H-NS represses transcription of the flagellin gene *lafA* of lateral flagella in *Vibrio parahaemolyticus*

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H-NS represses transcription of the flagellin gene *lafA* of lateral flagella in *Vibrio parahaemolyticus*

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Abstract

Swarming motility is ultimately mediated by the proton-powered lateral flagellar (*laf*) system in *Vibrio parahaemolyticus*. Expression of *laf* is tightly regulated by a number of environmental conditions and regulatory factors. The nucleoid-associated DNA-binding protein H-NS is a small and abundant protein that is widely distributed in bacteria, and H-NS like proteins-dependent expression of *laf* genes have been identified in *V. cholerae* and *V. parahaemolyticus*. The data presented here shows that H-NS acts as a repressor of the swarming motility in *V. parahaemolyticus*. A single σ^{28} -dependent promoter was detected for *lafA* encoding the flagellin of the lateral flagella, and its activity was directly repressed by the H-NS. Thus, H-NS represses swarming motility by directly acting on *lafA*. Briefly, this work revealed a novel function for H-NS as a repressor of the expression of *lafA* and swarming motility in *V. parahaemolyticus*.

Key words: *Vibrio parahaemolyticus*; swarming; lateral flagella; H-NS

16 Flagella are elongated, curved and membrane anchored proteinaceous structures
17 with a diversity of roles in infection processes, biofilm formation and motility
18 (Chaban et al. 2015). Motility correlated with flagella can be classified into two
19 categories: swimming and swarming. Swimming is an individual behaviour that
20 allows bacteria to swim in aqueous environment, while swarming is the movement of
21 a group of bacteria over solid surfaces, or viscous environment (Harshey 2003).

22 *Vibrio parahaemolyticus* expresses two distinct types of flagella adapted for life
23 under different circumstances (McCarter 2004). The sodium motive force powers
24 polar flagellum for swimming, while the proton motive force powers the peritrichous
25 lateral flagella (*laf*) for swarming (McCarter 2004). The polar flagellum is expressed
26 continuously, whereas the *laf* is induced by surface growth, iron limitation, calcium
27 presence, and inhibition of polar flagella (McCarter and Silverman 1989; Kawagishi
28 et al. 1996; Gode-Potratz et al. 2011). When *Vibrio parahaemolyticus* encounters a
29 surface or viscous environment, the polar flagellum is impaired while *laf* is induced.
30 Many of the *laf* genes are classes of σ^{54} -dependent as well as σ^{28} -dependent (Stewart
31 and McCarter 2003). LafK, a σ^{54} -dependent transcription factor, acts as an activator
32 of *laf* genes (Stewart and McCarter 2003). The *scrABC* locus, encoding a
33 diguanidylate cyclase/phosphodiesterase GGDEF-EAL domain protein, appears to
34 activate *laf* expression, any mutation of the three genes decrease swarming and *laf*
35 genes expression (Boles and McCarter 2002). Quorum sensing also involves in
36 regulating *laf* genes (Jaques and McCarter 2006; Wang et al. 2013). In addition,
37 overexpression of *swrZ* or mutation of *swrT* represses *laf* genes expression and

38 swarming motility (Jaques and McCarter 2006).

39 The nucleoid-associated DNA-binding protein H-NS is a small and abundant
40 protein that is involved in chromosome organization, gene regulation, and bacterial
41 genome evolution (Dan and Loparo 2015). Low concentrations of H-NS can
42 recognize and bind to the A+T-rich DNA sequences, then multimerize into higher
43 order complexes that form bridges between adjacent DNA helices to organize bacteria
44 chromosomes or repress gene transcription (Fang and Rimsky 2008). The A+T% of
45 horizontally transferred DNA is generally higher than the resident genome DNA.
46 Repression of the horizontally transferred genes by H-NS can promote these foreign
47 genes integration into the existing regulatory networks (Fang and Rimsky 2008). In
48 contrast, bacteria also evolve a set of anti-silencing mechanisms to derepress silenced
49 foreign genes to benefit from their expression (Navarre et al. 2007; Fang and Rimsky
50 2008).

51 Previous studies showed that H-NS acts as a repressor of major virulence gene loci
52 in *V. parahaemolyticus* including T6SS1, T6SS2, T3SS1, and Vp-PAI (Salomon et al.
53 2014; Sun et al. 2014; Zhang et al. 2016). The present study shows that H-NS is a
54 repressor of swarming motility through acting on *lafA* encoding the flagellin of the
55 lateral flagella in *V. parahaemolyticus*.

