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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

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

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

swarming motility (Jaques and McCarter 2006).

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

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

Materials and Methods

Bacterial strains and growth conditions

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% Bacto™ 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

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

86 **Electrophoretic mobility shift assay (EMSA)**

87 EMSA was designed, performed and analyzed as previously described (Gao et al.
88 2011; Sun et al. 2014). Briefly, the ^{32}P-labeled DNA probes were incubated with the
89 increasing amounts of His-H-NS at room temperature for 20 min. The products were
90 then loaded onto a native 4 % polyacrylamide gel, and analyzed with the Fuji Medical
91 X-ray film. The assay was done at least two times.

92 **DNase I footprinting**

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

99 **Statistical method**

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

104

105 **Results**

106 **Mutation of *hns* increases swarming motility**

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

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

H-NS represses the transcription of *lafA*

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

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

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

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

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

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

Structural organization of *lafA* promoter region

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

Discussion

In the present work, H-NS acts on *V. parahaemolyticus* swarming motility and *lafA* transcription were elucidated by using a series of experiments. The results show 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

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

Acknowledgements

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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

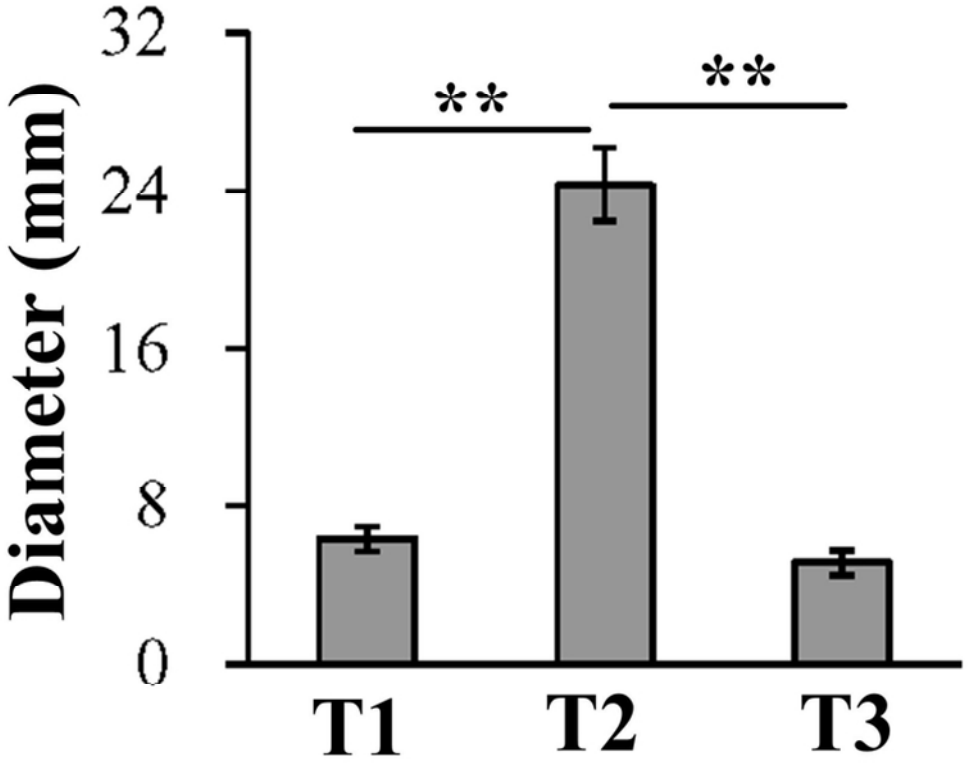

Fig. 1 The hns gene mutant and complemented strain effect on the swarming motility

