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Role of the Histone-Like Nucleoid Structuring Protein in the Regulation of *rpoS* and RpoS-Dependent Genes in *Vibrio cholerae*▽†

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Production of the Zn-metalloprotease hemagglutinin (HA)/protease by *Vibrio cholerae* has been reported to enhance enterotoxicity in rabbit ileal loops and the reactogenicity of live cholera vaccine candidates. Expression of HA/protease requires the alternate sigma factor σ^S (RpoS), encoded by *rpoS*. The histone-like nucleoid structuring protein (H-NS) has been shown to repress *rpoS* expression in *Escherichia coli*. In *V. cholerae* strains of the classical biotype, H-NS has been reported to silence virulence gene expression. In this study we examined the role of H-NS in the expression of HA/protease and motility in an El Tor biotype strain by constructing a Δ*hns* mutant. The Δ*hns* mutant exhibited multiple phenotypes, such as production of cholera toxin in nonpermissive LB medium, reduced resistance to high osmolarity, enhanced resistance to low pH and hydrogen peroxide, and reduced motility. Depletion of H-NS by overexpression of a dominant-negative allele or by deletion of *hns* resulted in diminished expression of HA/protease. Epistasis analysis of HA/protease expression in Δ*hns*, Δ*rpoS*, and Δ*hns* Δ*rpoS* mutants, analysis of RpoS reporter fusions, quantitative reverse transcription-PCR measurements, and ectopic expression of RpoS in Δ*rpoS* and Δ*rpoS* Δ*hns* mutants showed that H-NS posttranscriptionally enhances RpoS expression. The Δ*hns* mutant exhibited a lower degree of motility and lower levels of expression of *flaA*, *flaC*, *cheR-2*, and *motX* mRNAs than the wild type. Comparison of the mRNA abundances of these genes in wild-type, Δ*hns*, Δ*rpoS*, and Δ*hns* Δ*rpoS* strains revealed that deletion of *rpoS* had a more severe negative effect on their expression. Interestingly, deletion of *hns* in the *rpoS* background resulted in higher expression levels of *flaA*, *flaC*, and *motX*, suggesting that H-NS represses the expression of these genes in the absence of σ^S. Finally, we show that the cyclic AMP receptor protein and H-NS act along the same pathway to positively affect RpoS expression.

Cholera is an acute diarrheal disease characterized by the passing of voluminous rice water stool. *Vibrio cholerae* of serogroups O1 and O139 continues to cause seasonal cholera outbreaks that affect highly populated regions in Asia, Africa, and Latin America. The bacterium can persist outside the human host and alternates between planktonic and biofilm community lifestyles. *V. cholerae* is a highly motile organism with a single sheathed polar flagellum. It colonizes the small intestine and expresses a variety of virulence determinants, such as the toxin-coregulated pilus (TCP) and cholera toxin (CT), encoded by *tcpA* (major subunit) and *ctxA*, respectively (13, 27). *V. cholerae* also secretes a Zn-dependent metalloprotease that displays a broad range of potentially pathogenic activities: hemagglutinin/protease (HA/protease), encoded by *hapA* (14, 18, 34, 59–61). HA/protease contributes to the reactogenicity of live cholera vaccine candidates and enhances enterotoxicity in the rabbit ileal loop model (4, 15, 51). Transcription of *hapA* requires the quorum-sensing regulator HapR (25), the cyclic AMP receptor protein (CRP) (3, 49, 31), and the alternative sigma factor S (RpoS), encoded by *rpoS* (49, 62).

The histone-like nucleoid structuring protein (H-NS), a global regulator of environmentally controlled gene expression, belongs to a family of small nucleoid-associated proteins that also include its paralog StpA, Fis, the heat-unstable protein (HU), and integration host factor (IHF) (13). Mutations that inactivate *hns* are highly pleiotropic, suggesting that H-NS influences a broad spectrum of physiological processes (1, 21). *Escherichia coli* H-NS (137 amino acids) consists of an N-terminal domain extending to residue 65 with oligomerization activity, connected by a flexible linker to a nucleic acid binding domain beginning at residue 90, whereas *V. cholerae* H-NS contains an additional oligomerization domain located within the first 24 amino acids of the protein's N terminus (1, 12, 44). Both oligomerization and DNA binding are required for the biological activity of H-NS (54). It has been suggested that H-NS functions primarily as a transcriptional repressor by binding to highly curved AT-rich DNA regions (46, 57).