56

57 **Materials and Methods**

58 **Bacterial strains and growth conditions**

59 The wild-type (WT) *V. parahaemolyticus* RIMD 2210633, the nonpolar *hns*

deletion mutant (*Δhns*) and its complemented mutant were previously described (Makino et al. 2003; Sun et al. 2014). Strains were grown on HI plates (2.5% BactoTM Heart Infusion and 1.5% agar) at 37°C (Zhang et al. 2012). When necessary, the antibiotics were used: gentamicin 50 µg/ml and chloromycetin 5 µg/ml. The concentration of arabinose was used at 0.1% for induction.

Swarming motility assay

The swarming motility assay was performed as previously described (Wang et al. 2013). Briefly, 2 µl of each culture was spotted onto a solid swarm plate, and the diameter of the colony zone was measured after being incubated at 37 °C for 24-48 h.

Quantitative reverse transcription PCR (qRT-PCR)

Isolation of total RNAs, preparation of cDNAs, and the qRT-PCR assay were performed and analyzed as previously described (Gao et al. 2011). The experiments were done at least three times. Primers used in this work are listed in Table 1.

Primer extension assay

Primer extension and sequencing reaction assays were performed as previously described (Gao et al. 2011; Sun et al. 2014). The products were analyzed in a 6% polyacrylamide-8 M urea gel, and the results were detected by the Fuji Medical X-ray film.

LacZ fusion and β-galactosidase assay

Construction of the *lacZ* strains and measurement of the β-galactosidase activity in the bacteria cellular extracts were performed as previously described (Sun et al. 2014). The experiment was done at least three times.

Preparation of purified His-H-NS protein

The recombinant 6×His-tagged H-NS protein (His-H-NS) was prepared as

described previously (Sun et al. 2014). The concentration of purified His-H-NS was concentrated to approximately 0.3 mg/ml.

Electrophoretic mobility shift assay (EMSA)

EMSA was designed, performed and analyzed as previously described (Gao et al. 2011; Sun et al. 2014). Briefly, the ³²P-labeled DNA probes were incubated with the increasing amounts of His-H-NS at room temperature for 20 min. The products were then loaded onto a native 4 % polyacrylamide gel, and analyzed with the Fuji Medical X-ray film. The assay was done at least two times.

DNase I footprinting

The DNase I footprinting was carried out as previously described (Gao et al. 2011; Sun et al. 2014). Briefly, after being incubated with the increasing amounts of His-H-NS, the ³²P-labeled DNA probes were digested by the optimized RQ1 RNase-Free DNase I (Promega). The digested DNA fragments were then analyzed in a 6% polyacrylamide-8 M urea gel, and the radioactive species were detected by the Fuji Medical X-ray film. The assay was done at least two times.

Statistical method

The results of LacZ fusion, qRT-PCR and swarming motility assays were expressed as mean ± standard deviation. Paired Student's t-test was employed to calculate the statistically significant differences, $p < 0.01$ was considered to indicate statistical significance.

Results

Mutation of *hns* increases swarming motility

In this work, the regulation of swarming motility by H-NS was investigated in *V.*

108 *parahaemolyticus* RIMD2210633. As shown in Fig. 1, when the swarming motility of
109 Δhns /pBAD33 was tested on the solid swarming plate, it was found that mutation of
110 *hns* lead to increased swarming motility compared to that of WT/pBAD33. In contrast,
111 when the *hns* mutant was complemented (Δhns /pBAD33-*hns*), swarming motility was
112 reduced to almost the same level as that of WT/pBAD33, suggesting that H-NS acts
113 as a repressor of the swarming motility in *V. parahaemolyticus*.

114 **H-NS represses the transcription of *lafA***

115 Swarming motility ultimately depends on proton-powered lateral flagella function
116 (Shinoda and Okamoto 1977). Thus, we investigated the effects of H-NS upon the
117 expression of *lafA*, encoding the flagellin of the lateral flagella (Stewart and McCarter
118 2003). The qRT-PCR experiment was performed to quantify the mRNA levels of *lafA*
119 in WT and Δhns (but not WT/pBAD33 and Δhns /pBAD33). The results (Fig. 2a)
120 revealed that the mRNA level of *lafA* was significantly enhanced in Δhns compared to
121 that in WT. The primer extension assay was further employed to detect the yield of the
122 primer extension product of *lafA* in WT and Δhns (Fig. 2b). This assay detected a
123 single transcriptional start site for *lafA*, which was located at 91 bp upstream of
124 translational start site and has been confirmed as σ^{28} -dependent (Stewart and
125 McCarter 2003). Additionally, a heavy primer extension product was detected in Δhns ,
126 indicating the negative regulation of *lafA* by H-NS.

