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Role of the Histone-Like Nucleoid Structuring Protein （H-NS） in the Regulation of Virulence Factor Expression and Stress Response in *Vibrio vulnificus*

ABDELAZIZ ELGAML[1,2], AND SHIN-ICHI MIYOSHI[1]*

[1]*Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama University,
1-1-1, Tsushima-Naka, Kita-Ku, Okayama 700-8530, Japan*
[2]*Microbiology and Immunology Department, Faculty of Pharmacy, Mansoura University,
Elgomhouria Street, Mansoura City 35516, Egypt*

Temperature is one of the important parameters regulating the expression of virulence factors in bacteria. The global regulator, a histone-like nucleoid structuring protein （H-NS）, is known to play a crucial role in this regulation. In the present study, we first clarified the role of H-NS in the temperature-dependent regulation of virulence factor production in *Vibrio vulnificus*, including that of the cytolytic toxin （*V. vulnificus* hemolysin: VVH） and the proteolytic enzyme （*V. vulnificus* protease: VVP）. The expression of *hns* itself was subjected to temperature regulation, where *hns* was expressed more at 26℃ than at 37℃. VVH production and the expression of its gene *vvhA* were increased by disruption of the *hns* gene. H-NS appeared to affect the *vvhA* expression by the well-documented transcriptional silencing mechanism. On the other hand, *hns* disruption resulted in the reduction of VVP production and the expression of its gene *vvpE*. H-NS was suggested to positively regulate *vvpE* expression through the increase in the level of the *rpoS* mRNA. Moreover, H-NS was found to contribute to the survival of *V. vulnificus* in stressful environments. When compared to the wild type strain, the *hns* mutant exhibited reduced survival rates when subjected to acidic pH, hyperosmotic and oxidative stress.

Key words：*Vibrio vulnificus* / Temperature / H-NS / Hemolysin / Metalloprotease / Stress response.

INTRODUCTION

Vibrio vulnificus is a common Gram-negative halophilic estuarine bacterium. However, this species is an opportunistic human pathogen causing rapidly progressing fatal septicemia when raw or undercooked seafood contaminated with the pathogen is consumed, or necrotizing wound infections when open wounds are exposed to contaminated water （Oliver and Kaper, 2001; Jones and Oliver, 2009）. This bacterium preferentially affects individuals who are heavy drinkers of alcohol and patients with underlying hepatic diseases or other immunocompromised conditions such as haemochromatosis and β-thalassaemia （Morris, 1988; Hlady and Klontz, 1996; Strom and Paranjpye, 2000）.

A range of virulence factors have been proposed to play a role in the pathogenesis of *V. vulnificus*, among which, the 56-kDa cytolytic toxin designated as *V. vulnificus* hemolysin （VVH） and the 45-kDa proteolytic enzyme designated as *V. vulnificus* protease （VVP） are significantly important （Milton, 2006）. VVH exhibits powerful hemolytic and cytolytic activities （Gray and Kreger, 1985）, and it also causes vasodilation that may play a role in hypotensive septic shock （Kook et al., 1996）. On the other hand, VVP causes serious hemorrhagic skin damage through digestion of the vascular basement membrane, especially type IV collagen forming the framework of the membrane （Miyoshi et al., 2001）. It also induces edema formation through the induction of exocytotic histamine release from mast cells （Miyoshi et al., 2003） and activation of the factor XII-plasma kallikrein-kinin cascade （Miyoshi et al., 2004）.

＊Corresponding author. Tel: +81-86-251-7966, Fax: +81-86-251-7926, E-mail : miyos-s（a）cc.okayama-u.ac.jp

During the infection process, *V. vulnificus* must sense and withstand changes in environmental factors between the natural reservoir and the human host. One very important difference in many respects is the environmental temperature (Lee et al., 2007). The regulation systems for the production of VVH and VVP, and expression of their genes (*vvhA* and *vvpE*, respectively) are subjected to temperature-dependent regulation. Namely, VVH production, as well as *vvhA* expression, is maximum at 37℃ (around the human intestinal temperature). On the other hand, both VVP production and *vvpE* expression are maximum at 26℃ (around the estuarine temperature in the summer season in Japan) (Kawase et al., 2004; Elgaml et al., 2014).

Temperature-dependent regulation of virulence genes has been extensively studied in many pathogenic bacterial species such as *Clostridium perfringens*, *Escherichia coli*, *Shigella* and *Salmonella* species, and the global regulator, a histone-like nucleoid structuring protein (H-NS), is known to play important roles in this regulation (Hurme and Rhen, 1998; Klinkert and Narberhaus, 2009).

H-NS is a member of the family of small nucleoid-associated proteins including the factor for inversion stimulation, the heat-unstable protein, and the integration host factor (Dorman and Deighan, 2003; Dorman, 2004). H-NS affects the expression of a large number of unrelated genes encoding housekeeping proteins as well as virulence factors, and it plays a fundamental role in the bacterial response to the environmental temperature (Hurme and Rhen, 1998; Klinkert and Narberhaus, 2009). The *hns* mutants in many pathogenic bacterial species are highly pleiotropic, suggesting that H-NS influences a broad spectrum of physiological processes (Atlung and Ingmer, 1997; Hommais et al., 2001; Atlung and Hansen, 2002). H-NS consists of an N-terminal oligomerization domain connected by a flexible linker to a C-terminal nucleic acid binding domain (Atlung and Ingmer, 1997; Cerdan et al., 2003; Nye and Taylor, 2003; Dorman, 2004). Both the oligomerization and DNA binding domains are required for the biological activity of H-NS, which includes DNA condensation and regulation of transcription (Spurio et al., 1997; Dame et al., 2001). In transcriptional regulation, H-NS has been shown to be a silencer protein, which negatively affects gene expression by the direct binding to promoter sites of the target genes, and to prefer highly curved AT-rich sequences (Owen-Hughes et al., 1992; Ueguchi and Mizuno, 1993; Lang et al., 2007). H-NS itself is subjected to the temperature control, since the formation of the higher-order functional oligomers and the DNA binding capacity are reduced at 37℃ (Ono et al., 2005). On the other hand, H-NS is also known to affect positively the expression of a broader spectrum of genes by increase in the mRNA stability (Brescia et al., 2004).

