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The Histone-Like Nucleoid Structuring Protein (H-NS) Is a Negative Regulator of the Lateral Flagellar System in the Deep-Sea Bacterium *Shewanella piezotolerans* WP3

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Although the histone-like nucleoid structuring protein (H-NS) is well known for its involvement in the adaptation of mesophilic bacteria, such as *Escherichia coli*, to cold environments and high-pressure stress, an understanding of the role of H-NS in the cold-adapted benthic microorganisms that live in the deep-sea ecosystem, which covers approximately 60% of the earth's surface, is still lacking. In this study, we characterized the function of H-NS in *Shewanella piezotolerans* WP3, which was isolated from West Pacific sediment at a depth of 1,914 m. An *hns* gene deletion mutant (WP3Δ*hns*) was constructed, and comparative whole-genome microarray analysis was performed. H-NS had a significant influence (fold change, >2) on the expression of a variety of WP3 genes (274 and 280 genes were upregulated and downregulated, respectively), particularly genes related to energy production and conversion. Notably, WP3Δ*hns* exhibited higher expression levels of lateral flagellar genes than WP3 and showed enhanced swarming motility and lateral flagellar production compared to those of WP3. The DNA gel mobility shift experiment showed that H-NS bound specifically to the promoter of lateral flagellar genes. Moreover, the high-affinity binding sequences of H-NS were identified by DNase I protection footprinting, and the results support the "binding and spreading" model for H-NS functioning. To our knowledge, this is the first attempt to characterize the function of the universal regulator H-NS in a deep-sea bacterium. Our data revealed that H-NS has a novel function as a repressor of the expression of genes related to the energy-consuming secondary flagellar system and to swarming motility.

The histone-like nucleoid structuring protein (H-NS), which was originally identified as a small, heat-stable protein factor that stimulates bacteriophage DNA transcription (1), is widely distributed in Gram-negative bacteria (2). By recognizing and selectively silencing the expression of xenogeneic DNA sequences (3–6), H-NS differentially regulates horizontally acquired and core-genome genes (7). As a multifunctional bacterial modulator, the phenotypes that result from *hns* mutations are highly pleiotropic and involve diverse functions, such as conjugative transfer (8), outer membrane protein expression (9), fimbrial gene transcription (10), lipopolysaccharide production (11), motility and osmolarity (12), biofilm formation and exopolysaccharide biosynthesis (13), and the superinfection of bacteriophages and induction of the clustered regularly interspaced short palindromic repeat (CRISPR)-*cas* system (14–16), especially in the environmental adaptation of some pathogenic bacteria (17–21). Given the importance of maintaining fitness when laterally acquired genes are uncontrollably expressed, mutations in *hns* are lethal in *Salmonella enterica* serovar Typhimurium (22) and *Yersinia enterocolitica* (23).

In an *Escherichia coli hns* mutant, the expression of approximately 5% of genes is altered, and one-third of these genes encode proteins that are usually involved in bacterial adaptation to changes in environmental conditions (24). The *hns* gene belongs to the cold shock regulon, and a cold shock transcriptional enhancer was identified in the promoter region of *hns* (25). Temperature and osmolarity impact the formation of active H-NS tetramers (26), and H-NS–DNA-binding conformations directly respond to pH and temperature *in vivo* (27) and *in vitro* (28). Sixty-nine percent of the temperature-regulated genes in *E. coli*, including those related to the iron and nutrient acquisition systems, the general stress response, biofilm formation, and cold shock, are controlled by H-NS (29, 30). H-NS was also shown to be an essential part of a thermally controlled mechanism of gene regulation in *S. enterica* and to be responsible for the expression of 77% of the thermoregulated genes by microarray analysis (31). Moreover, evidence supports a significant role for H-NS in the cold and high-pressure adaptation of *E. coli*, as the ability of *E. coli* to cope efficiently with a cold environment (12°C and 25°C) has been shown to be significantly impaired upon *hns* mutation (32, 33). Moreover, H-NS was reported to be involved in osmosensitivity and survival in the stationary phase (34, 35) and was proposed to be one of the environmental sensors (temperature and osmolarity) of bacterial cells (36). Although the global regulator H-NS has been extensively investigated with respect to the environmental adaptation of mesophilic bacteria, such as *E. coli* (37–41), the function of H-NS in extreme environments, such as the

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype/characteristics[a]	Reference or source
Strains		
E. coli		
WM3064	Donor strain for conjugation; Δ*dapA*	80
C41(DE3)	Recombinant protein expression host	GE Healthcare
S. piezotolerans WP3		
WP3	Wild type, GenBank accession no. CP000472	Lab stock
WP3Δ*hns*	WP3 with deletion of the *hns* gene	This work
WP3Δ*hns*-C	WP3Δ*hns* with pSW2-*hns*	This work
Plasmids		
pRE112	Allelic-exchange vector; Cm^r *sacB*	48
pSW2	Shuttle vector for complementation; Cm^r, derived from the filamentous bacteriophage SW1	81
pET28a	Kan^r, His-tag protein expression vector	GE Healthcare
pRE112-*hns*	pRE112 containing the PCR fragment for deletion of the *hns* gene	This work
pSW2-*hns*	pSW2 containing *hns* and its upstream promoter region	This work
pET28a-*hns*	pET28a containing the coding region of the *hns* gene	This work

[a] Cm^r, chloramphenicol resistance; Kan^r, kanamycin resistance; GST, glutathione *S*-transferase.

deep sea, which represents a large portion of the Earth's ecosystem (42), remains unknown.