127 **Mutation of *hns* greatly increases the promoter activity of *lafA***

128 The LacZ fusion and β -galactosidase assay was employed to test the action of
129 H-NS on the promoter activity of *lafA*. The results showed that the promoter activity
130 of *lafA* in Δhns was significantly enhanced than that in WT, indicating a negative

131 regulation of *lafA* by H-NS (Fig. 3).

132 **Direct binding of H-NS to the *lafA* promoter**

133 The A+T% of 304bp upstream of *lafA* is 63.16%, which is higher than the average
134 of the whole genome in this bacterium (Makino et al. 2003). Thus, we conducted the
135 EMSA and DNase I footprinting assays to investigate the binding activity of H-NS to
136 *lafA* promoter DNA. As shown in Fig. 4a, the EMSA results revealed that His-H-NS
137 was able to bind to the *lafA* promoter DNA fragment in a dose-dependent manner *in*
138 *vitro*, and the His-H-NS proteins at all amounts used could not bind to the 16S rDNA
139 fragment as the negative control. As further determined by DNase I footprinting (Fig.
140 4b), His-H-NS protected a single region from 205 to 40 bp upstream of *lafA* against
141 DNase I digestion in a dose-dependent manner. Taken together, H-NS represses *lafA*
142 transcription in a direct manner.

143 **Structural organization of *lafA* promoter region**

144 Collection of the translation/transcription start sites, H-NS site, core promoter -10
145 and -35 elements, and Shine-Dalgarno (SD) sequence (ribosomal binding site)
146 enabled us to depict the structural organization of *lafA* promoter region characterized
147 herein (Fig. 5).

148

149 **Discussion**

150 In the present work, H-NS acts on *V. parahaemolyticus* swarming motility and
151 *lafA* transcription were elucidated by using a series of experiments. The results show
152 that mutation of *hns* significantly increased the swarming motility of *V.*

153 *parahaemolyticus* (Fig. 1), suggesting a negative correlation between H-NS and
154 swarming. Meanwhile, the transcription of *lafA* was hugely repressed by H-NS (Fig.
155 2 and 3). Primer extension detected a single transcriptional start site for *lafA*, which
156 is located at 91 bp upstream of *lafA* (Fig. 2). DNase I footprinting detected a single
157 protected region from 205 bp to 40 bp upstream for *lafA* against DNase I digestion
158 (Fig. 4). The H-NS site overlaps the transcription start site as well as the core
159 promoter-10 and -35 elements (Fig. 5), which would block the entry of RNAP and
160 thus represses *lafA* transcription.

161 Despite being described as a widely distributed repressor, H-NS has been identified
162 as a positive regulatory factor for the biogenesis of flagella. Studies in *E. coli* showed
163 that the *hns* mutant exhibits immotile phenotype on semisolid medium predominantly
164 due to the downregulation of the flagellar master regulators FlhD and FlhC (Bertin et
165 al. 1994; Soutourina et al. 1999). Another study suggested that, H-NS-dependent
166 regulation of flagellar synthesis is mediated by repressing *hdfR*, encoding a LysR
167 family regulatory protein represses flagellar genes expression (Ko and Park 2000).
168 Moreover, cross-linking assays revealed that H-NS can interact with the flagellar
169 torque generating rotor protein FliG to form a complex to increase in flagellum
170 rotational speed (Donato and Kawula 1998; Paul et al. 2011). However, Eun A. Kim
171 and David F. Blair proved H-NS influences motility via numerous regulatory
172 molecules besides FlhDC, but has little or no effect on FliG organization (Kim and
173 Blair 2015). Studies in *V. cholera* demonstrated that the *hns* mutant also exhibited
174 reduced motility due to low FlrA production (Amalendu Ghosh 2006). Two H-NS-like

175 proteins have been reported to act as an activator of swarming behavior in *V. cholera*
176 and *V. parahaemolyticus*, respectively (Tendeng et al. 2000; Park et al. 2005).
177 However, a recent study in *Shewanella piezotolerans* WP3 demonstrated that H-NS
178 acts as a repressor of swarming motility through directly repressing the transcription
179 of *laf* genes including *lafA* (Jian et al. 2016). The data presented here revealed a novel
180 function for H-NS as a repressor of the expression of *lafA* and swarming motility in a
181 Kanagawa-positive *V. parahaemolyticus* strain RIMD2210633. Nevertheless, whether
182 other *laf* genes are directly repressed by H-NS, needs to be further characterized.

183

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- 277

Figure legends

Fig. 1 The *hns* gene mutant and complemented strain effect on the swarming motility. T1: WT/pBAD33; T2: Δhns /pBAD33; T3: Δhns /pBAD33-*hns*. The results were analyzed by Paired Student's t test. ** represent $p < 0.01$.