According to the genomic annotation, we found the H-NS homologue in *V. vulnificus*, for instance, in VV1_2923 of strain CMCP6, VV1346 of strain YJ016 and VVMO6_01867 of strain MO6-24/O. Since the H-NS protein affects a broad spectrum of physiological processes and plays a fundamental role in bacterial response to temperature, we decided to evaluate the role of H-NS in the temperature-dependent regulation of the expression of virulence factors including VVH and VVP, as well as in the response to stress factors in *V. vulnificus*.

MATERIALS AND METHODS

Bacterial strains, plasmids and cultivation media

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown in Luria-Bertani (LB) broth (1.0% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.0) or on LB plates containing 1.5% agar, and, when required, an appropriate antibiotic was added to the media as follows: chloramphenicol 10 μg/mL, streptomycin 50 μg/mL and kanamycin 50 μg/mL.

For cultivation of *V. vulnificus* strains, TYE broth (0.5% tryptone, 0.25% yeast extract, 2.0% NaCl, 25 mM K_2HPO_4, pH 7.5) was used. Thiosulfate-citrate-bile-salts-sucrose (TCBS) agar plates containing chloramphenicol 10 μg/mL were used to select *hns* or the *luxO* mutant.

Measurement of the bacterial growth

V. vulnificus strains were cultivated in TYE broth, and the growth was monitored by measuring the optical density at 600 nm (OD_{600}) of the cultures every 1 h. Then, the growth curves were drawn. Thereafter, the early log phase, late log phase and early stationary phase were determined.

Real time reverse transcription PCR

Cultures of *V. vulnificus* strains cultivated at 26℃ or 37℃ were collected at the early log, late log or early stationary phase and treated with RNAprotect Bacterial Reagent (Qiagen GmbH, Hilden, Germany). Then, total RNA was extracted from each culture by using RNeasy Mini Kit (Qiagen GmbH) according to the manufacturer's manual.

Real time reverse transcription PCR (RT-PCR) was conducted using an iScriptTM one-step RT-PCR kit with SYBR® green (Bio-Rad Laboratories, Berkeley, California, USA). The reaction mixtures were composed of 12.5 μL of SYBR® Green PCR Master Mix (Bio-Rad Laboratories), 0.75 μL of each of the forward and reverse primers (the final concentration of each primer

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant features[a]	Reference
Vibrio vulnificus		
CDC B3547	Human clinical isolate; virulent.	(Senoh et al., 2005)
HNSD713	CDC B3547 strain, *hns*::Cm[r].	This study
HNSR813	Revertant strain of HNSD713.	This study
AAD613	CDC B3547 strain, *luxO*::Cm[r].	This study
Escherichia coli		
SY327 λ *pir*	△(*lac pro*), *argE*(Am), *rif*, *nalA*, *recA56*, *rpoB*, λ *pir*, Sm[r], host for π-requiring plasmids.	(Miller and Mekalanos, 1988)
SM10 λ *pir*	*thi-1*, *thr*, *leu*, *tonA*, *lacY*, *supE*, *recA*::RP4-2-Tc::Mu, λ *pir*, *oriT* of RP4, Km[r]; conjugational donor.	(Simon et al., 1983)
Plasmids		
pKTN701	*R6K*-ori suicide vector for gene replacement; Cm[r].	(Nishibuchi et al., 1991)
pKTSD713	pKTN701 with *hns*; Cm[r].	This study
pKTAD613	pKTN701 with *luxO*; Cm[r].	This study

[a]Cm[r], chloramphenicol-resistant; Sm[r], streptomycin-resistant; Km[r], kanamycin-resistant.

was 200 nM), 0.5 μL of iScript™ one-step RT-PCR reverse transcriptase, and 0.5 μL of RNA (100 ng/μL), and nuclease free water was added to adjust the final volume to 25 μL. The primer sets used (*vvhA*, *vvpE*, *rpoS*) in the experiments are shown in Table 2.

The reaction for the reverse transcription was performed in the MiniOpticon™ real time PCR detection system (Bio-Rad Laboratories) at 50℃ for 10 min. Thereafter, the reverse transcriptase was inactivated by heating at 95℃ for 5 min, and PCR amplification of the target gene was performed for 40 cycles of denaturation at 95℃ for 10 s and annealing at an appropriate temperature for 30 s. DNA polymerization was conducted in the range of temperatures from 55℃ to 95℃ within 20 min to obtain the melting curve for determining the PCR amplification specificity. The RT reaction without the reverse transcriptase was used as a negative control for each gene. The 16S rRNA was used as an internal standard. The amount of each mRNA was determined by comparing the C_t value of each sample to that of the standard 16S rRNA.

Construction of *hns* and *luxO* knock-out mutants and their revertants

The mutants were constructed by the single crossover homologous recombination as described previously (Nishibuchi et al., 1991; Funahashi et al., 2002).