The deep-sea bacterium *Shewanella piezotolerans* WP3 was isolated from West Pacific sediment at a depth of 1,914 m, an environment with permanent low temperatures of approximately 2 to 4°C. The growth temperature range of WP3 is 0 to 28°C, with optimal growth occurring at 20°C (43, 44). Previously, fatty acid biosynthesis, RNA helicases, and lateral flagella were shown to play important roles in the cold adaptation of WP3 (45–47). In this study, the function of H-NS was characterized in this cold-adapted bacterium. A mutation in the H-NS gene did not affect WP3 growth, whereas a large number of genes were differentially expressed upon *hns* deletion. The expression of lateral flagellar genes was significantly upregulated in WP3Δ*hns*, and higher swarming motility was observed. In addition, the binding of H-NS to promoters of lateral flagellar genes were characterized by electrophoretic mobility shift assay (EMSA) and DNase protection assays. To our knowledge, this is the first demonstration that H-NS negatively regulates flagellar gene expression and motility; thus, we believe that H-NS contributes significantly to the fitness of WP3 in deep-sea environments.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and growth assay. All of the bacterial strains and plasmids that were used in this study are listed in Table 1. The *Shewanella* strains were cultured in modified 2216E marine medium (2216E) (5 g/liter tryptone, 1 g/liter yeast extract, 0.1 g/liter $FePO_4$, 34 g/liter NaCl) with shaking at 220 rpm. *E. coli* strain WM3064 was incubated in lysogeny broth (LB) medium (10 g/liter tryptone, 5 g/liter yeast extract, 10 g/liter NaCl) at 37°C with the addition of 50 μg/ml DL-α,ε-diaminopimelic acid (DAP) was added. For solid medium, 1.5% (wt/vol) agar-A (Bio Basic, Inc., Ontario, Canada) was added. The antibiotic chloramphenicol (Cm) (Sigma, St. Louis, MO, USA) was added to the medium at 25 μg/ml and 12.5 μg/ml for the *E. coli* and *Shewanella* strains, respectively, when required. The growth of the WP3 strains was determined using turbidity measurements at 600 nm with 2216E medium.

Construction of an *hns* gene deletion mutant and complemented strain. An *hns* deletion mutant was constructed, as described previously (47). First, the upstream and downstream fragments flanking both sides of the *hns* gene were amplified with PCR primer pairs (Table 2). These two fragments were used as the templates in a second fusion PCR, resulting in a fragment with a deletion in the *hns* gene. Next, the PCR product was cloned into pRE112 (48) as an XmaI-XbaI fragment, yielding pRE112-*hns*. This plasmid was transformed into *E. coli* WM3064 and then moved into WP3 by two-parental conjugation. The transconjugant was selected by chloramphenicol resistance and verified by PCR. The WP3 strain in which pRE112-*hns* had been inserted into the chromosome was plated on 2216E agar medium supplemented with 10% sucrose. One successful *hns* deletion mutant was screened and confirmed by PCR. For complementation, a *Shewanella-E. coli* shuttle vector, pSW2, was used, as previously described (49). In brief, the *hns* gene, along with its native promoter region, was amplified with *Pfu* DNA polymerase (Tiangen, Beijing, China). Both the PCR product and pSW2 were digested with MluI and XhoI and ligated to generate pSW2-*hns*. The recombinant plasmid was introduced into WM3064 and then into WP3Δ*hns* by conjugation. The complemented strain was confirmed by PCR and enzyme digestion of the reisolated plasmid.

RNA isolation and real-time qPCR. The WP3 strains were inoculated into 2216E medium, and the culture was collected immediately when the cells reached mid-exponential phase (optical density at 600 nm [OD_{600}], ~0.8). The samples were harvested by centrifugation and immediately placed in liquid nitrogen until RNA extraction. Total RNA extraction, reverse transcription, and real-time quantitative PCR (qPCR) were performed as previously described (49). The amount of target was normalized to that of the reference gene swp2079 (49), whose expression remains stable under various conditions relative to the calibrator (the transcription levels of the genes of WP3 were set as 1). The primer pairs (Table 2) used to amplify the selected genes via qPCR were designed using Primer Express software (Applied Biosystems, CA, USA).

Whole-genome microarray analysis. A microarray containing 95% of the total predicted gene content of WP3 was designed and manufactured (CapitalBio, Beijing, China). The preparation of fluorescent dye-labeled DNA, hybridizations, image acquisition, data processing, and clustering were performed as previously described (50). In short, total RNA was reverse transcribed with SuperScript II (Invitrogen, Carlsbad, CA, USA), and cDNA was labeled with Cy3 and Cy5 using Klenow enzyme (TaKaRa Bio, Inc., Japan), according to the manufacturer's instructions. Labeled cDNA was purified with a PCR purification kit (Macherey-Nagel, Düren, Germany) and resuspended in elution buffer. The microarray slides were hybridized with cDNA prepared from 3 biological replicates. As a measure of technical replication, the dye swap experiment was performed on each sample so that a total of 6 data points were available for every open reading frame (ORF) on the microarrays. A LuxScan 10K scanner and microarray scanner 2.3 software (CapitalBio) were used for array image acquisition. The linear normalization method was used for data analysis, based on the expression levels of WP3 housekeeping genes in combination with yeast external controls. The normalized data were log-transformed and loaded into MAANOVA in the R environment for multiple testing using a mixed-effects analysis of variance (ANOVA) model (51). Microarray spots with F-test P values of <0.001 were regarded as differentially expressed genes (DEGs). In addition, all of the DEGs were confirmed with the Significance Analysis of Microarrays (SAM) software (52).

Motility assay and TEM. For the motility assays, 1 μl of culture from each strain was placed on swimming plates (2216E medium with 0.3% agar; Eiken Chemical, Tokyo, Japan) or swarming plates (2216E medium with 0.7% agar). For the swimming and swarming motility assays, the plates were incubated at 20°C for 3 days and 4 days, respectively. Motility