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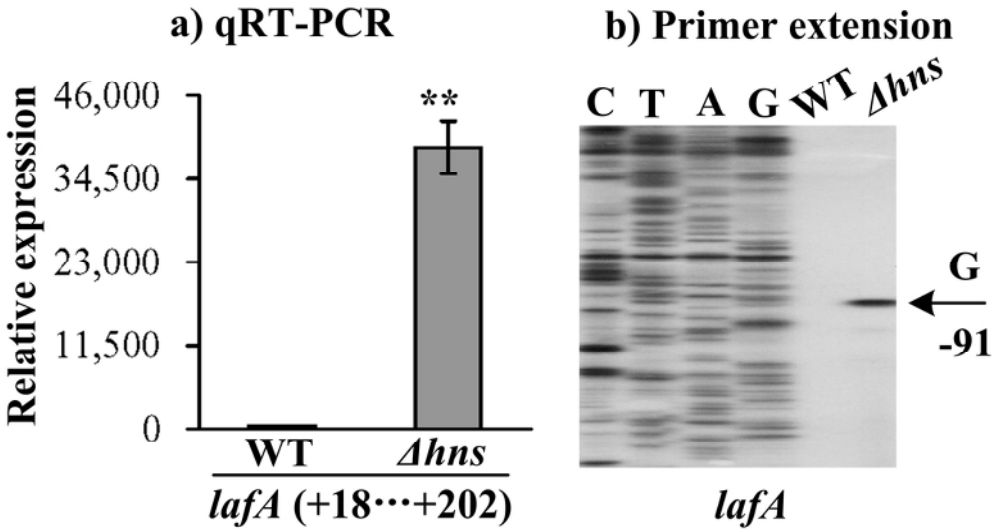

Fig. 2 H-NS represses the transcription of lafA

46x24mm (600 x 600 DPI)

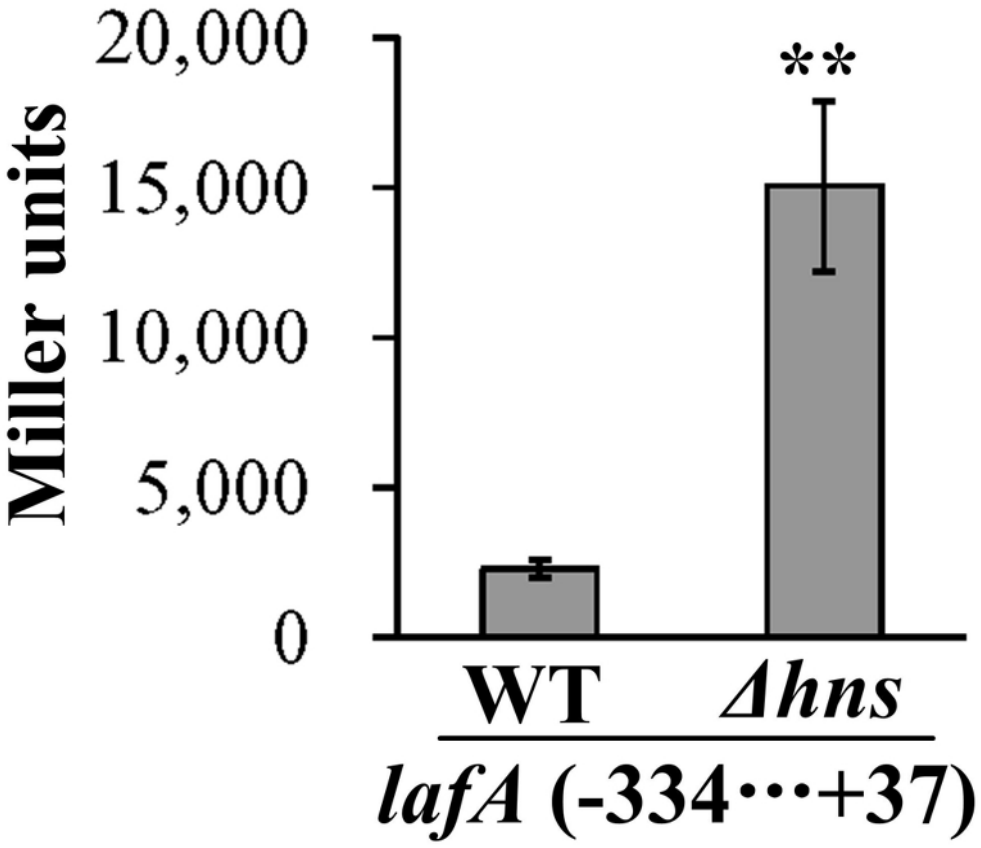

Fig. 3 H-NS represses the promoter activity of lafA

42x36mm (600 x 600 DPI)