Depletion of H-NS in *V. cholerae* of the classical biotype (by overexpression of a truncated *hns* allele lacking the DNA binding domain) induced pleiotropic effects such as the formation of mucoid colonies and reduced swarming in semisolid agar (56). Furthermore, *V. cholerae hns* mutants have been reported

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to exhibit diminished bile- and anaerobiosis-mediated repression of *ctxA* expression, motility, and intestinal colonization capacity (16, 30). H-NS has been shown to silence virulence gene expression by repressing transcription at different levels of the ToxR regulatory cascade, which include the *toxT*, *tcpA*, and *ctxA* promoters (45). At the level of the *ctxA* and *tcpA* promoters, the positive regulator ToxT binds to these promoters, displacing H-NS and allowing RNA polymerase (RNAP) to initiate transcription (63).

In *E. coli*, RpoS regulates the expression of more than 100 genes in response to starvation and other stresses such as osmotic shock, oxidative stress, and temperature (19). The intracellular concentration of σ^S is controlled at the levels of transcription, translation, and protein stability (19, 43, 58). At the level of transcription, the cyclic AMP-CRP complex has been shown to modulate the activity of an *rpoS-lacZ* transcriptional fusion by binding to CRP boxes in the *rpoS* promoter (19). Also, polyphosphate and guanosine tetraphosphate (ppGpp) have been shown to enhance *rpoS* transcription (19). At the level of translation, several *trans*-acting factors can bind to *rpoS* mRNA in response to high osmolarity, low temperature, and an acidic pH to enhance its translation (19). These factors include the RNA binding protein Hfq, the nucleoid-associated protein HU, and the small RNAs DsrA and RprA (19, 32). In addition, two riboregulators, OxyS and DsrA, enhance and diminish *rpoS* translation, respectively (19, 43, 58). At the protein level, the response regulator RssB is required for the degradation of σ^S by the housekeeping protease ClpXP (2, 19, 43, 58). H-NS has been shown to participate as a negative factor in the expression of RpoS by promoting *rpoS* mRNA degradation (6) and increasing the activity of RssB to enhance the proteolysis of σ^S (64).

V. cholerae rpoS mutants are more sensitive to starvation, high osmolarity, and oxidative stress than the wild type (62), suggesting an analogy to the role of RpoS in *E. coli*. However, very little is known about the regulation of RpoS expression in *V. cholerae*. Recently, it has been shown that multiple motility and chemotaxis genes are downregulated in an *rpoS* mutant, suggesting that RpoS could facilitate mucosal escape during infection by enhancing motility (42). Furthermore, *V. cholerae rpoS* mutants are affected in intestinal colonization (39), suggesting that RpoS could contribute to the ability of vibrios to overcome host-specific stresses in the gut. Since both H-NS and RpoS have been shown to affect cholera pathogenesis, we decided to examine the interaction between these global regulators. In this study we show that H-NS exerts positive regulation over RpoS and multiple RpoS-dependent genes, including *hapA*. Furthermore, we provide evidence that H-NS can affect the expression of some motility and chemotaxis genes through distinct and overlapping effects: (i) as a positive factor by posttranscriptionally enhancing RpoS expression and (ii) as a repressor or negative factor in the absence of σ^S. Finally, we show that CRP and H-NS act on the same pathway to enhance the expression of RpoS.

MATERIALS AND METHODS

Strains and media. The *V. cholerae* and *E. coli* strains used in this study are shown in Table 1. For CT production, *V. cholerae* strains were grown in AKI (23) and LB media at 30°C. For HA/protease production, vibrios were grown in Bacto tryptic soy broth (TSB) at 37°C with shaking (250 rpm). For motility determination, strains were stabbed into LB medium containing 0.3% agar (swarm agar) and incubated at 30°C for 16 h. Plasmid DNA was introduced into *V. cholerae* by electroporation (33). Culture media were supplemented with ampicillin (Amp; 100 μg/ml), kanamycin (Km; 50 μg/ml), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 20 μg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG; 20 μg/ml), or polymyxin B (PolB; 100 U/ml) as required.