TABLE 2 Primers used in this study

Primer name	Sequence (5′-3′)	Description
hnsUR	GGCTCTAGAGGCCAATACTGAGTACAACAAC	*hns* deletion
hnsDL	TGGGTTCTTAAAGGGCGTCCGATTAAAAAAAATTGATAAAGG	*hns* deletion
hnsDR	GCCCTTTAAGAACCCAGCATTTGCT	*hns* deletion
Chlfor	ATGCCCGGGCTCTGAATGGATAGCGCCAT	Mutant conformation
Chlrev	TTTACCAGAGGTCGCAGAA	Mutant conformation
hnsCFor	CAGCCAAGTATAGTCACCAATT	Mutant complementation
hnsCRev	ATACACGCGTAGGGCTTCCTTCTTACGTCTCCCAGCTCGAGCTGGGTTCTTAAAGGGCAAA	Mutant complementation
HNSexpFor	ACTTAGGATCCATGAGCGAATTTTTAGATATTTTG	H-NS expression
HNSexpRev	AAAGAGAGCTCTTAGATAAGGAAGTCTTCCATAGT	H-NS expression
PmotYFor	GACAACTTAATGACGCAG	EMSA
PmotYRev	TCCTCAGTTTTATCCCTT	EMSA
PlafBFor	TTCTTCCCCCCACCCAATTT	EMSA
PlafBRev	TACGATCCTCCAATCTAGCT	EMSA
PlafAFor	GTTCAGTCTCTTTACGCTTA	EMSA
PlafARev	AACGTGTTCCTTTAGGCTTT	EMSA
PmotY-DF-For	6-FAM-GACAACTTAATGACGCAG	DNase I footprinting
PlafB-DF-For	6-FAM-TTCTTCCCCCCACCCAATTT	DNase I footprinting
lafARTFor	AAACAGCCAGCCGTAACGTT	qPCR
lafARTRev	TGCACCATCTGCAGTTTGGA	qPCR
fliA2RTFor	TTTTGGCCATCGAAGACATG	qPCR
fliA2RTRev	GCCTTGCGGACTCGAGTAAC	qPCR
pepNRTFor	TTAAGGCAATGGAAGCTGCAT	qPCR
pepNRTRev	CGTCTTTACCCGTTAATGATACGA	qPCR
swp3093RTFor	GATGTTGCGTACGCGATGTC	qPCR
swp3093RTRev	TCTGCATAGCCTGTTAAGTCCAAA	qPCR
swp0265RTFor	TGGCGAGCATGTCACTACAGA	qPCR
swp0265RTRev	GGGCTGATTTGCCATCCA	qPCR
swp2979RTFor	TTTGCTGTCGTTGCAGATGAG	qPCR
swp2979RTRev	TCAGTCGATTCTTGCGTTCGT	qPCR
swp5089RTFor	GCTTCAGGCAACCGCATAG	qPCR
swp5089RTRev	TGCAACCTCATCAGCACGTT	qPCR
swp4459RTFor	CTTGGTCGTTGGAGCTGTACAG	qPCR
swp4459RTRev	CCGCAGTATTCCCCACCATAT	qPCR
swp2844RTFor	CGGATTTGAGCGCACGTT	qPCR
swp2844RTRev	CAAGATCTGCGCCAATGACA	qPCR

was assessed by examining the migration distance of the bacteria from one side of the colony edge to the other (defined as swimming diameter and maximal swarming distance). For each strain, the assays were performed on at least four different days, with three independent cultures spotted onto three plates each day. The data were analyzed using two-tailed Student's *t* test with the Excel software (Microsoft Corporation, USA). For transmission electron microscopy (TEM), bacteria were grown on swarming agar plates and suspended in a 1% (wt/vol) sterile NaCl solution. The samples were placed onto a carbon-coated grid (200-mesh) and air dried. The grid was then examined using a Tecnai G2 BioTWIN microscope (FEI Company, Eindhoven, the Netherlands).

Protein expression and purification. The expression plasmids were constructed using the expression vector pET28a (Novagen, Madison, WI, USA). The coding region of the *hns* gene was PCR amplified from WP3 genomic DNA with *Pfu* DNA polymerase using the primer pair HNSpETExpFor/HNSpETExpRev. The PCR product was gel purified and then ligated into the pET28a vector at the BamHI and SacI sites. *E. coli* C41(DE3) cells were transformed with this recombinant plasmid and selected on LB medium containing kanamycin. The positive clones were confirmed by enzyme digestion and DNA sequencing. C41 cells harboring pET28a-*hns* were propagated in 5 ml of LB with kanamycin overnight at 37°C. The bacteria were then inoculated in 1 liter of fresh LB supplemented with kanamycin (50 μg/ml) and rotated at 200 rpm at 37°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.5 mM) was added when the culture was in exponential phase (OD_{600}, 0.8 to ~1.0). The bacteria were sedimented by centrifugation at 7,700 × *g* for 10 min at 4°C and suspended in 10 ml of binding buffer (150 mM NaCl, 20 mM imidazole, 20 mM Tris-HCl [pH 8.0]). The suspension was then sonicated on ice with a microtip probe for 10 min; during this period, each 10-s sonication was performed at 20-s intervals. The bacterial lysates were centrifuged at 10,000 × *g* for 20 min at 4°C, and His-tagged proteins were purified from the soluble fraction with Ni-Sepharose high-performance resin by gravity flow, according to the manufacturer's instructions (GE Healthcare, Milwaukee, WI, USA). The protein was eluted in elution buffer (150 mM NaCl, 500 mM imidazole, 20 mM Tris-HCl [pH 8.0]), and imidazole was removed using HiTrap desalting columns (GE Healthcare). The purity of the protein was examined by SDS-PAGE, and protein concentrations were determined by the Bradford assay, with bovine serum albumin (BSA) as the standard.

EMSA. DNA probes were generated by PCR using the primers listed in Table 2 and purified with a Cycle-Pure kit (Omega Bio-Tek, Norcross, GA, USA). These fragments were mixed with different concentrations of purified protein for 30 min at 20°C. The 20-μl reaction mixture contained 40 mM KCl, 12.5 mM Tris (pH 7.5), 125 μM $MnCl_2$, 1.25 mM $MgCl_2$, 5% (vol/vol) glycerol, 0.5 mM dithiothreitol (DTT), 5 μg/ml BSA, and 10 ng/μl poly(dI-dC). Protein-DNA complexes were resolved on 6% nondenaturing polyacrylamide gels at 20°C with 0.5× Tris-borate-EDTA buffer (TBE) as the running buffer. The DNA was stained with GelRed (Biotium, Inc., CA, USA) and visualized with a gel imaging system (Tanon, Shanghai, China).

DNase I footprinting assay. The DNA probes for the DNase I footprinting assay were amplified 5′ 6-carboxyfluorescein (FAM)-labeled primers (Table 2), using WP3 genome DNA as the template. The FAM-labeled probe was purified by the gel extraction kit (Tiangen Biotech, Beijing, China) and quantified with a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA). For each assay, 1.5 pmol probe was incubated with different amounts of H-NS in a total volume of 20 μl in the EMSA buffer. After incubation for 30 min at 20°C, 10 μl of solution containing 0.015 U of DNase I (TaKaRa Bio, Inc., Kyoto, Japan) was added, and the sample was further incubated for 1 min at room temperature. The reaction was stopped by incubation for 10 min at 80°C. Digested samples were first extracted with phenol-chloroform and then precipitated with ethanol, and the pellets were dissolved in 20 μl of Milli-Q water as previously described (82). For preparation of the DNA ladders, the fmol DNA cycle sequencing system (Promega, WI, USA) was used. The volumes of the sequencing reactions were enlarged to 12 μl with 15 ng of *motY* and *lafB* promoter regions as the template, and 5 pmol of 5′FAM-labeled primer was used for the sequencing reaction. The sequencing samples were precipitated with ethanol, dried, and dissolved in 5 μl of Milli-Q water. For both digested DNA fragments and sequencing products, 1 μl of each sample was added to 8.5 μl of Hi-Di formamide and 0.5 μl of GeneScan 500 LIZ size standards and was analyzed with a 3730 DNA analyzer (Applied Biosystems, CA, USA). The electropherograms were then analyzed with the GeneMarker software (SoftGenetics, PA, USA) to determine the protected sequences in the DNase I digestion map.

