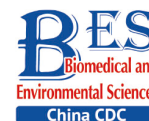


Original Article



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

XUE Xing Fan^{1,✉}, ZHNAG Miao Miao^{1,✉}, SUN Jun Fang², LI Xue², WU Qi Min², YIN Zhe³,
YANG Wen Hui³, NI Bin¹, HU Ling Fei³, ZHOU Dong Sheng^{3,✱}, LU Ren Fei^{2,✱}, and
ZHANG Yi Quan^{2,✱}

1. School of Medicine, Jiangsu University, Zhenjiang 212013, Jiangsu, China; 2. Department of Clinical Laboratory, Affiliated Nantong Hospital 3 of Nantong University, Nantong 226006, Jiangsu, China; 3. State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China

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

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INTRODUCTION

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium that primarily causes seafood-associated gastrointestinal illness in humans^[1]. *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 conditions^[1,2]. *V. parahaemolyticus* employs various specific structures, such as flagella, type-IV pili, and exopolysaccharide (EPS), to form mature biofilms^[2]. EPS is the main component of the biofilm matrix^[2]. In *V. parahaemolyticus*, the *cpsA-J* operon is responsible for the synthesis of EPS^[3]. The deletion of *cpsA-J* led to smooth colonies on plates, whereas the wild-type (WT) strain formed wrinkled colonies^[3]. Previous studies showed that the

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✉These authors contributed equally to this work.

✱Correspondence should be addressed to ZHANG Yi Quan, E-mail: zhangyiquanq@163.com; LU Ren Fei, E-mail: rainman78@163.com; ZHOU Dong Sheng, E-mail: dongshengzhou1977@gmail.com

Biographical notes of the first authors: XUE Xing Fan, male, born in 1997, Master's Degree Candidate, majoring in gene regulation of pathogenic bacteria; ZHANG Miao Miao, female, born in 1998, Master's Degree Candidate, majoring in gene regulation of pathogenic bacteria.

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*^[4-10].

C-di-GMP is a ubiquitous secondary messenger involved in regulating multiple behaviors of bacteria, including biofilm formation, motility, and virulence^[11]. Increased intracellular c-di-GMP promotes biofilm formation and motility inhibition^[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^[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^[4,6]. 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*^[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^[9].

H-NS is an abundant nucleoid-associated DNA-binding protein involved in chromosome folding and gene regulation in Gram-negative bacteria^[16]. H-NS prefers to bind AT-rich DNA sequences and has been generally described as a negative regulator of gene expression^[16]. In *V. parahaemolyticus*, H-NS is a negative regulator of the major virulence determinants, including T3SS1, T3SS2, TDH, T6SS1, and T6SS2^[17,18]. The deletion of *hns* led to enhanced swarming motility and *laf* expression in *V. parahaemolyticus*^[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*^[8,20]. However, the regulatory mechanisms remain not fully understood. The data on crystal violet (CV) staining presented here showed that H-NS acted as a repressor of biofilm formation by *V. parahaemolyticus* RIMD2210633. H-NS was a negative regulator of intracellular c-di-GMP synthesis and transcription of *scrA*, *scrG*, VP0117, VPA0198, VPA1176, VP0699, and VP2979, encoding a set of proteins containing GGDEF and/or EAL domains.

MATERIALS AND METHODS

Construction and Complementation of the *hns* Mutant

V. parahaemolyticus strain RIMD2210633 was used as the WT^[21]. Non-polar *hns* deletion mutant (Δhns) and complementary Δhns (C- Δhns) were constructed in our previous study^[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^[17]. Table 1 lists all the primers used in the current study.

Growth Conditions

The *V. parahaemolyticus* strains were similarly cultured as described previously^[9]. 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 μ L 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 μ g/mL gentamicin, 5 μ g/mL chloramphenicol, or 0.1% arabinose.