The 250 bp region of *hns* was amplified by PCR using a primer set *hns*-2, a forward primer containing the recognition sequence for *Sal*I (GTCGAC) and a reverse primer containing the recognition sequence for *Eco*RI (GAATTC) (Table 2), and digested with *Sal*I and *Eco*RI. The *Sal*I-*Eco*RI-digested PCR product was inserted into a similarly digested vector pKTN701 (Nishibuchi et al., 1991). The hybrid plasmid obtained was transformed into *E. coli* SY327 λ *pir*, then into *E. coli* SM10 λ *pir*. Thereafter, it was transferred to *V. vulnificus* CDC B3547 by conjugation, and the conjugates were cultivated on TCBS agar plates containing chloramphenicol 10 μg/mL. One suitable *hns* mutant named strain HNSD713 was selected by 48 h cultivation at 37℃. The revertant named strain HNSR813 was obtained by repeated sub-culturing of strain HNSD713 in LB broth without chloramphenicol at 37℃. Disruption and reversion of the *hns* gene were confirmed by PCR using the primer set *hns*-3 (Table 2).

Inactivation of the *luxO* gene was also done by similar method. The 781 bp region of *luxO* was amplified by PCR using the primer set *luxO*-1, a forward primer containing the recognition sequence for *Xba*I (TCTAGA) and a reverse primer containing the recognition sequence for *Eco*RI (GAATTC) (Table 2), and digested with *Xba*I and *Eco*RI. The *Xba*I-*Eco*RI digested PCR product was inserted into the suicide vector pKTN701 by the same method as described above, and the recombinant plasmid obtained was used for construction of the *luxO* disruptant named strain AAD613 by the same method as described above. Our recent studies using other *V. vulnificus* strains showed that this genetic method caused inactivation of the *luxO* gene (Elgaml et al., 2013, 2014).

TABLE 2. Oligonucleotide primers used

Gene/Primer	Accession number		Nucleotide sequence 5' ~ 3'	Position
hns-1	CP002469	Forward	AACTGTTGTTGCAGAGCGTC	102－121
		Reverse	AGCAAGTGCGCTGATTAGTG	221－240
hns-2[a]	CP002469	Forward	GCGTCGACCAACTGTTGTTGCAGAGCGT	101－120
		Reverse	GCGAATTCGGTGTACGACCTTGACCAGT	331－350
hns-3	CP002469	Forward	TCTGTTGAACATTCGCAGCC	21－40
		Reverse	TCTTCTAGAGATTTACCCGC	376－395
hns-up	AE016795	Forward	GATGAATGCCATGGTTGCAG	64－83
		Reverse	CCTGCTTTCTGGTTTGATGG	387－406
hns-down	AE016795	Forward	GCGGAGAATGTTGTACCAAC	63－82
		Reverse	TGGGCAGACATGGTACTTTG	458－477
luxO-1[a]	BA000037	Forward	GCTCTAGAGGTCACGGTAAACAACGCTATC	354－375
		Reverse	GCGAATTCGTTGTTAAGCACCACCACATTAC	1112－1134
luxO-2	BA000037	Forward	CCCTCTATCGCTCCTATCTCATGC	68－91
		Reverse	GTTCAAAGGGCTGGTTCAAAGGAG	1166－1189
luxO-up	CP002469	Forward	GGAGTCTGCATACAAGATCG	361－380
		Reverse	TCGATCCTGTACTGGAAGTG	754－773
luxU	BA000037	Forward	CAGAAGAAAATTGCCTCGTTGACAG	49－73
		Reverse	TGCTACCACATCTTGCGTTTCATC	295－318
16S rRNA-1	X76333	Forward	CATGATAGCTTCGGCTCAAA	171－190
		Reverse	TAGTGCTATTAACACTACCAC	447－467
16S rRNA-2	X76333	Forward	GATAGCTTCGGCTCAAAGAG	174－193
		Reverse	TTCCAGTGTGGCTGATCATC	295－314
vvhA	AB124803	Forward	AAGTGCGTGCTACACACAAG	115－134
		Reverse	CATCTGACGTTGCTGATTGC	219－238
vvpE	DQ923325	Forward	ATCCGTTCAAGCTCGTAGTC	120－139
		Reverse	CGACAACGGCAGTATCAAAC	240－259
rpoS	AE016795	Forward	ACTTCGCGAGGAGTTTGATG	120－139
		Reverse	TTCATCGCCTCGTAATGCAC	236－255

[a] The artificial restriction enzyme site is indicated by the underline.

Assay of the activity of VVH and VVP

V. vulnificus strains were grown in TYE medium until the early log phase, late log phase and early stationary phase. Then, cell free culture supernatants were prepared from these cultures by centrifugation (at 12,000 *g* for 5 min at 4℃) and filtration (through 0.2 μm Millipore filter).

The activity of VVH was assayed with 1% sheep erythrocytes as described by Shinoda et al. (1985). Briefly, the sample (0.6 mL) was allowed to act on the erythrocytes (0.6 mL) at 37℃ for 2 h in 20 mM Tris-HCl buffer containing 0.9% NaCl (pH 7.5). Thereafter, the reaction mixtures were centrifuged at 1000 *g* for 5 min, and the amount of hemoglobin released from the disrupted erythrocytes was determined by measuring the absorbance of the supernatant at 540 nm. One hemolysin unit (HU) was defined as the amount of VVH eliciting 50% hemoglobin release.