Microarray sequence accession number. The microarray data have been deposited in the NCBI Gene Expression Omnibus (GEO) and can be accessed via the GEO series accession no. GSE57905.

RESULTS

Construction of the *hns* gene deletion mutant and growth assay. In WP3, H-NS contains 129 amino acids, with a predicted molecular mass of 14.6 kDa. WP3 H-NS has high identity (38.7%) and similarity (60.6%) with H-NS in *E. coli* (15.5 kDa, 137 amino acids) (53). The protein sequence alignment results showed that WP3 H-NS exhibits >70% identity with other *Shewanella* strains and that both the N-terminal protein interaction domain and the C-terminal nucleic acid binding domain are conserved (see Fig. S1 in the supplemental material). The intact *hns* coding region was deleted from the WP3 genome, and the mutated strain was designated WP3Δ*hns*. Subsequently, the complemented strain WP3Δ*hns*-C was developed by introducing the recombinant shuttle vector pSW2-*hns* into WP3Δ*hns*.

The possible impact of H-NS on the growth of WP3 was initially investigated (see Fig. S2 in the supplemental material). In contrast to the results observed with *E. coli*, in which the ability to grow at 12°C and 25°C was strongly impaired by *hns* insertion mutations (32), a deficiency in the growth of WP3Δ*hns* was not observed. This result also contrasts with results demonstrated in *S. enterica* serovar Typhimurium, as the *hns* mutant displayed a severely reduced growth rate (4).

Transcriptome profiling of WP3Δ*hns* by comparative microarray analysis. Whole-genome microarray analysis was performed to identify possible H-NS regulatory targets by comparing the gene transcription profiles of WP3 and WP3Δ*hns* at 20°C. Overall, 554 genes, accounting for 11.2% of the total number of WP3 genes, were found to be differentially expressed between these two strains (see Table S1 in the supplemental material). To validate the microarray data, 7 genes, including some that were upregulated, downregulated, or unchanged, were selected for real-time qPCR. The same samples were used for the microarray and qPCR. The relative mRNA levels for each gene were calculated and log-transformed. The correlation coefficient (R^2) between the microarray and qPCR data was 0.9691 (see Fig. S3 in the supplemental material), demonstrating that the microarray data were reliable and could be used for follow-up analysis.

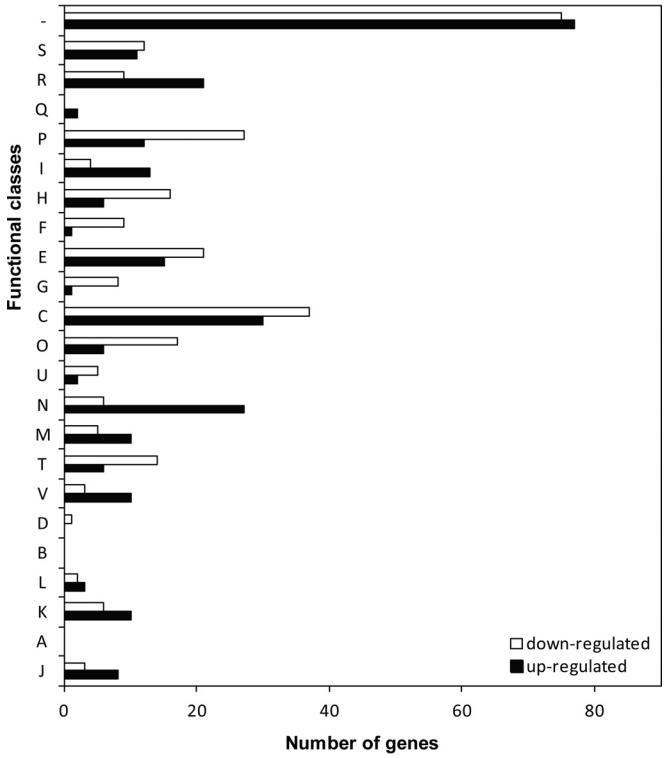

FIG 1 Numeric and functional annotation of the differentially expressed genes according to their COG categories. The bars indicate the number of genes in each group that showed significant changes in expression at 20°C after deletion of the *hns* gene in WP3. The genes were divided into functional categories according to NCBI (http://www.ncbi.nlm.nih.gov/COG/). The functional categories are abbreviated as follows: J, translation, ribosomal structure, and biogenesis; A, RNA processing and modification; K, transcription; L, replication, recombination, and repair; B, chromatin structure, and dynamics; D, cell cycle control, cell division, and chromosome partitioning; V, defense mechanisms; T, signal transduction mechanisms; M, cell wall/membrane/envelope biogenesis; N, cell motility; U, intracellular trafficking, secretion, and vesicular transport; O, posttranslational modification, protein turnover, and chaperones; C, energy production and conversion; G, carbohydrate transport and metabolism; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; H, coenzyme transport and metabolism; I, lipid transport and metabolism; P, inorganic ion transport and metabolism; Q, secondary-metabolite biosynthesis, transport, and catabolism; R, general function prediction only; S, function unknown; and −, no COG identified.

Functional classification of the differentially expressed genes was performed according to the Clusters of Orthologous Groups (COG) database (Fig. 1). Approximately 27.4% of the genes that exhibited altered expression had unknown functions. The genes that exhibited increased expression were associated with energy production and conversion (67/239 [number of genes with a significant change at the transcriptional level/total number of genes in the specific pathway]), amino acid transport and metabolism (36/250), inorganic ion transport and metabolism (39/172), and cell motility (35/134).