CV Staining

The CV staining was carried out as previously described^[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 OD₆₀₀ 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 μ g/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 OD₆₀₀ values. The surface-attached cells were stained with 0.1% CV. The bound CV was dissolved with 20% ethanol, and the OD₅₇₀ values were measured. The relative biofilm formation was calculated with the formula: $100 \times \text{OD}_{570} / \text{OD}_{600}$.

Quantification of c-di-GMP

The intracellular c-di-GMP levels were measured similarly as previously described^[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 OD₆₀₀ bacterial suspensions were centrifuged at 10,000 $\times g$ 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 $\times g$ 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 $2^{-\Delta\Delta Ct}$ method.

LacZ Fusion and β -galactosidase Assay

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

Table 1. Oligonucleotide primers used in this study

Target	Primers (forward/reverse, 5'–3')	Reference
Protein expression		
<i>hns</i>	AGCGGGATCCATGTCAGAGCTGACTAAAACAC/AGCGAAGCTTTTAGATTAGGAAATCGTCTAG	[17]
qPCR		
<i>scrA</i>	CACACCACGAACACATTGC/TCAATAGCGTCACGGAATGC	[9]
<i>scrG</i>	AAGCCGTGGTGGAAGAAGG/GCGTGTGAGTGC GTTGG	
VP0117	GACCACCTCAATAGTTATCTG/TAAGTAGGCTTGGACATCTC	
VPA0198	GCATCAGAATCAGCAAGAC/ATGCTTAGCTCCTCTTCTTC	
VPA1176	GCCATATTCCAAACTCGTTGTG/TGCGTAAGCCAAGTTGATGAG	
VP0699	CTGACACATCGTGATACTTC/TTGATGTTGCAGCTCTTG	
VP2979	GCAACTCTCAAGTCATCATC/CAACAACCGTCTTCTATGG	
LacZ fusion		
<i>scrA</i>	GCGCGTCGACCATCAAGCCATTTATGAAAC/GCGCGAATTCGTCGGCTGCGATTAGTCTG	[9]
<i>scrG</i>	GCGCGTCGACTAGCACGCTTGTTGGAC/GCGCGAATTCAGGGAAATGAAGTAATCATGC	
VP0117	GCGCTCTAGACTCACACAACACTTTCTG/GCGCGAATTCAGACAATCACACCGATAG	
VPA0198	GCGCGTCGACCTCTGGTTCATTGTCTTG/GCGCGAATTCGCTTGCTGATTCTGATG	
VPA1176	AAAGTCGACTCAGGTACGCTTGCTTCAC/GGGGAATTCGTTGCTTGGTAGTGGTAATAG	
VP0699	GCGCGTCGACGGAGAATACCTAGCAGAG/GCGCGAATTCAGTATCACGATGTGTCAG	
VP2979	GCGCTCTAGATTTCTATCCGTTGGCTAC/GCGCGAATTCCTGACTTACATCGTGGAC	
EMSA		
<i>scrA</i>	GAGCGTATATCCAAGTGGTTTG/GTCGGCTGCGATTAGTCTG	[9]
<i>scrG</i>	TAGCACGCTTGTTGTTGGAC/CAGGGAAATGAAGTAATCATGC	
VP0117	CTCACACAACACTTTCTG/GCGCGAATTCAGACAATCACACCGATAG	
VPA0198	CTCTGGTTCATTGTCTTG/GTCTTGCTGATTCTGATG	
VPA1176	TCAGGTACGCTTGCTTCAC/CGTTGCTTGGTAGTGGTAATAG	
VP0699	GGAGAATACCTAGCAGAG/AGTATCACGATGTGTCAG	
VP2979	TTTCTATCCGTTGGCTAC/CTGACTTACATCGTGGAC	
16S rDNA	GACACGGTCCAGACTCCTAC/GGTGCTTCTTCTGTCGCTAAC	

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 λ pir (Epicenter) 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 μ g/mL chloramphenicol and incubated at 37 °C with shaking at 200 rpm to reach an OD₆₀₀ 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* BL21 λ DE3 to express 6 $\times$ 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 $\pm$ 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