The activity of VVP was assayed with azocasein (Sigma-Aldrich, St. Louis, MO, USA) as described by Miyoshi et al. (1987). Briefly, the sample was allowed to act at 30℃ for an appropriate time on 1.0 mg of azocasein in a total volume of 0.6 mL of 50 mM Tris-HCl buffer (pH 8.0). The reaction was stopped by the addition of 1.4 mL of 5% trichloroacetic acid. After centrifugation at 5000 *g* for 5 min, an aliquot of the supernatant was withdrawn and mixed with the same volume of 0.5 M NaOH. Thereafter, the absorbance at 440 nm was

measured. One protease unit (PU) was defined as the amount of VVP hydrolyzing 1 µg of the substrate in 1 min.

Western blot analysis

V. vulnificus strains were grown in TYE medium until the early log phase, late log phase and early stationary phase. Then, cell free culture supernatants were prepared from these cultures by centrifugation (at 12,000 *g* for 5 min at 4℃) and filtration (through 0.2 µm Millipore filter). Thereafter, proteins in the sample were precipitated by mixing with the same volume of 25% trichloroacetic acid, and collected by centrifugation for 5 min at 15000 *g* at 4℃. Thereafter, the proteins collected were washed with 100% ethanol, treated with the buffer containing 2% sodium dodecyl sulfate (SDS) at 100℃ for 5 min and subjected to SDS-PAGE on the PhastSystem™ using a PhastGel™ Gradient 10-15 (GE Health Bio-Sciences, Little Chalfont, Buckinghamshire, United Kingdom). After SDS-PAGE, the proteins separated were transferred to a polyvinylidene difluoride (PVDF) membrane (GE Health Bio-Sciences). The membrane with the bound proteins was then incubated with rabbit IgG antibody against VVH or VVP, and the antigen-antibody complex was visualized using the antibody against rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and the chromogenic substrate 4-methoxy-1-naphthol and hydrogen peroxide.

Survival assay

The culture of *V. vulnificus* (100 µL) grown until the middle log phase in TYE broth at 26℃ was added to fresh TYE medium (900 µL) and subjected to one of the test stress conditions described below, and incubated at 26℃. At indicated time points, an aliquot was withdrawn to determine the survival rate. The stress conditions tested were acidic (pH 4.0), hyperosmotic (2.7 M NaCl) or oxidative (2 mM H_2O_2). In these experiments, non-stress conditions were defined as pH 7.5, 0.35 M NaCl and 0 mM H_2O_2, respectively.

Statistical analysis

To evaluate the significance of difference in the results, all experiments were repeated at least three times, the data were analyzed by the Student *t*-test, and the *P* value less than 0.05 was considered significantly different.

RESULTS

Temperature-dependent expression of *hns*

Although the *hns* gene was significantly expressed at both 26℃ and 37℃, its expression was significantly
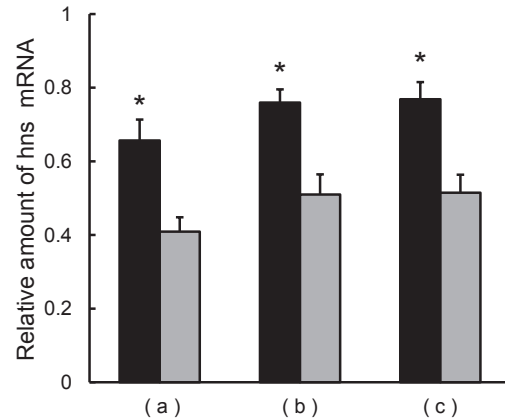

FIG. 1. Effects of cultivation temperatures on the expression of *hns* in *V. vulnificus*.
V. vulnificus strain CDC B3547 was cultivated in TYE broth at 26℃ (black bar) or 37℃ (grey bar), total RNA was extracted at the early log phase (a), late log phase (b) and early stationary phase (c), and the level of *hns* mRNA was measured by real time RT-PCR. The amount of mRNA was represented using the amount of 16S rRNA as 1.00. The data represent the mean + S.D. of three experiments. The asterisk (*) indicates the significant difference (P < 0.05) between cultivation at 26℃ and at 37℃.

higher at 26℃ (Fig.1) indicating that *V. vulnificus* H-NS works more effectively at 26℃.

Construction of the *hns* knock-out mutant and its revertant

H-NS is a silencer protein with a fundamental role in the bacterial response to the environmental temperature (Owen-Hughes et al., 1992; Ueguchi and Mizuno, 1993; Lang et al., 2007). As shown in Fig.1, *hns* was expressed more at 26℃. Therefore, it is considered that H-NS may down-regulate *vvhA* expression, because VVH production is apparently lower at 26℃ (Elgaml et al., 2014). On the other hand, H-NS also enhances the expression of the genes by increase in the mRNA stability (Brescia et al., 2004). In line with this, it is suggested that H-NS may positively regulate *vvpE* expression because VVP is highly produced at 26℃ (Kawase et al., 2004; Elgaml et al., 2014). To support this hypothesis, we constructed the *hns* mutant (strain HNSD713) and its revertant (strain HNSR813) from the wild type strain CDC B3547. The disruption and reversion of the *hns* gene were confirmed by PCR (data not shown). Moreover, the mRNA level of *VV1_2922* and *VV1_2924*, which are located upstream and downstream to *hns*, respectively, were examined by real time RT-PCR. The results showed the comparable expression of both genes among the three strains, indicating that disruption of *hns* did not affect the upstream and downstream genes (data not shown).