A large portion of the H-NS-regulated genes were located in clusters; these genes included the downregulated swp0428 to swp0431 (encoding fumarate reductase), swp0851 to swp0854

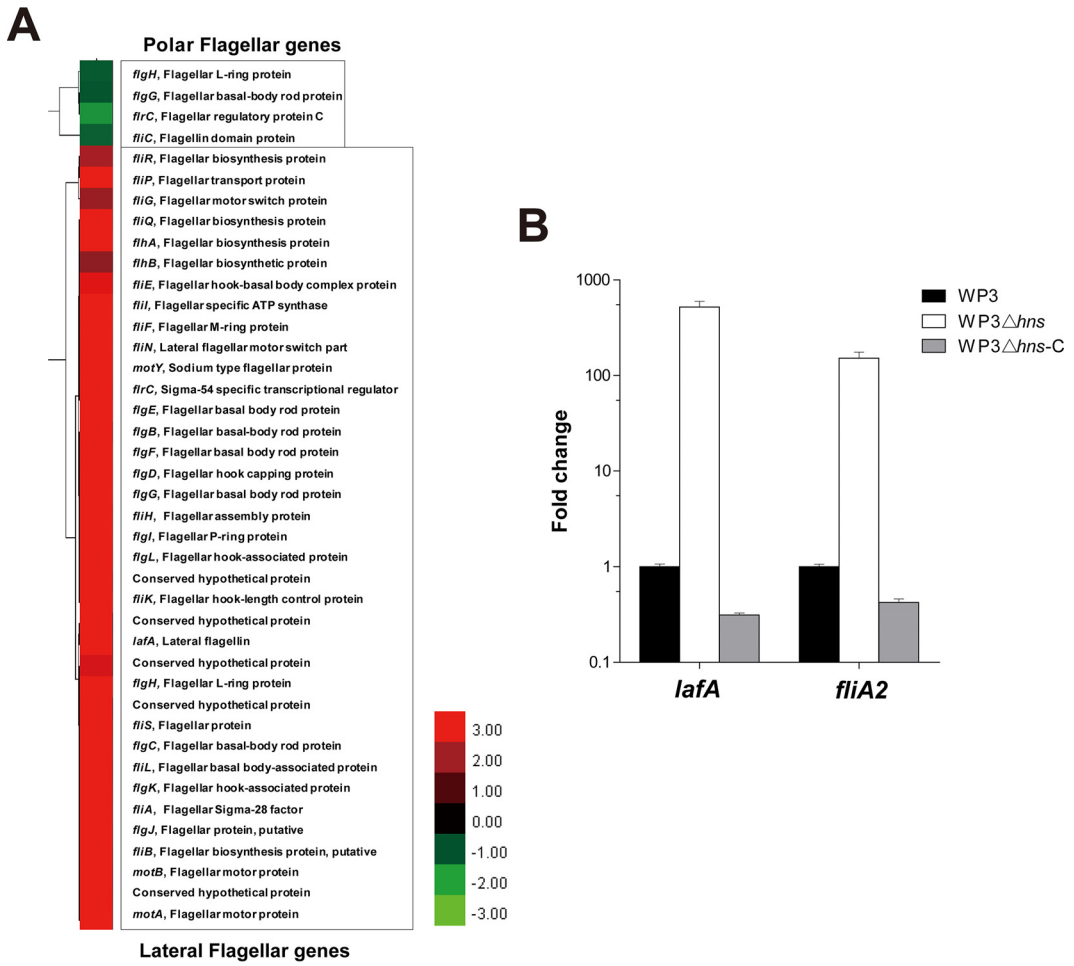

FIG 2 Differential expression of flagellar genes in the *hns* gene mutant. (A) Hierarchical cluster plot showing the gene expression levels of selected genes related to flagella. Red and green indicate genes that were induced and repressed, respectively. (B) Relative transcriptional levels of the lateral flagellar genes *lafA* and *fliA2* in the *hns* gene mutant and complemented strains. The transcription level of the WP3 wild-type strain was set as 1. The data shown represent two independent experiments, and the error bars indicate the standard deviations.

(encoding sulfite reductase), swp2039 to swp2043 (*ccmABDE*), swp2646 to swp2649 (*hypACD*), swp3360 to swp3363 (encoding conserved hypothetical proteins), swp5002 to swp5009 (encoding molybdenum cofactor biosynthesis proteins), and swp5023 to swp5037 (encoding formate dehydrogenase and twin-arginine translocation pathway signal), as well as the upregulated swp0132 to swp0136 (encoding histidine ammonia-lyase and urocanase), swp0931 to swp0941 (encoding NADH-ubiquinone oxidoreductase and Na^+/H^+ antiporter), swp1167 to swp1171 (encoding NADH-ubiquinone oxidoreductase), swp1588 to swp1593 (encoding sulfate adenylyltransferase and acetyltransferase), swp4931 to swp4945 (encoding flavoprotein, dehydrogenase, lipase, and carnitine racemase), swp5082 to swp5126 (encoding the lateral flagellar system), and swp5156 to swp5162 (encoding ATP synthase and ATPase). This type of clustered gene regulation by H-NS has also been identified within pathogenicity-associated islands in *E. coli* (6). These differentially expressed WP3 genes, which were identified in clusters, are likely cotranscribed, with common promoters under the control of H-NS. Alternatively, the regulation might be mediated by secondary regulators under the control of H-NS.

Interestingly, the microarray data identified 37 upregulated genes belonging to the lateral flagellar gene cluster (Fig. 2A); these genes were the most highly expressed (3.4- to 45.9-fold) in a comparison of WP3Δ*hns* to WP3. In contrast, only 4 genes encoding polar flagellar proteins were moderately downregulated (2.0- to 3.5-fold). All of these data indicate that H-NS specifically regulates the gene expression of WP3 lateral flagella.

Involvement of H-NS in lateral flagellar gene expression and WP3 swarming motility. Two representative genes from the lateral flagellar gene cluster, *lafA* and *fliA2*, which encode lateral flagellin and flagellum-specific transcription factor sigma-28, respectively, were selected to confirm the H-NS-mediated regulation of the lateral flagellar gene cluster. The relative mRNA expression levels of *lafA* and *fliA2* were measured by qPCR, and consistent with the microarray data, these two genes were upregulated 521.3- and 151.7-fold, respectively, in WP3Δ*hns* compared to the WP3 strain (Fig. 2B). As expected, the complementation of WP3Δ*hns* returned the expression of *lafA* and *fliA2* to wild-type levels.

