a condition may be also due to the global influence of H-NS[26]. Specifically, studies showed that H-NS induced *opaR* expression and polar
flagellum production but repressed the transcription of lateral flagellar genes in *V. parahaemolyticus* RIMD2210633\textsuperscript{[19,29,30]}. Flagella-mediated motilities promote not only the initial adhesion of biofilm formation but also the active dispersal of biofilms\textsuperscript{[31]}; OpaR is an inhibitor of biofilm formation by *V. parahaemolyticus* TIMD2210633\textsuperscript{[9]}. OpaR may have some effects on the H-NS-mediated inhibition of biofilm formation in *V. parahaemolyticus* RIMD2210633. Although regulatory mechanisms

![Graphs and images]

**Figure 3.** H-NS repressed the transcription of the seven genes associated with c-di-GMP metabolism in *V. parahaemolyticus*. The negative and positive numbers indicate the nucleotide positions upstream and downstream of indicated genes, respectively. (A) qPCR. After incubation at 37 °C for 6 h on HI plates, the bacterial cells were harvested, and total RNA was extracted using TRIzol Reagent. qPCR assay was used to determine the relative mRNA levels for target genes in Δhns and WT using the classical 2−ΔΔCt method. (B) LacZ fusion. The promoter DNA region of each target gene was cloned into the pHRP309 and then transferred into Δhns and WT to test the promoter activity in cellular extracts.
Figure 4. Two-plasmid reporter assay in *E. coli*. The *E. coli* 100λpir containing the pBAD33 plasmid or recombinant pBAD33-*hns* plasmid and a LacZ fusion reporter plasmid were grown in LB broth containing 0.2% arabinose until the mid-log phase (OD$_{600}$ = 1.2). The bacterial cells were collected and assayed for *lacZ* expression using the β-galactosidase assay. The negative and positive numbers indicate the nucleotide positions upstream and downstream of indicated genes, respectively.

Figure 5. Binding of His-H-NS to the target DNA fragments. The regulatory DNA region of each target gene was incubated with increased amounts of His-H-NS and then subjected to 6% (w/v) polyacrylamide gel electrophoresis. The results were detected by EB staining and a UV transilluminator. A schematic representation of the EMSA design is shown below. The negative and positive numbers denote the nucleotide positions upstream and downstream of indicated genes, respectively.
may be different, the negative regulation of H-NS on biofilm formation has been confirmed in V. cholerae[32,33].

The increased c-di-GMP content results in enhanced biofilm formation and motility inhibition[2]. The data presented here showed that the deletion of hns significantly increased the intracellular c-di-GMP level relative to that in the WT strain (Figure 2). The genome of V. parahaemolyticus RIMD2210633 contains more than 50 genes encoding proteins that may be associated with the c-di-GMP metabolism[31], among which the transcriptions of scrABC, scrG, VP0117, VPA0198, VPA1176, VP0699, and VP2979 are directly regulated by the master QS regulator OpaR[30]. Here, we showed that H-NS can bind the promoter–proximal DNA regions of scrA, scrG, VP0117, VPA0198, VPA1176, VP0699, and VP2979 to repress their transcription (Figures 3, 4, and 5). Notably, OpaR showed the reverse regulation of scrG and VP2979 compared with those of H-NS regulation[30]; OpaR and H-NS exerted the same effects on c-di-GMP production. However, such findings should be easy to understand because the effect degrees of dozens of putative enzymes on intracellular c-di-GMP metabolism should be inconsistent under various genetic backgrounds (or growth conditions). Except for scrA and scrG, the functions of the other 5 target genes are completely unknown, and the data presented here showed that H-NS repression of the intracellular c-di-GMP levels in V. parahaemolyticus may occur via the direct regulation of the transcription of enzyme genes involved in the synthesis of c-di-GMP. The H-NS binding sites are usually long and overlap with the core –10 and –35 elements[37,38]. Thus, H-NS is usually thought to repress gene transcription by directly interfering with the action of RNA polymerase.

 Altogether, this work reported that H-NS was a negative regulator of the biofilm formation by V. parahaemolyticus RIMD2210633. H-NS directly repressed the transcription of scrA, scrG, VP0117, VPA0198, VPA1176, VP0699, and VP2979, which encode a group of GGDEF and/or EAL type proteins and thus decrease the intracellular c-di-GMP level. Therefore, H-NS repression of the biofilm formation by V. parahaemolyticus RIMD2210633 may occur via the repression of the production of intracellular c-di-GMP. However, the regulation mechanisms of H-NS on the biofilm formation by V. parahaemolyticus still need to be further studied.

Received: December 24, 2021; 
Accepted: March 29, 2022

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