The growth of *V. vulnificus*

Disruption of *hns* resulted in the decreased growth of *V. vulnificus* (Fig.2). The *hns* mutant strain HNSD713 exhibited slow growth compared to the wild type strain CDC B3547. On the other hand, the growth of strain HNSR813, the revertant from strain HNSD713, was the same as that of the wild type strain (Fig.2). This finding indicates that H-NS may be a critical factor for sufficient
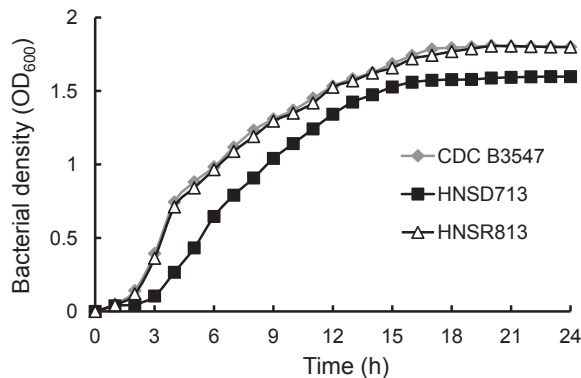

FIG. 2. Effect of *hns* disruption on the growth of *V. vulnificus*. Strain CDC B3547 (grey diamond), HNSD713 (black square) and HNSR813 (white triangle) were cultivated in TYE broth at 26℃, and the bacterial growth (OD$_{600}$) was measured every 1 h. Data represent the mean of three experiments.

bacterial growth.

The *vvhA* expression and VVH production

The wild type strain CDC B3547, the *hns* mutant HNSD713, and the revertant HNSR813 were compared for *vvhA* expression (Fig.3A) and VVH production (Fig.3B and 3C). Transcription of *vvhA* was significantly increased by disruption of the *hns* gene. The usual transcription level was restored in the revertant, which showed a comparable expression level of *vvhA* to that of the wild type strain (Fig.3A). In addition, VVH production was also increased by the *hns* mutation, and the usual level of production was restored in the revertant (Fig.3B and 3C). The *vvhA* gene makes an operon with the up-stream gene *vvhB* (senoh et al., 2005). By using the Virtual Footprint program (http://www.prodoric.de/vfp/), the putative H-NS binding site could be identified within the promoter region of *vvhBA* (Fig.4). Therefore, it is suggested that H-NS negatively regulates VVH production at the transcriptional stage of the *vvhA* gene.

The *vvpE* expression and VVP production

The wild type strain, the *hns* mutant and the revertant were compared for *vvpE* expression (Fig.5A) and VVP production (Fig.5B and 5C). Transcription of *vvpE* was significantly decreased by the *hns* disruption, while the usual level of transcription was restored in the revertant

FIG. 3. Effect of *hns* disruption on *vvhA* expression (A), VVH activity (B) and VVH production (C) in *V. vulnificus*. (A) Strain CDC B3547 (grey bar), HNSD713 (black bar) and HNSR813 (white bar) were cultivated in TYE broth at 26℃, and total RNA was extracted at the early log phase (a), late log phase (b) and early stationary phase (c), and the level of *vvhA* mRNA was measured by real time RT-PCR. The amount of mRNA was represented using the amount of 16S rRNA as 1.00. (B) Culture supernatants were prepared from the early log phase (a), late log phase (b) and early stationary phase (c) of the growth of strain CDC B3547 (grey bar), HNSD713 (black bar) and HNSR813 (white bar). The activities of VVH (HU/mL) in the culture supernatants were determined using 1% sheep erythrocytes, and the specific hemolysin activity (HU/OD$_{600}$) was calculated. In (A) and (B), the data represent the mean + S.D. of three experiments. The asterisk (*) indicates the significant difference (P < 0.05) between strain HNSD713 and both strain CDC B3547 and HNSR813. (C) The proteins in the culture supernatants were precipitated by the addition of the same volume of 25% tricloroacetic acid. The proteins precipitated were then washed by 100% ethanol, dissolved in SDS sample buffer and boiled for 5 min, and an aliquot of the sample prepared was subjected to SDS-PAGE. Thereafter, the proteins were transferred to a PVDF membrane, and VVH antigens were detected with the antibody against purified VVH. Lane 1; strain CDC B3547, lane 2; strain HNSD713 and lane 3; strain HNSR813.

−236   −211
TATATTAGATCACTTTTAAAACAATA

−210   −35   −10   −35   −141
ATAGATCAGATATTAATCTGTTGATTTT<u>GTGATA</u>ATGAGCCAAAAAATACTTT<u>TATTTTATTTATAT</u>GAA

−140   −10   **H-NS**   −71
ATATTTTCAGGAT<u>TATT</u>**AATAAATAGC**CAACAGGATTTTGGTGCATATCTATTCTCAAGGACGAACCAAA

−70   **H-NS**   −35   −10   RBS   −1
CAATC**TCCATACAAA**TATT<u>AATG</u>TTATGGAGAAAATAAC<u>AATAAT</u>AACCCTTACTCGTA<u>ATGAGG</u>AATCT