The polar flagellum-directed swimming motility and lateral flagellum-directed swarming motility of WP3 and WP3Δ*hns* were monitored. In accordance with our microarray and qPCR data,

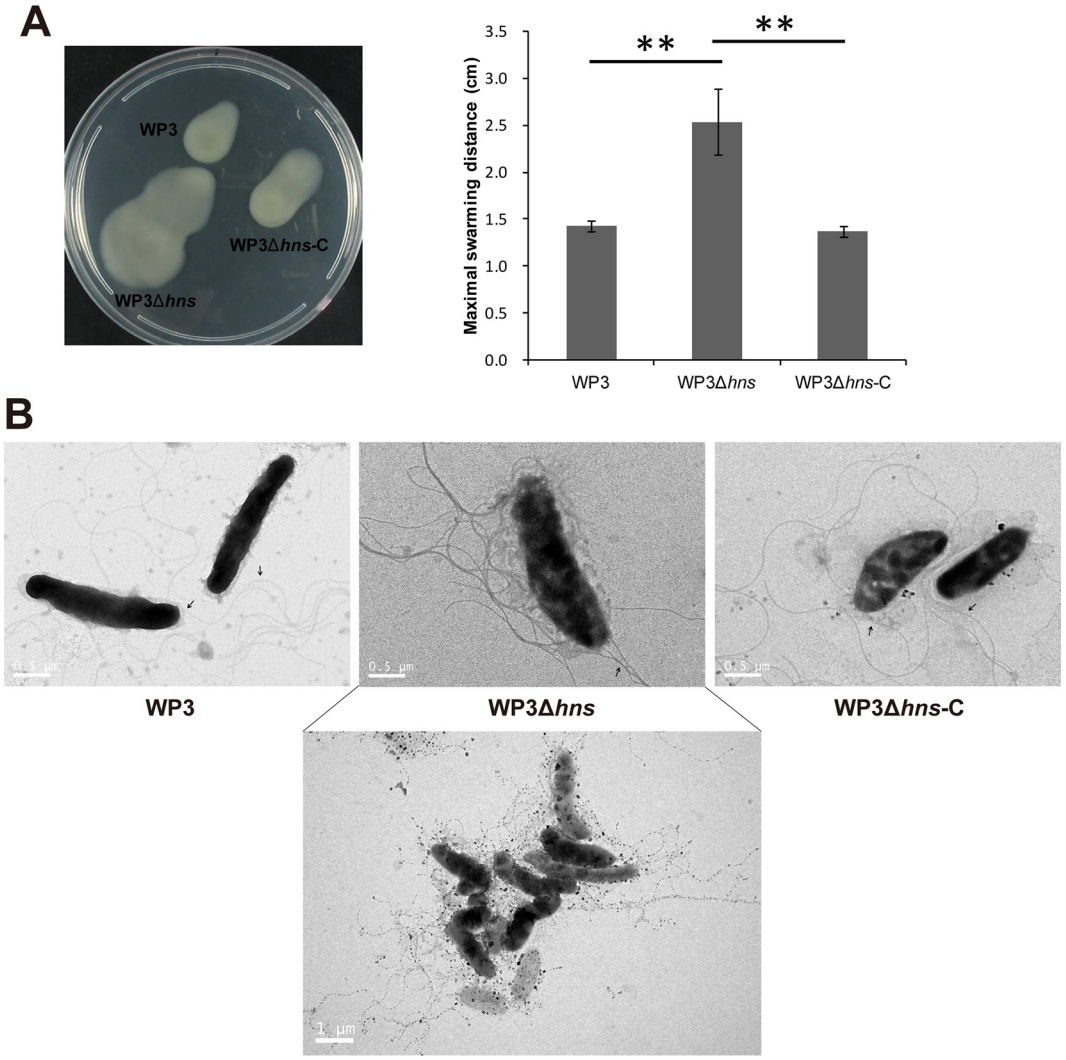

FIG 3 Influence of H-NS on swarming motility and flagellar production of WP3. (A) Swarming motility assays of the *hns* gene mutant and complemented strain. The data shown represent at least three independent experiments, and the error bars indicate the standard deviations. The data were analyzed by Student's *t* test; **, $P < 0.01$. (B) Transmission electron microscopic observation of WP3Δ*hns* swarming cells that were cultured on swarming agar plates. The arrows indicate the polar flagellum, and scale bars are shown at the bottom left.

there was no significant difference between the swimming motilities of WP3 and WP3Δ*hns* (see Fig. S4 in the supplemental material). However, the complemented strain showed a decreased swimming diameter compared with that of the wild-type strain, suggesting that the overexpression of H-NS affected the functioning of polar flagella. Interestingly, the maximal swarming distance of WP3Δ*hns* was 177% greater than that of WP3, indicating that motility was significantly influenced by deletion of the *hns* gene (Fig. 3A). Moreover, the TEM results showed that more lateral flagella were produced in WP3Δ*hns* than in the wild type and the *hns* gene-complemented strain (Fig. 3B). Taken together, our data demonstrate that H-NS serves as a negative regulator that modulates lateral flagellar gene expression and swarming motility.

H-NS binds to the promoter of lateral flagellar genes. To further illustrate how H-NS regulates the expression of lateral flagellar genes, an EMSA was performed to verify binding of the H-NS protein to the promoter region of the genes of the flagellar gene cluster. The expression vector pET28a-*hns* was constructed, and a His-tagged recombinant H-NS protein was purified. The shifted bands were observed in a 6% nondenaturing polyacrylamide gel after H-NS and the promoter region of *flrB* (positive control) were mixed, suggesting that H-NS is a functional DNA-binding protein (see Fig. S5 in the supplemental material). As expected, H-NS bound to the promoter regions of two lateral flagellar operons, including two regulatory genes, *flrC* and *fliA* (Fig. 4), indicating that H-NS is involved in the regulatory hierarchy of the WP3 lateral flagellar system. Moreover, H-NS bound to *lafA*, which encodes the flagellin protein of lateral flagella (Fig. 4B). These results suggest that H-NS regulates the expression of lateral flagellar structural genes through direct binding with the promoter regions. In general, H-NS is thought to control the gene transcription of the WP3 lateral flagellar system through different regulatory tiers, either by acting as a master regulator of the expression of regulatory flagellar genes or by directly binding to the promoter of structural flagellar genes.