Construction of mutants. Deletion mutants were constructed by allelic exchange using strain C7258 as a wild-type precursor. *V. cholerae* target sequences were amplified from genomic DNA of strain C7258 by using the Advantage PCR system (BD Biosciences Clontech). All primers (Table 2) were designed based on the DNA sequence of the *V. cholerae* N16961 genome, downloaded from the TIGR database (http://cmr.tigr.org). Amplification products were directionally cloned into pUC19 using *E. coli* TOP10 as the host and were confirmed by sequencing of both DNA strands with M13 forward and reverse primers. In all cases, mutants were obtained by cloning a chromosomal DNA fragment containing a deletion of the target gene in pCVD442 (11). The resulting suicide vectors were constructed in *E. coli* S17-1λ*pir* (9) and mobilized into the corresponding *V. cholerae* receptor strain by conjugation. Exconjugants were selected on LB agar containing PolB, Amp, or Km as required. Next, sucrose selection was used to isolate segregants retaining the mutant allele. Using this approach, strain C7258 and the isogenic mutants AJB50 (C7258 Δ*rpoS*), AJB51 (C7258 Δ*hapR*), and WL7258 (C7258 Δ*crp*), described previously (31), were modified by deletion of the chromosomal *lacZ* gene. The corresponding *lacZ* deletion mutants (Table 1) were confirmed by DNA sequencing. A similar strategy was followed to construct the *hns* deletion/insertion (Δ*hns*::Km) mutant AJB80 (Table 1) starting from C7258Δ*lacZ*. Strain AJB80, containing the Δ*hns*::Km allele, was confirmed by Southern blotting and sequencing of the *V. cholerae* chromosome-Km junctions. Strains AJB81 (Δ*lacZ* Δ*hns*::Km Δ*rpoS*) and AJB82 (Δ*lacZ* Δ*hns*::Km Δ*crp*) (Table 1) were similarly obtained from AJB80 by allelic exchange using the suicide vectors pCVDΔRpoS2 and pCVDΔCRP (31), respectively. Again, mutants were confirmed by DNA sequencing. A detailed description of the strain construction procedures is provided as supplemental material.

Plasmid constructions. The complete *hns* open reading frame (ORF) was amplified from strain C7258 and cloned into pUC19 to yield plasmid pHNS (Table 1). A truncated *hns* allele (*hns*^{90}) was amplified from C7258 to generate a DNA fragment encoding the first 90 amino acids of H-NS. The truncated allele was cloned into plasmid pTTQ18 (55) to create the expression vector pTTHNS90 (Table 1), expressing *hns*^{90} from the Tac promoter. Similarly, the complete *rpoS* ORF was amplified and cloned into pTTQ18 to yield the expression vector pTTRpoS (Table 1). The RpoS reporter plasmid pPerA-LacZ (Table 1) was constructed by cloning the *perA* promoter region downstream of the *rrnB* T_1T_2 transcription terminator into pTT3 (Table 1) and subsequently subcloning the terminator-promoter fragment upstream of a promoterless *lacZ* gene into plasmid pKRZ1 (48). An identical strategy was used to construct a HapR reporter plasmid. In this case, we used the *lux* promoter amplified from cosmid pBB1 containing the *Vibrio harveyi lux* operon (41) to generate plasmid pLux-LacZ (Table 1). The construction of the *rpoS-lacZ* promoter fusion pRpoSLac5 (Table 1) has been described previously (49).

RT-PCR. *V. cholerae* strains were grown as described in each figure legend, and total RNA was isolated using the RNeasy kit (Qiagen Laboratories). The RNA samples were analyzed by quantitative real-time reverse transcription-PCR (qRT-PCR) using the iScript two-step RT-PCR kit with Sybr green (Bio-Rad Laboratories) as described previously (31). Relative expression values (*R*) were calculated as $2^{-(\Delta C_t \text{ target} - \Delta C_t \text{ reference})}$ where C_t is the fractional threshold cycle. *recA* mRNA was used as a reference. The following primer combinations were used: Bfr146 and Bfr431 for *bfr* mRNA, CheR377 and CheR587 for *cheR-2* mRNA, Cyt835 and Cyt948 for *c551* mRNA, FlaA40 and FlaA291 for *flaA* mRNA, FlaC405 and FlaC592 for *flaC* mRNA, KatB594 and KatB792 for *katB* mRNA, MotX253 and MotX374 for *motX* mRNA, PerA607 and PerA863 for *perA* mRNA, RecA578 and RecA863 for *recA* mRNA, RpoS576 and RpoS1003 for *rpoS* mRNA, and ToxR369 and ToxR498 for *toxR* mRNA. A control mixture lacking reverse transcriptase was run for each reaction to exclude chromosomal DNA contamination.

To assess the longevity of *rpoS* mRNA in the wild-type and Δ*hns*::Km mutant backgrounds, the strains were grown to an optical density at 600 nm (OD_{600}) of 1.5, and rifampin was added (150 μg/ml) to block transcription. Samples were taken at different time points after the addition of rifampin, and *rpoS* mRNA was detected using the Titanium One-Step RT-PCR kit by following the manufacturer's instructions, with 20 ηg of total RNA and 25 cycles of amplification. Since less *rpoS* mRNA is produced in the Δ*hns*::Km mutant than in the wild type (see below), two additional cycles were provided for the mutant to generate a stronger starting signal.