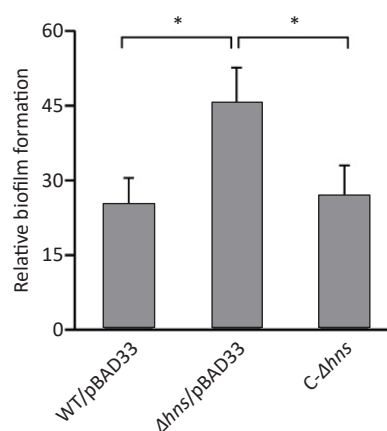


Figure 1. H-NS repressed biofilm formation by *V. parahaemolyticus*. Biofilms of *V. parahaemolyticus* were measured by CV staining. * $P < 0.01$.

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 λ pir, which also contained a *lacZ* fusion reporter plasmid^[25]. 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* 100 λ pir containing the recombinant pBAD33-*hns* were significantly lower than those in *E. coli* 100 λ pir containing the empty 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.

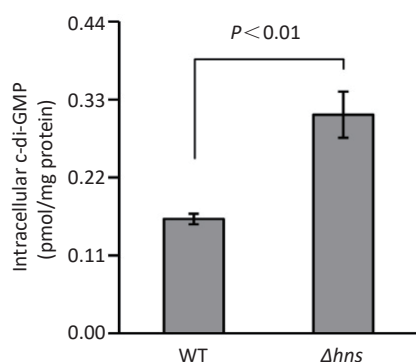


Figure 2. Intracellular c-di-GMP levels in *V. parahaemolyticus*. The data are expressed as the mean $\pm$ SD of at least three independent experiments. WT: wild-type.

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^[16]. 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^[19,29,30]. Flagella-mediated motilities promote not only the initial adhesion of biofilm formation but also the active dispersal of biofilms^[31];

OpaR is an inhibitor of biofilm formation by *V. parahaemolyticus* TIMD2210633^[9]. OpaR may have some effects on the H-NS-mediated inhibition of biofilm formation in *V. parahaemolyticus* RIMD2210633. Although regulatory mechanisms