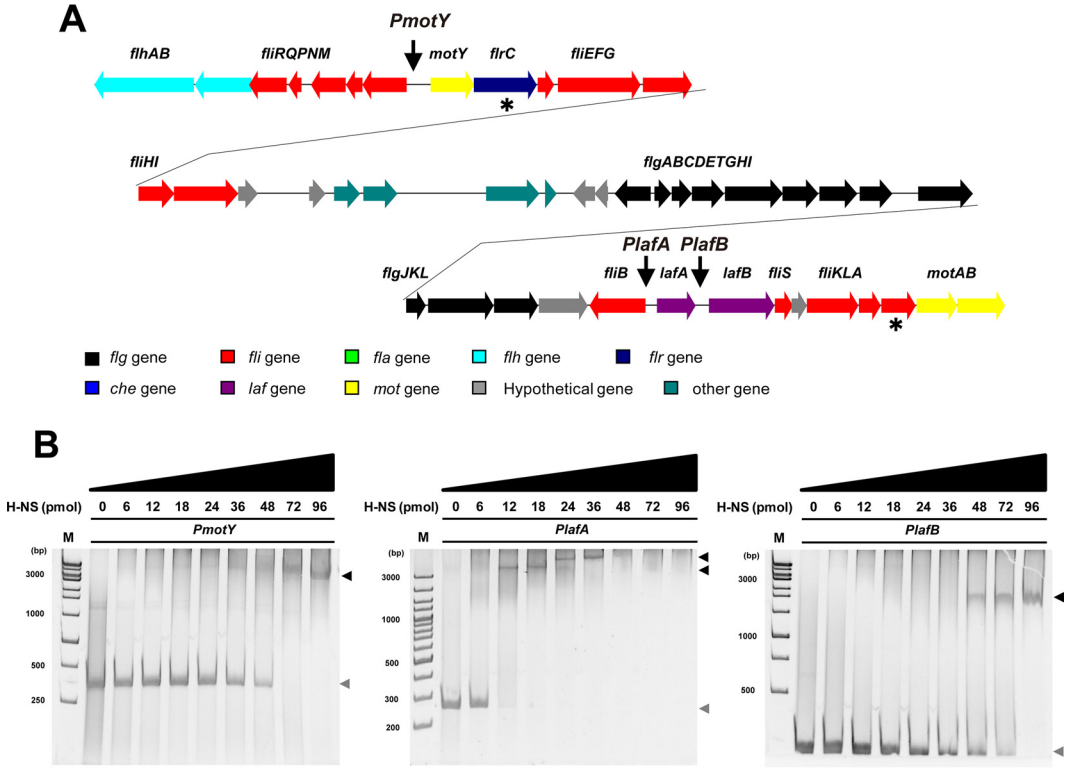

FIG 4 Binding assay of H-NS with the promoter of flagellar genes. (A) The lateral flagellar gene cluster of WP3. Different colors are used to represent genes with different functions. The asterisks indicate regulatory genes, and the arrows show the promoter regions used in the binding assay. (B) Binding of H-NS to the promoter of the lateral flagellar genes *motY*, *lafA*, and *lafB*. The DNA probe was preincubated with increasing concentrations of the purified H-NS protein, as indicated. The negative control consisted of DNA without the addition of protein. The black and gray arrows indicate the shifted DNA-protein complex and free DNA, respectively. Lane M, molecular size marker (in bp).

Mapping H-NS binding sites in the promoter of lateral flagellar genes *motY* and *lafB*. In an effort to more closely characterize the binding sites of H-NS in the promoter of WP3 lateral flagellar genes, we used a DNase I footprinting assay to identify the H-NS binding sites. DNA fragments covering the promoter regions of *motY* and *lafB* that were end labeled with 6-carboxyfluorescein (FAM) were mixed with H-NS protein and then subjected to DNase I digestion. The digested fragments were separated by capillary electrophoresis and peak heights on the chromatograms. After that, the protected regions were identified by comparing sequence patterns in the absence or presence of H-NS (0.9 and 3.6 μM). The results of these experiments revealed that H-NS binds to a 20-bp region and a 27-bp region of P*motY* and P*lafB* at a low concentration (0.9 μM), respectively. Notably, the addition of a high concentration (3.6 μM) of H-NS to the binding reaction led to the promoters being almost completely protected by H-NS. These results indicated that H-NS first binds to the high-affinity sites, and this binding facilitates the subsequent recruitment of other H-NS molecules to adjacent sites of lower affinity. Moreover, we identified a 10-bp binding motif (TA GATCGATT) in the high-affinity binding sites of P*lafB*, which shared high identity with the conserved sequence TCGATAAATT in *E. coli* (6). Additionally, the high-affinity binding sites in P*motY* were found to be AT rich (G+C content, 22%), although no conservative binding motif was identified.

DISCUSSION

In this study, transcriptomic analysis of the *hns* mutation was performed, and a large number of genes involved in various functions were differentially expressed (Fig. 1), indicating that H-NS plays a global regulatory role in the deep-sea bacterium WP3. The majority of the differentially expressed genes were related to energy and metabolism, indicating the significance of H-NS in the allocation and utilization of energy and nutrients in this cold-adapted microorganism. Considering the low supply of energy that is available in low-temperature environments because of the decreased affinity for substrate uptake and biochemical reaction rates (54–56), the regulatory function of H-NS might be crucial for the adaptation of WP3 to the energy- and nutrient-limited benthic environment.

The impacts of H-NS on flagellar biogenesis and motility have been documented in the literature and can be classified into two types of mechanisms. The first mechanism involves the role of H-NS as a transcriptional regulator. In an early study, H-NS was shown to positively regulate genes involved in the biogenesis of flagella in *E. coli* (57), and *hns* mutants have been shown to be nonmotile due to repression of the *flhDC* master operon and a complete lack of flagellin synthesis (58). Moreover, a similar phenotype was also reported in *Vibrio cholerae* (59) and *Vibrio parahaemolyticus* (60). The Δ*hns* mutant exhibited lower levels of *flaA*, *flaC*, and *motX* expression than the wild-type strain of *V. cholerae* (61). Further analyses showed an interaction between H-NS and the general stress sigma factor RpoS; this interaction involved a reduction in the occupancy of H-NS at the *flrA* and *rpoN* promoters (62). The second mechanism involves the direct interaction between the H-NS protein and the flagellar torque-generating ro-

tor protein FliG, which enhances the organization of FliG subunits and motor performance (63, 64). In addition, two other genes, *ycgR* and *yhjH*, take part in motor function, along with H-NS (65). Interestingly, the cyclic diguanylate monophosphate (c-di-GMP) binding protein YcgR was identified as a "brake" protein in the control of flagellar motor direction and speed, thereby affecting the swimming and swarming motilities of *E. coli* and *Salmonella* strains (66–68). The interaction between H-NS and YcgR homologs in WP3 needs to be further investigated.