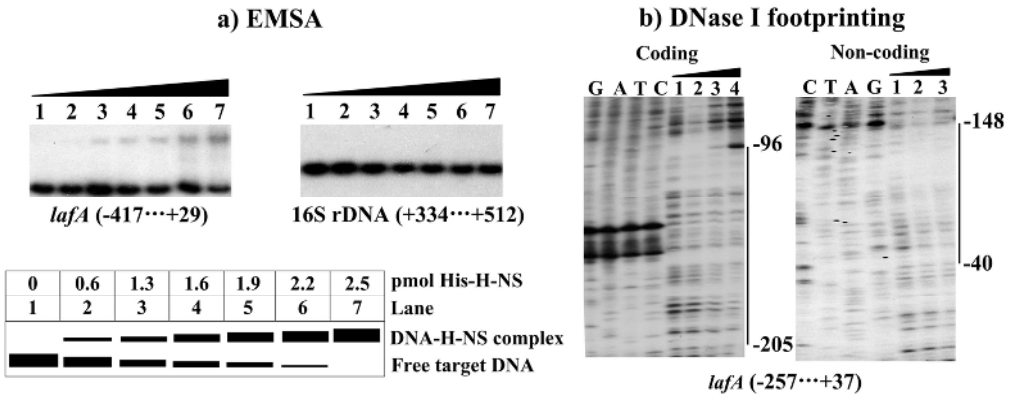

Fig. 4 DNA binding analysis

62x25mm (600 x 600 DPI)

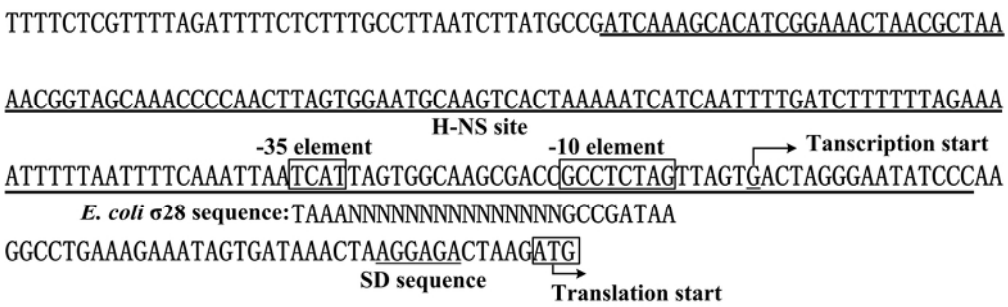

Fig. 5 Organization of lafA promoter DNA region

45x13mm (600 x 600 DPI)

Table 1. Primers used in this study

targets	Primers (5'→3', sense/anti-sense)
Construction of mutant	
hns	GTGACTGCAGACTTATGATGAGAACCAATGC/CAAGAACGATTAGATTAGGAATTAGTCAGCTCTGACATAACG
	CGTTATGTCAGAGCTGACTAATTCCTAATCTAATCGTTCTTG/GTGAGCATGCAGAGTGGGCTGATATGGTG
	GTGACTGCAGACTTATGATGAGAACCAATGC/GTGAGCATGCAGAGTGGGCTGATATGGTG
Construction of complemented mutant	
hns	GATCCCGGGAGGAGGAATTCACCATGTCAGAGCTGACTAAAACAC/GACGTCGACTTAGATTAGGAAATCGTCTAG
Protein expression	
hns	AGCGGGATCCATGTCAGAGCTGACTAAAACAC/AGCGAAGCTTTTAGATTAGGAAATCGTCTAG
Primer Extension	
lafA	/AAGTGATGCGTAGTTAGTGTG
LacZ fusion	
lafA	GCGCGTCGACCTGTCTTTCCGTTCCTTTGATG/CGCGAATTCCAAGTGATGCGTAGTTAGTGTG
qRT-PCR	
lafA	CACTAACTACGCATCACTTG/CGTTACGCATTGCTACAG
EMSA	
lafA	CTGTCTTTCCGTTCCTTTGATG/CAAGTGATGCGTAGTTAGTGTG
DNase I footprinting	
lafA	GCTTTTTTACTTTTCTCGTTTTAG/CAAGTGATGCGTAGTTAGTGTG