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H-NS is an activator of exopolysaccharide biosynthesis genes transcription in *Vibrio parahaemolyticus*

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ABSTRACT

Vibrio parahaemolyticus is capable of surviving in biofilm communities attached to biotic and abiotic surfaces. The exopolysaccharide (EPS) plays a key role in the maturing of the biofilm. The VPA1403-1412 (*cpsA-J*) operon is responsible for EPS production in *V. parahaemolyticus*. The expression of *cpsA-J* is controlled by ScrABC, intracellular concentration of c-di-GMP, CpsS-CpsR-CpsQ regulatory cascade, and quorum sensing. The data presented here showed that H-NS activates the EPS-dependent bacterial colony morphology and the transcription of *cpsQ* and *cpsA-J*. H-NS has negative regulatory activity on *scrABC* transcription, and thereby may result in enhancing the intracellular concentration of c-di-GMP. Thus, a regulatory circuit involved in regulating *cpsA-J*/EPS production by H-NS, ScrABC and CpsQ was identified in *V. parahaemolyticus*.

1. Introduction

Vibrio parahaemolyticus, a Gram-negative halophilic bacterium and a human pathogen, is capable of surviving in biofilm communities attached to biotic and abiotic surfaces. Biofilms are surface-associated microbial aggregates that are enclosed in extracellular polymer matrix. The mature biofilm depends upon some special structures, such as flagella, pili, and exopolysaccharide (EPS) biosynthesis, and other regulatory processes, such as quorum sensing and c-di-GMP [1]. The genes responsible for EPS production in *V. parahaemolyticus* are located on chromosome II, i.e., the VPA1403-1412 (*cpsA-J*) operon [2,3]. Expression of *cpsA-J* is correlated with biofilm-associated changes in colony morphology. Deletion of this operon resulted in a smooth colony morphology in *V. parahaemolyticus*, whereas the wild-type strain displayed rugose colony morphology with a raised and wrinkled central area [2]. The *scrABC* signaling pathway has been shown to be involved in regulating the expression of EPS genes. ScrC is a GGDEF-EAL motif-containing protein that acts to degrade c-di-GMP in the presence of ScrA and ScrB [4,5]. Mutations in the *scrABC* operon were shown to increase EPS genes expression, leading to a crinkly colony morphology [4–6]. Mutation of *cpsQ* suppresses the phenotype of *scr* mutants. CpsQ is a c-di-GMP-binding regulatory protein that was found to have direct and positive control of *cpsA* transcription [7,8]. Although not essential, CpsR has been shown to be required for the increased EPS genes expression in *scrA ΔopaR* background strain [8]. CpsS appears to act as a repressor. The *cpsS* mutant showed greatly elevated *cpsA* transcription [8]. Furthermore, CpsS represses *cpsR*; CpsR activates *cpsQ*; CpsQ in turn suppresses *cpsS* but activates its own gene [7]. The master quorum sensing regulator OpaR acts as an activator of *cpsA*, because the reporter fusions to *cpsA* showed10-fold decreased transcription in the *opaR* mutant strain compared to a wild type strain [8]. Thus, the production of EPS is tightly regulated by *scr* signaling pathway, intracellular concentration of c-di-GMP, CpsS-CpsR-CpsQ regulatory cascade, and quorum sensing in *V. parahaemolyticus*.

The histone-like nucleoid structuring protein (H-NS) is a small and abundant nucleoid-associated protein that has two distinct biological functions: DNA folding and control of gene transcription [9]. In regulation of transcription, H-NS has a propensity to recognize A/T-rich and curved DNA sequences, and it has been commonly described as a transcriptional repressor [9]. In *V. parahaemolyticus*, H-NS serves as a major repressor of virulence through directly acting on the three virulence loci T3SS1, Vp-PAI, and T6SS2 [10]. Salomon et al., also showed that H-NS acts as a negative regulator of T6SS1 under various environmental conditions [11]. The *hns* mutants exhibited severely impaired biofilm formation, and failed to produce both EPS and polar flagella [12]. Thus, H-NS may act as a transcriptional activator of *cpsA-J*

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operon and polar flagella genes. In the present work, we report that H-NS promotes EPS production and *cpsA-J* transcription; H-NS also activates *cpsQ*, but represses *scrABC*. Thus, a regulatory circuit identified was involved in regulating EPS production in *V. parahaemolyticus*.

2. Materials and methods

2.1. Bacteria strains and growth conditions

The *V. parahaemolyticus* wild-type strain RIMD 2210633 (WT), *hns* non-polar mutant (*Δhns*) and complementary*Δhns* (C-*Δhns*) were used in the present study [10]. In order to counteract the effects of arabinose and chloramphenicol on bacterial growth, the empty complementary vector pBAD33 was also transferred into WT and *Δhns* to generate WT/pBAD33 and *Δhns*/pBAD33.

V. parahaemolyticus was grown in HI broth [2.5% Bacto heart infusion (BD Bioscience)] at 37 °C with shaking at 250 rpm overnight. The cell cultures were 40-fold diluted with the PBS buffer (pH 7.2), and then 100 μl of the diluted cells were spread onto a HI plate [2.5% Bacto heart infusion (BD Bioscience), and 1.5% bacteriological grade agar] with a diameter of 5 cm. After 8 h of growth at 37 °C, cells were harvested from the plate. Bacterial cultivations were done with at least three biological replicates. When required, the culture medium was supplemented with 50 μg/ml gentamicin, 5 μg/ml chloramphenicol, or 0.1% arabinose.