FIG. 4. Putative H-NS binding sites within the promoter region of the *vvhBA* operon.
The promoter region of the *vvhBA* operon (Genbank accession number M34670) was analyzed by using the Virtual Footprint program (http://www.prodoric.de/vfp/). Putative H-NS binding sites are shown as bold letters in the boxes and labeled as H-NS. Additionally, possible promoters within the sequence are underlined and labeled as -10 or -35, and the putative ribosomal binding site is underlined and labeled as RBS.
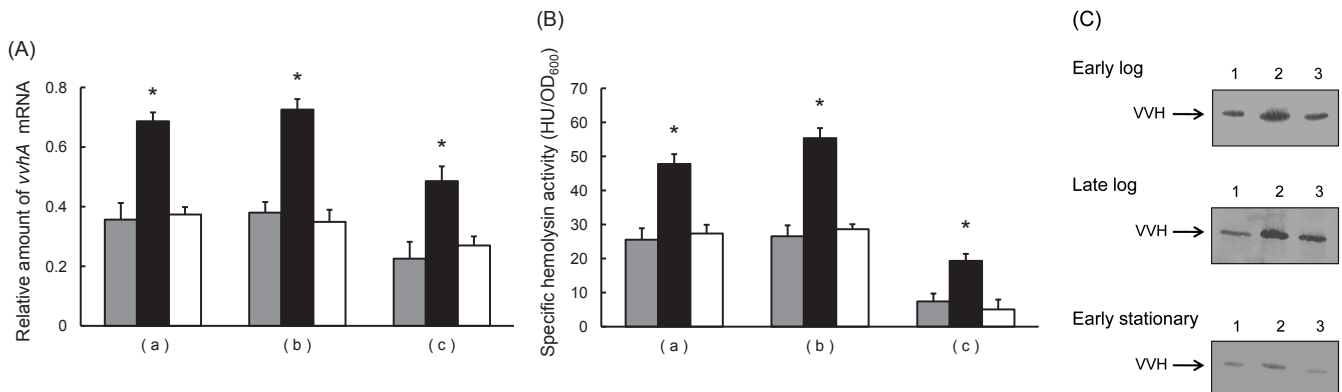
(A)

(B)

(C)

FIG. 5. Effect of *hns* disruption on *vvpE* expression (A), VVP activity (B) and VVP production (C) in *V. vulnificus*. (A) Strain CDC B3547 (grey bar), HNSD713 (black bar) and HNSR813 (white bar) were cultivated in TYE broth at 26℃, and total RNA was extracted at the early log phase (a), late log phase (b) and early stationary phase (c), and the level of *vvpE* mRNA was measured by real time RT-PCR. The amount of mRNA was represented using the amount of 16S rRNA as 1.00. (B) Culture supernatants were prepared from the early log phase (a), late log phase (b) and early stationary phase (c) of growth of strain CDC B3547 (grey bar), HNSD713 (black bar) and HNSR813 (white bar). The activities of VVP (PU/mL) in the culture supernatants were determined using azocasein as the substrate, and the specific protease activity (PU/OD$_{600}$) was calculated. In (A) and (B), the data show the mean + S.D. of three experiments. The asterisk (*) indicates the significant difference (P < 0.05) between strain HNSD713 and both strain CDC B3547 and HNSR813. (C) The proteins in the culture supernatants were precipitated by the addition of the same volume of 25% tricloroacetic acid. The proteins precipitated were then washed by 100% ethanol, dissolved in SDS sample buffer and boiled for 5 min, and an aliquot of the sample prepared was subjected to SDS-PAGE. Thereafter, the proteins were transferred to a PVDF membrane, and VVP antigens were detected with the antibody against purified VVP. Lane 1; strain CDC B3547, lane 2; strain HNSD713 and lane 3; strain HNSR813.

(Fig.5A). Moreover, VVP production was apparently decreased by *hns* mutation. The difference between the wild type strain and the mutant was not significant during the log phase; however, it became evident during early stationary phase. The reduced protease production revealed by the mutant was reversed in the revertant (Fig.5B and 5C). These results prove that the *vvpE* transcription and VVP production might be under the positive regulation of H-NS.

The level of the *rpoS* mRNA
It is well documented that VVP production in *V. vulnificus* is positively regulated by the stationary-phase sigma factor, RpoS (Jeong et al., 2001, 2003). It was recently reported that in *Vibrio cholerae*, H-NS enhances the production of RpoS by increasing the stability of the *rpoS* mRNA, which in turn increases the production of the hemagglutinin/protease (Silva et al., 2008; Wang et al., 2012). It is thereby suggested that *V. vulnificus* H-NS can increase *vvpE* expression and consequently VVP production through enhancement of RpoS production. Consistent with this, we next studied the effect of *hns* disruption on the level of the *rpoS* mRNA.

As shown in Fig.6, the level of the *rpoS* mRNA was significantly decreased by the *hns* mutation, and this decreased level of transcription was reversed in the
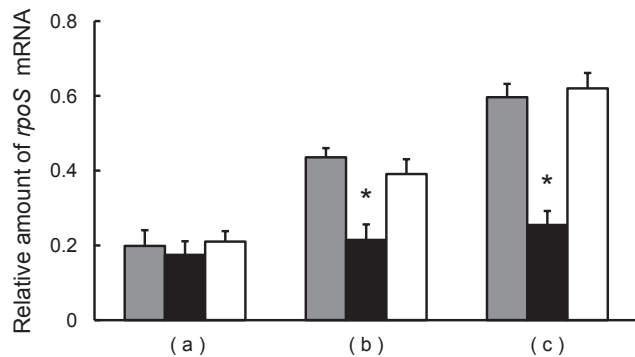
FIG. 6. Effect of *hns* disruption on the level of the *rpoS* mRNA in *V. vulnificus*.
Strain CDC B3547 (grey bar), HNSD713 (black bar) and HNSR813 (white bar) were cultivated in TYE broth at 26℃, and total RNA was extracted at the early log phase (a), late log phase (b) and early stationary phase (c), and the level of *rpoS* mRNA was measured by real time RT-PCR. The amount of mRNA was represented using the amount of 16S rRNA as 1.00. The data represent the mean + S.D. of three experiments. The asterisk (*) indicates the significant difference (P < 0.05) between strain HNSD713 and both strain CDC B3547 and HNSR813.

revertant (Fig.6). These results suggest that H-NS indirectly stimulates the *vvpE* transcription in *V. vulnificus*.