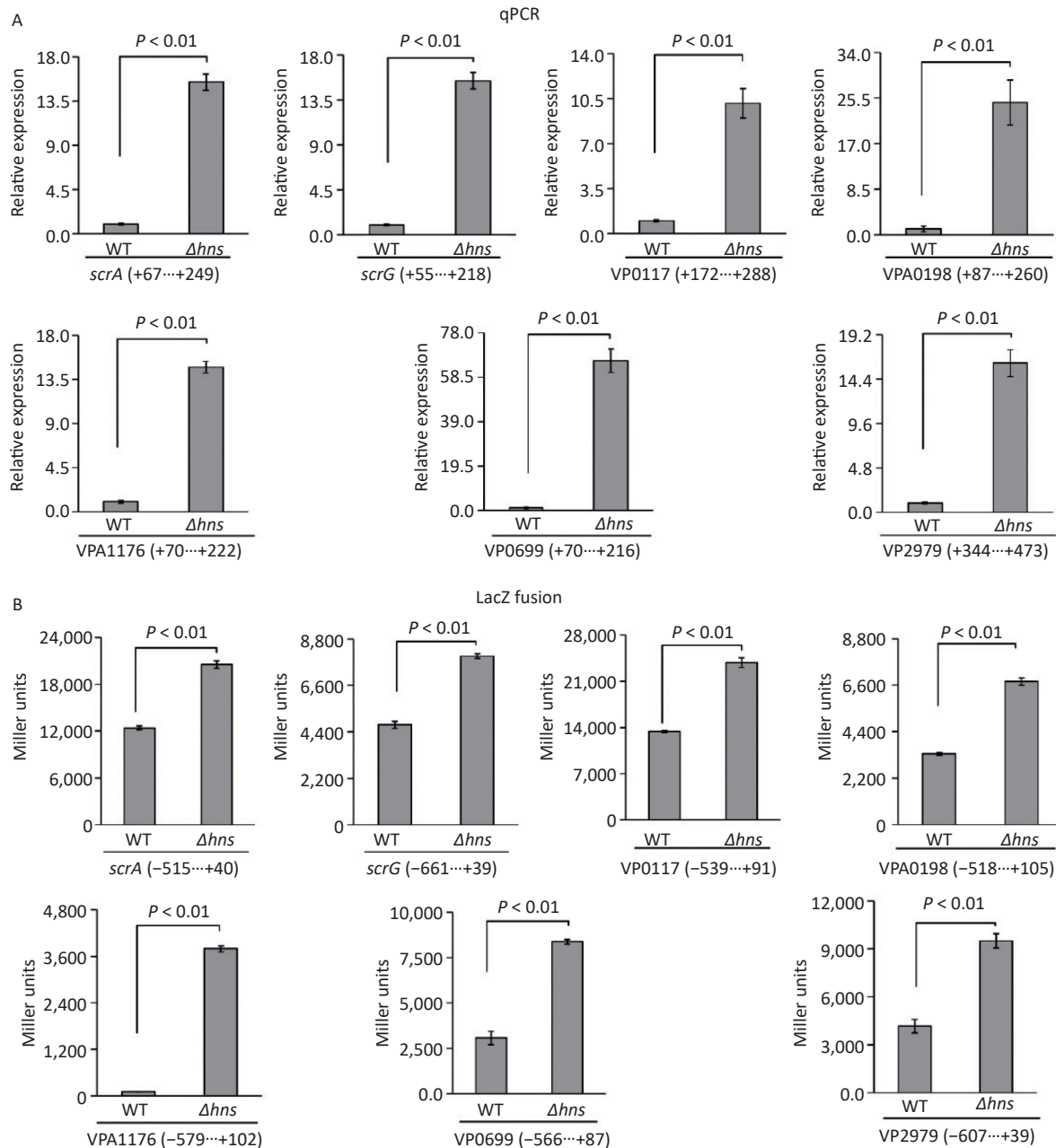


Figure 3. H-NS repressed the transcription of the seven genes associated with c-di-GMP metabolism in *V. parahaemolyticus*. The negative and positive numbers indicate the nucleotide positions upstream and downstream of indicated genes, respectively. (A) qPCR. After incubation at 37 °C for 6 h on HI plates, 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 $2^{-\Delta\Delta Ct}$ 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.