TABLE 1. Strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
Escherichia coli		
TOP10	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC)* ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ*(ara leu)7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
S17-1λPir	F$^-$ *recA hsdR* RP4-2 (Tc::Mu) (Km::Tn*7*) lysogenized with λ*pir* phage	9
Vibrio cholerae		
C7258	Wild type, El Tor biotype	Peru, 1991
C7258Δ*lacZ*	C7258 *lacZ* deletion mutant	This study
AJB80	C7258Δ*lacZ* Δ*hns*::Km	This study
AJB50Δ*lacZ*	C7258Δ*lacZ* Δ*rpoS*	This study
AJB51Δ*lacZ*	C7258Δ*lacZ* Δ*hapR*	This study
WL7258Δ*lacZ*	C7258Δ*lacZ* Δ*crp*	This study
AJB81	AJB80 Δ*rpoS*	This study
AJB82	AJB80 Δ*crp*	This study
Plasmids		
pHNS12	0.9-kb SacI-BamHI DNA fragment 5′ of the *hns* ORF amplified with primers Hns120 and Hns1018 in pUC19	This study
pHNS34	0.8-kb BamHI-SphI DNA fragment 3′ of the *hns* ORF amplified with primers Hns1467 and Hns2289 in pUC19	This study
pΔHNS	BamHI-SphI fragments from pHNS34 in pHNS12	This study
pΔHNSK	1.2-kb BamHI Km^r cassette from pUC4K in pΔHNS	This study
pCVDΔHNSK	SacI-SphI Δ*hns*::Km fragment from pΔHNSK in pCVD442	This study
pHNS	0.4-kb BamHI-HindIII DNA fragment carrying the *hns* ORF amplified with primers Hns162 and HnsD2 in pTTQ18	This study
pTTHNS90	0.3-kb BamHI-HindIII fragment encoding 90 amino acids of the H-NS N terminus amplified with primers Hns162 and Hns429 in pTTQ18	This study
pTTRpoS	1.0-kb EcoRI-HindIII fragment carrying *rpoS* amplified with primers RpoS489 and RpoS1511 in pTTQ18	This study
pTT3	XbaI-PstI DNA fragment containing the *rrnB* T_1T_2 transcription terminator in pUC19	7, 52
pTT3Lux	0.3-kb SphI-HindIII DNA fragment carrying the *V. harveyi luxC* promoter amplified with primers LuxC84 and LuxC434 in pTT3	This study
pLux-LacZ	0.5-kb KpnI-HindIII fragment containing *rrnB* T_1T_2 and the *luxC* promoter fused to a promoterless *lacZ* gene in pKRZ1	This study
pTT3PerA	0.9-kb SphI-HindIII fragment carrying *perA* amplified with primers PerA498 and PerA987 in pTT3	This study
pPerA-LacZ	1.1-kb KpnI-HindIII fragment containing *rrnB* T_1T_2 and the *perA* promoter fused to a promoterless *lacZ* gene in pKRZ1	This study
pRpoSLac5	A SphI-HindIII DNA fragment containing the *rpoS* promoter region was cloned between the *rrnB* T_1T_2 transcription terminator and a promoterless *lacZ* gene in pKRZ1	49
pLacZ12	0.9-kb SacI-BamHI DNA fragment 5′ of the *lacZ* ORF amplified with primers LacZ6 and LacZ859 in pUC19	This study
pLacZ34	0.8-kb BamHI-SphI DNA fragment 3′ of the *lacZ* ORF amplified with primers LacZ3236 and LacZ4029 in pUC19	This study
pΔLacZ	0.8-kb BamHI-SphI DNA fragment from pLacZ34 in pLacZ12	This study
pCVDΔLacZ	1.6-kb SacI-SphI fragment from pΔLacZ in pCVD442	This study

Stress response assays. *V. cholerae* strains were grown for 18 h (to stationary phase) in LB medium at 37°C. The cells were centrifuged, washed, and resuspended in 1 volume of LB medium. The bacterial suspension was then inoculated into fresh LB medium that either contained 2.4 M NaCl or 3 mM hydrogen peroxide or was brought to pH 4.5 so as to obtain a cell density of 10^6 to 10^7 cells per ml. Cultures were incubated at 37°C with shaking (250 rpm), and samples were taken at different time points for dilution plating.

Determination of CT levels. CT levels were determined by a GM_1 enzyme-linked immunosorbent assay using a standard curve of pure CT (Sigma Chemical Co.) as described previously (31).