Independence of the quorum sensing system

We next studied the interaction of the H-NS system with the quorum sensing (QS) system, another global regulation system. In *V. vulnificus*, like the H-NS system, the QS system works more strongly at 26℃. The QS system is well known to inhibit VVH production but to stimulate VVP production (Kim et al., 2003; Elgaml et al., 2013, 2014). Therefore, we carried out the disruption of *luxO* encoding the central response regulator of QS system to examine if H-NS is under the control of the QS system or not. When the *luxO* mutant was compared to the wild type strain, no significant difference in the transcription of the *hns* gene was observed (data not shown). Moreover, we tested if H-NS regulates SmcR, the master transcriptional regulator in the QS system (Shao and Hor, 2001; Kim et al., 2003; Kawase et al., 2004), or not. When the *hns* mutant was compared to the wild type strain, no significant difference in the transcription of the *smcR* gene was found (data not shown). These findings demonstrate that the H-NS and QS systems are independent of each other.

The survival of *V. vulnificus*

To study the role of H-NS in the ability of *V. vulnificus* to surviving at diverse environmental stresses, the wild type strain, the *hns* mutant and the revertant were exposed to various challenges, and survival rates were
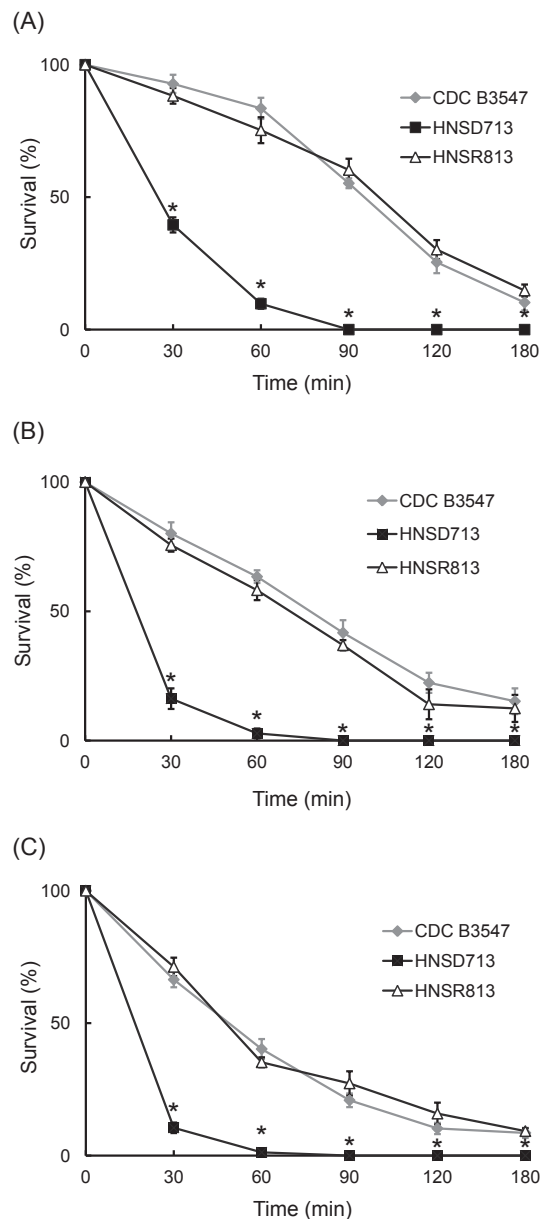

FIG. 7. Survival of *V. vulnificus* subjected to acidic, hyperosmotic or oxidative stress.
V. vulnificus strain CDC B3547 (grey diamond), HNSD713 (black square) and HNS813 (white triangle) were grown to the middle log phase at 26℃ and subjected to acidic (pH 4.0, A), hyperosmotic (2.7 M NaCl, B), or oxidative (2 mM H_2O_2, C) conditions in TYE broth. At different time points, an aliquot of the bacterial culture was withdrawn to determine the survival rate. The data represent the mean + S.D. of three experiments. The asterisk (*) indicates the significant difference (P < 0.05) between strain HNSD713 and both strains CDC B3547 and HNSR813.

determined. Over the time period of 180 min, the *hns* mutant (strain HNSD713) was significantly less resistant to the acidic condition of pH 4.0 (Fig.7A). In the

hyperosmotic condition, 2.7 M NaCl, H-NS was seen to enhance the bacterial survival markedly. The cell numbers of strain HNSD713 was rapidly dropped, while those of CDC B3547 (the wild type strain) and HNSR813 (the revertant) remained at high levels (Fig.7B). Strain HNSD713 was also more sensitive to 2 mM H_2O_2 (Fig.7C). These results apparently indicated that *V. vulnificus* H-NS is important for the bacterial survival under the stressful conditions.