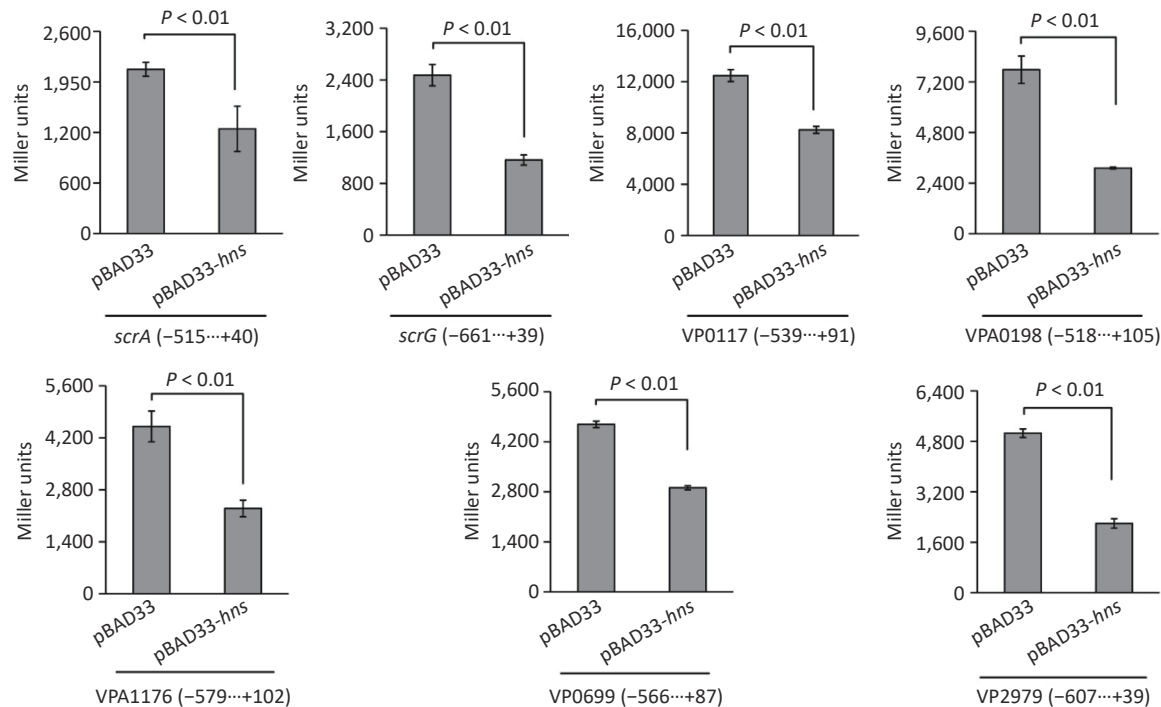


Figure 4. Two-plasmid reporter assay in *E. coli*. The *E. coli* 100λpir containing the pBAD33 plasmid or recombinant pBAD33-*hns* plasmid and a LacZ fusion reporter plasmid were grown in LB broth containing 0.2% arabinose until the mid-log phase (OD₆₀₀ = 1.2). The bacterial cells were collected and 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.

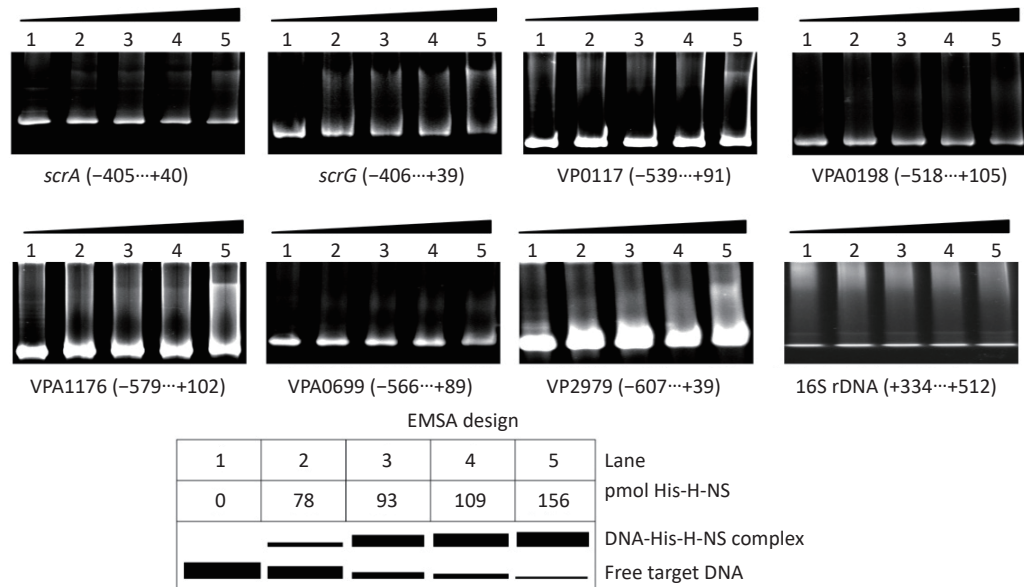


Figure 5. Binding of His-H-NS to the target DNA fragments. The regulatory DNA region of each target gene was incubated with increased amounts of His-H-NS and then subjected to 6% (w/v) polyacrylamide gel electrophoresis. The results were detected by EB staining and a UV transilluminator. A schematic 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^[21], among which the transcriptions of *scrABC*, *scrG*, VP0117, VPA0198, VPA1176, VP0699, and VP2979 are directly regulated by the master QS regulator OpaR^[9]. 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^[9]; 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^[17,19]. 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.

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