2.2. Colony morphology

The bacterial cells were cultivated overnight in HI broth. The cell cultures were 50-fold diluted into 3 ml of Difco Marine (M) broth 2216 (BD Bioscience) containing 0.1% arabinose and 5 μg/ml chloramphenicol, and incubated without shaking at 30 °C for 4 d, then mixed thoroughly, and 2 μl of each strain of the cultured cell was spotted on the HI plate for the rugose colony morphology assay [13].

2.3. RNA isolation and quantitative real-time PCR (qRT-PCR)

Total bacterial RNAs were extracted using the TRIzol Reagent (Invitrogen, USA). The contaminated genome DNA in the total RNAs was removed by using the Ambion's DNA-*free*™ Kit according to the manufacturer's instructions. cDNAs were generated by using 8 μg of total RNAs and 3 μg of random hexamer primers. The SYBR Green qRT-PCR assay was performed and analyzed as previously described [14]. The relative mRNA levels were determined based on the standard curve of 16S rRNA (reference gene) expression [10]. All the primers used were listed in Table 1.

2.4. LacZ fusion and β-galactosidase assay

The promoter DNA region of each target gene was amplified and cloned into the corresponding restriction endonuclease sites of pHRP309 plasmid harboring a promoterless *lacZ* reporter gene and a gentamicin resistance gene [15]. After being verified by DNA sequencing, the recombinant plasmid was transferred into WT and *Δhns* strains, respectively. The β-Galactosidase activity in cellular extracts from cultivated cells was measured using the β-Galactosidase Enzyme Assay System (Promega, USA) according to the manufacturer's instructions.

2.5. Experimental replicates and statistical methods

The LacZ fusion and qRT-PCR assays were performed with at least three independent bacterial cultures, and the values were expressed as mean ± standard deviation (SD). Paired Student's *t*-test was used to calculate statistically significant differences, $p < .01$ was considered to indicate statistical significance. The colony morphology assay was done with at least two independent times.

Table 1
Oligonucleotide primers used in this study.

Targets	Primer sequences (5′-3′)
Construction of mutants	
hns	GTGACTGCAGACTTATGATGAGAACCAATGC/ CAAGAACGATTAGATTAGGAATTAGTCAGCTCTGACATAACG
	CGTTATGTCAGAGCTGACTAATTCCTAATCTAATCGTTCTTG/ GTGAGCATGCAGAGTGGGCTGATATGGTG
	GTGACTGCAGACTTATGATGAGAACCAATGC/ GTGAGCATGCAGAGTGGGCTGATATGGTG
Construction of complementary strain	
hns	GATCCCGGGAGGAGGAATTCACCATGTCAGAGCTGACTAAAACAC/ GACGTCGACTTAGATTAGGAAATCGTCTAG
qRT-PCR	
cpsA	GAGAGCGGCAACCTATATCG/GCGGTCAAACAAAGGGTAAAC
scrA	CACACCACGAACACATTGC/TCAATAGCGTCACGGAATGC
LacZ fusion	
cpsA	GCGCGTCGACCTTCCCTGTAAATAAGTCATCC/ GCGCGAATTCAAGCGAACTCCATCTCATAAG
cpsR	GCGCGTCGACGACAACCCATTCTTCACA/ GCGCGAATTCGTACCGCCAACTACCACAAG
cpsQ	GCGCGTCGACCAGACGGGCATTGATAAG/ GCGCGAATTCCATTAGGATTTCAGGCTTTT
scrA	GCGCGTCGACCATCAAGCCATTTTATGAAAC/ GCGCGAATTCGTCGGCTGCGATTAGTCTG

3. Results

3.1. Mutation of hns affects V. parahaemolyticus colony morphology

V. parahaemolyticus can alter its colony morphology when grown on agar plate due to the synthesis of abundant EPS [13]. In the present study, the results showed that *Δhns*/pBAD33 developed much smoother colony morphology than that of WT/pBAD33 and *C-Δhns*, but WT/pBAD33 and *C-Δhns* exhibited similar colony morphology results (Fig. 1). This observation suggested that *Δhns*/pBAD33 produced much less EPS relative to WT/pBAD33.

3.2. H-NS activates the expression of EPS genes

To examine whether the difference in colony morphology between *Δhns*/pBAD33 and WT/pBAD33 is due to transcriptional regulation of EPS genes expression, the mRNA level of *cpsA* in *Δhns* and WT (but not *Δhns*/pBAD33 and WT/pBAD33) was detected using qRT-PCR. The mRNA level of *cpsA* was significantly decreased in *Δhns* relative to WT (Fig. 2a), suggesting a positive regulation manner of EPS genes by H-NS in *V. parahaemolyticus*. To further test the action of H-NS on the target promoter, the promoter DNA region of *cpsA* was amplified and cloned into the pHRP309 plasmid that harbors a promoterless *lacZ* reporter gene, and then transformed into *Δhns* and WT. The β-galactosidase activity of *cpsA* in *Δhns* was much lower relative to that in WT (Fig. 2b), indicating the positive correlation of H-NS and EPS genes expression in *V. parahaemolyticus*. In brief, *V. parahaemolyticus* H-NS acts as a transcriptional activator of EPS genes.

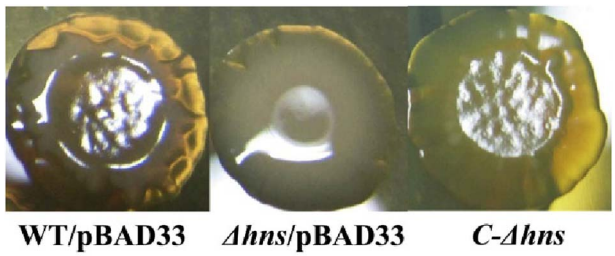

Fig. 1. Bacterial colony morphology. Bacteria cells were spotted on HI plate, and incubated at 30 °C for 7 days.