DISCUSSION

Temperature has a profound effect on many cellular processes, and therefore bacteria must possess molecular thermosensing devices in order to adjust to changes in the environmental temperature (Hurme and Rhen, 1998; Klinkert and Narberhaus, 2009). Transfer of the bacterium from a natural reservoir to an infectious host offers a number of cues, which induce the signal transmission to turn on the virulence potentials. Signals from the changing environmental factors between the natural reservoir and host are relayed to specific genes by cognate signal transduction systems, resulting in the expression of genes including specific virulence factor genes required for *in vivo* survival and growth at an appropriate time and in a tightly regulated fashion. The most important difference between the natural reservoir and host in many respects is the environmental temperature (Heithoff et al., 1997; Lee et al., 1999; Kim et al., 2007; Lee et al., 2007).

Specific silencer proteins, which bind to bent DNA and modulate the pre-existing DNA loops, are likely to play important roles in the temperature-dependent regulation of virulence genes. Among these silencer proteins is H-NS (Hurme and Rhen, 1998; Klinkert and Narberhaus, 2009). Since H-NS is an important global regulator involved in transcriptional regulation of a variety of environmentally responsive genes in many Gram-negative bacteria (Beloin and Dorman, 2003; Poore and Mobley, 2003; Tendeng and Bertin, 2003; Chen and Wu, 2005; Ghosh et al., 2006; Navarre et al., 2006; Corbett et al., 2007; Lithgow et al., 2007; Olekhnovich and Kadner, 2007; Banos et al., 2008; Silva et al., 2008; Liu et al., 2012; Wang et al., 2012; Mou et al., 2013), we decided to evaluate the role of H-NS in the temperature-dependent regulation of virulence factors as well as the response to stress factors in *V. vulnificus*.

Here, we found that H-NS itself was subjected to the temperature regulation in *V. vulnificus*. By real time RT-PCR, *hns* was indicated to be expressed more at 26℃ than at 37℃. We therefore hypothesized that H-NS can down-regulate VVH production because less VVH is produced at 26℃. Disruption of *hns* resulted in a significant increase in *vvhA* expression and VVH production. In the transcriptional regulation, H-NS has been shown to block the gene expression by the binding to the promoter site of the target gene because it prefers highly curved AT-rich sequences (Owen-Hughes et al., 1992; Ueguchi and Mizuno, 1993; Lang et al., 2007). Indeed, we could find the putative curved AT-rich sequence and the high scoring H-NS binding site within the promoter region of *vvhBA*, which supported that H-NS can directly down-regulate the expression of *vvhBA*.

In contrast, by disruption of *hns*, both *vvpE* expression and VVP production were significantly decreased, indicating that H-NS is a positive regulator of VVP production. In *V. cholerae*, H-NS enhances the production of RpoS by increasing the stability of *rpoS* mRNA, which results in the increased production hemagglutinin/protease (Silva et al., 2008; Wang et al., 2012). Since it is well-documented that VVP production is positively regulated by RpoS (Jeong et al., 2001, 2003), we next studied the effect of *hns* disruption on *rpoS* expression. The level of the *rpoS* mRNA was significantly decreased in the *hns* mutant; therefore, it is thought that the enhanced level of *rpoS* mRNA causes the increase in VVP production. This regulatory mechanism of H-NS in both *V. cholerae* and *V. vulnificus* is in contrast to that in *E. coli*, in which H-NS negatively affects the expression of the *rpoS* gene (Silva et al., 2008). This odd result clearly suggests that the regulation of *rpoS* expression has diverged to promote long-term colonization of different ecological niches. While *E. coli* is most commonly found in the gastrointestinal tracts of warm-blooded animals, both *V. cholerae* and *V. vulnificus* can persist for longer periods in the aquatic ecosystems.

Like other Gram-negative bacteria, *V. vulnificus hns* mutant exhibited a slow growth phenotype. It has been suggested that the inadequate overexpression of numerous cellular proteins and defective chromosome replication contribute to the slow growth of *hns* mutants (Kaidow et al., 1995; Atlung and Hansen, 2002; Hengge-Aronis, 2002). Moreover, H-NS was shown to possibly affect the survival of *V. vulnificus* in stressful conditions. Namely, our *hns* mutant was more sensitive than the wild type and revertant strains to acidic, high osmotic or oxidative conditions. Most striking was the high sensitivity to the oxidative stress. Reactive oxygen species may result from the oxidative burst of host macrophages during infection or from UV irradiation of water. Therefore, oxidative resistance plays a role in the persistence of pathogens in the human host and in survival in the aquatic environments (Storz and Zheng, 2000; Nowakowska and Oliver, 2013). Moreover, these results may emphasize the importance of H-NS in the ability of *V. vulnificus* to withstand the higher osmolarity condi-

tions in the marine environment (Nowakowska and Oliver, 2013; Rao et al., 2013) or higher acidic conditions in the human stomach (Rhee et al., 2002, 2005).

Taken together, our data suggest that H-NS regulates the expression of *vvhA* and *vvpE* genes encoding VVH and VVP, respectively, which are responsible for the enormous tissue damage during *V. vulnificus* infection. Namely, *V. vulnificus* produces a significant amount of VVP only in the interstitial tissue of limbs, where the temperature is lower than in the small intestine and the blood-stream, and the VVP produced causes serious hemorrhagic and edematous skin damage (Miyoshi, 2006). On the other hand, a sufficient amount of VVH is produced in the small intestine, which results in the acceleration of the bacterial invasion into the blood-stream. In the case of VVH, H-NS appears to affect the *vvhA* expression by the well-documented transcriptional silencing mechanism. However, in the case of VVP, the gene expression is positively affected by H-NS through enhancing the *rpoS* mRNA level. Moreover, as a global regulator, H-NS may be involved in the regulation of a variety of processes required for withstanding diverse environmental stresses.

ACKNOWLEDGMENT

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