Fig. 2 H-NS represses the transcription of *lafA*. The negative and positive numbers indicated the nucleotide positions upstream and downstream of *lafA*, respectively. **a) qRT-PCR.** The *lafA* transcripts in Δhns and WT were detected. ** $p < 0.01$. **b) Primer extension.** An oligonucleotide primer was designed to be complementary to the RNA transcript of *lafA*. The primer extension products were analyzed with 8 M urea-6% acrylamide sequencing gel. The transcriptional start site was indicated by arrow with nucleotide and position. Lanes C, T, A, and G represented the Sanger sequencing reactions.

Fig. 3 H-NS represses the promoter activity of *lafA*. The promoter DNA region of *lafA* was cloned into pRBR309 vector, and then transformed into WT or Δhns to determine the β -galactosidase activity (miller units) in cellular extracts. The negative or positive numbers indicated the nucleotide positions upstream or downstream of *lafA*, respectively. ** $p < 0.01$

Fig. 4 DNA binding analysis. The negative and positive numbers indicated the nucleotide positions upstream and downstream of *lafA*, respectively. **a) EMSA.** The radioactively labeled DNA fragment from 417 bp upstream to 29 bp downstream of *lafA* was incubated with increasing amounts of purified His-H-NS protein, and then

303 subjected to 4% (w/v) polyacrylamide gel electrophoresis. Shown below is the
304 schematic representation of the EMSA design. **b) DNase I footprinting.** Labeled
305 coding or non-coding DNA probes was incubated with increasing amounts of purified
306 His-H-NS (Lanes 1, 2, 3, and 4 containing 0, 44.2, 66.3, and 77.3 pmol, respectively),
307 and then subjected to DNase I footprinting assay. The footprint regions were indicated
308 with vertical bars. Lanes G, A, T, and C represented the Sanger sequencing reactions.

309

310 **Fig. 5 Organization of *lafA* promoter DNA region.** Shown were translation and
311 transcription start, SD sequence, H-NS site (underlined nucleotide sequence), -10 and
312 -35 core promoter elements, and *E. coli* σ^{28} sequence (Liu and Matsumura 1996).

313

Fig. 1 The hns gene mutant and complemented strain effect on the swarming motility
33x26mm (600 x 600 DPI)

Fig. 2 H-NS represses the transcription of *lafA*

46x24mm (600 x 600 DPI)

Fig. 3 H-NS represses the promoter activity of *lafA*

42x36mm (600 x 600 DPI)

Fig. 4 DNA binding analysis

62x25mm (600 x 600 DPI)

Fig. 5 Organization of lafA promoter DNA region

45x13mm (600 x 600 DPI)

Table 1. Primers used in this study

targets	Primers (5'→3', sense/anti-sense)
Construction of mutant	
<i>hns</i>	GTGACTGCAGACTTATGATGAGAACCAATGC/CAA
	GAACGATTAGATTAGGAATTAGTCAGCTCTGACAT
	AACG
	CGTTATGTCAGAGCTGACTAATTCCTAATCTAATCG
<i>hns</i>	TTCTTG/GTGAGCATGCAGAGTGGGCTGATATGGT
	G
	GTGACTGCAGACTTATGATGAGAACCAATGC/GTG
	AGCATGCAGAGTGGGCTGATATGGTG
Construction of complemented mutant	
<i>hns</i>	GATCCCGGGAGGAGGAATTCACCATGTCAGAGCT
	GACTAAAACAC/GACGTCGACTTAGATTAGGAAAT
	CGTCTAG
Protein expression	
<i>hns</i>	AGCGGGATCCATGTCAGAGCTGACTAAAACAC/A
	GCGAAGCTTTTAGATTAGGAAATCGTCTAG
Primer Extension	
<i>lafA</i>	/AAGTGATGCGTAGTTAGTGTG
LacZ fusion	
<i>lafA</i>	GCGCGTCGACCTGTCTTTCCGTTCCCTTTGATG/CGC
	GAATTCCAAGTGATGCGTAGTTAGTGTG
qRT-PCR	
<i>lafA</i>	CACTAACTACGCATCACTTG/CGTTACGCATTGCTA
	CAG
EMSA	
<i>lafA</i>	CTGTCTTTCCGTTCCCTTTGATG/CAAGTGATGCGTA
	GTTAGTGTG
DNase I footprinting	
<i>lafA</i>	GCTTTTTTACTTTTCTCGTTTTAG/CAAGTGATGCG
	TAGTTAGTGTG