Swarming is defined as the multicellular movement of bacteria across a solid surface, powered primarily by multiple lateral flagella (69). This phenotype has been observed in a large number of bacteria, indicating that it is an important means of surface colonization in natural habitats (70). H-NS has been reported to play a role as an activator in the regulation of lateral flagellar and swarming motility (60, 71). To our knowledge, our study provides the first evidence that H-NS negatively regulates lateral flagellar gene expression and swarming motility. In addition to WP3, 7 lateral flagellar systems have been identified in 23 sequenced *Shewanella* genomes, including those of *S. putrefaciens* CN-32, *S. denitrificans* OS217, *S. baltica* OS155, *S. halifaxensis* HAW-EB4, *S. pealeana* ANG-SQ1, *S. sediminis* HAW-EB3, and *Shewanella* sp. strain W3-18-1. The lateral flagellar system is remarkably different from the polar flagellar system, which implies that it has been acquired by horizontal gene transfer (72). Furthermore, the dinucleotide relative abundance value, δ*, and the G+C variation were analyzed with the δρ-Web program (73). Although no significant difference in G+C content was observed between the lateral flagellar gene cluster (45.75%) and the whole WP3 genome (43.3%), the value of δ* for this cluster was shown to be higher than that of the majority of the other genomic fragments (95.28%). All of these data suggest that the lateral flagellar gene cluster of WP3 consists of exogenetic DNA. In addition, evidence suggests that secondary flagellar systems originated early and were subsequently lost from most bacterial genomes (74, 75). Therefore, we cannot exclude the possibility that the lateral flagellar system in WP3 is evolutionarily ancient and has been maintained throughout evolutionary history. Interestingly, gene transcription of the lateral and polar flagellar systems in WP3 was demonstrated to increase in response to low temperature and high hydrostatic pressure, respectively (46), indicating that the regulation of the flagellar system is well matched to the inhabited environment. Thus, the lateral flagellar system may have been incorporated into the regulatory network of H-NS, which functions as a master modulator for environmental adaptation.

The binding of H-NS to DNA has been exclusively studied (39, 76), and the results indicate that H-NS silences extensive regions of the bacterial genome by binding first to nucleating high-affinity sites and then spreading along AT-rich DNA (6). In this study, the H-NS binding sites in the lateral flagellar genes (*motY* and *lafB*) were identified by DNase I footprinting assay (Fig. 5), and the results also support the binding and spreading model. It noteworthy that these two high-affinity sites were found to be either AT rich or to contain a conserved H-NS nucleation motif. From this aspect, a more comprehensive understanding of the regulatory mechanism of H-NS in deep-sea bacteria will be gained by identifying more high-affinity binding motifs in the WP3 genome.

In bacteria, motility is an important quality for environmental adaptation; nevertheless, the use of a flagellum, especially multiple lateral flagella, can constitute a considerable metabolic burden for

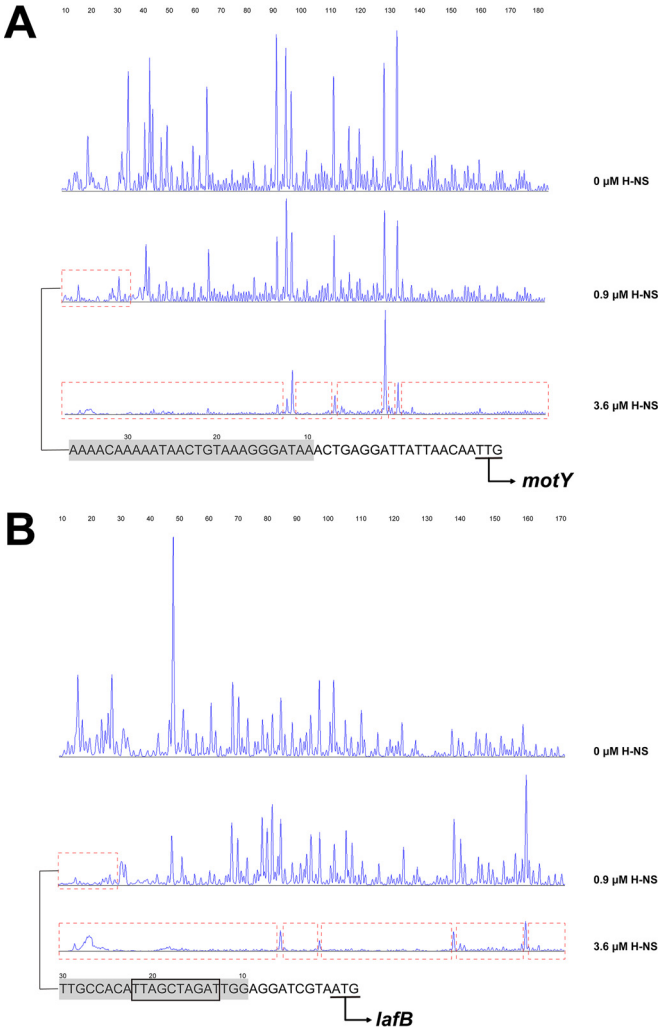

FIG 5 Identification of the sequence of the H-NS-protected regions of the *motY* (A) and *lafB* (B) promoter by DNase I protection footprinting. A concentration of 0.075 μM probe P*motY* and P*lafB* covering the entire promoter region of *motY* and *lafB* was incubated with H-NS (at 0.9 μM and 3.6 μM) in the EMSA buffer. The promoter fragments were labeled with 6-carboxyfluorescein (FAM) dye. The regions protected by H-NS from DNase I cleavage are indicated with red dotted boxes. The sequences of the high-affinity H-NS binding region are shown at the bottom (with the protected region in gray shading), and the start codons are underlined. The black box denotes the conserved H-NS binding motif.

cells due to the large number of genes and large amount of energy required for flagellar biosynthesis and function (77). The energy saved and reduced power consumption might confer additional advantages to the microorganisms, such as growth and stress resistance (78). Swarming motility should be rigorously regulated because of the trade-offs that must occur due to the limitations of cellular resources and physical or chemical constraints (79). The discovery that the horizontally transferred lateral flagellar system was negatively regulated by the H-NS protein provides useful insight into the ecological roles of this global modulator in the widely distributed deep-sea environment.

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