Enzyme assays. Production of HA/protease was measured using an azocasein assay as described previously (3). One azocasein unit is the amount of enzyme that produces an increase of 0.01 OD unit in this assay. β-Galactosidase activity was measured as described previously (40) using the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG). Specific activities are given in Miller units and calculated as [1,000 ($OD_{420}/t \times v \times OD_{600}$)] where t is the reaction time and v is the volume of enzyme extract per reaction.

RESULTS

Construction of a *V. cholerae* El Tor biotype *hns* deletion/insertion mutant. We have constructed an *hns* deletion/insertion mutant (Δ*hns*::Km) of the El Tor biotype strain C7258Δ*lacZ*. The mutant was confirmed by Southern blot analysis and DNA sequencing. Since it has been reported that H-NS negatively influences virulence gene expression in classical biotype *V. cholerae*, we first determined whether or not H-NS had a similar effect in the El Tor biotype mutant. The El Tor biotype Δ*hns*::Km mutant AJB80 produced 15.7 mg/liter/OD_{600} unit of CT in AKI cultures, compared to 2.6 mg/liter/OD_{600} unit produced by its wild-type precursor (C7258Δ*lacZ*) under identical conditions. Furthermore, while the wild-type strain did not

TABLE 2. Primers

Primer name	Sequence[a]
Bfr146	5′-TTGATGAGATGAAACACGCTGACCA
Bfr431	5′-GCTTGCAGATAATTTTGGATACCCG
CheR377	5′-TAGAAGTGCAGCAAAAACGTCCGG
CheR587	5′-CCATTAAGTTTTGCGGACGGAAGT
Cyt835	5′-GCTGTGGATATCATGGGGCGTTTA
Cyt948	5′-TCGTTGGATGGCGGTAAAATGGGT
FlaA40	5′-GCACAACGTTATCTGACCAAAGGC
FlaA291	5′-GAGTTGGTACCGTTCGCCGATTG
FlaC405	5′-CAAGCTGCTCAACGGTACATTCG
FlaC592	5′-GCCTTCGCGTTTGTCTTTCAGAGT
Hns120	5′-GAAGAGCTCGAAGATGGTGAACGTA
Hns162	5′-GAAGGATCCGGAAATGGTAATGTCG
Hns429	5′-GGCCAAGCTTTTCGTTTTAGTTTCG
Hns1018	5′-GCAGGATCCATGAAGGTAAATCTCT
Hns1467	5′-GATGGATCCTGACAAGTTTTCGCTG
Hns2289	5′-GATGCATGCCCCTCTTTGACAAACA
HnsD2	5′-GGGAAAGCTTGGCAAAATTACAGAG
KatB594	5′-TACCGAACAAGGCAACTGGGATTT
KatB792	5′-GTTCGATAGCTTGCCGGTGTACT
LacZ6	5′-GCGGATCCAGCCGAGGAGTAAAGA
LacZ859	5′-GCGAGCTCCGAAAATGACTGTTGT
LacZ3236	5′-GCGGATCCAAAGCAAGAGCCA
LacZ 4029	5′-GCGCGCATGCAACTCGGCTATCGTCC
LuxC84	5′-GCGCAAGCTTAATCGATTTTCTTCAGTAG
LuxC434	5′-GCGCGCATGCACACTGTCACACATC
MotX253	5′-GGAGTGTGTGTGGATCAGGATGTT
MotX374	5′-GGATCGCGCGTTCTTTGTCTTGTT
PerA498	5′-GTTGCATGCCTTTACCACCTTGATC
PerA607	5′-GAAAATAGCCGTTACTCTGGTCAGC
PerA863	5′-CTAAGCCTTGTTCGTGCAGTTCAG
PerA987	5′-GCGAAGCTTATTGATTACTCCTTGC
RecA578	5′-GTGCTGTGGATGTCATCGTTGTTG
RecA863	5′-CCACCACTTCTTCGCCTTCTTTGA
RpoS576	5′-CGATTTTGAAGATGAAGCACTGGAAG
RpoS489	5′-GTTGAATTCGAGGCCGCTATGAGT
RpoS1003	5′-TGTTTGGTTCATCAGCGCACGTTC
RpoS1511	5′-GCGAAGCTTTTTTGGATGAGTCTGG
ToxR369	5′-GGAAACGGTTGAAGAAGAGATGGC
ToxR498	5′-TTATTCGTCACAACATTGGCTGGC

[a] Restriction sites used for directional cloning are underlined.

produce detectable CT in nonpermissive LB medium, the Δ*hns*::Km mutant produced 3.8 mg/liter/OD_{600} unit of CT in LB medium. Moreover, in contrast to its wild-type precursor, the Δ*hns*::Km mutant expressed significant autoagglutination, an indicator of TCP expression (8), in LB medium (data not shown). These phenotypes were complemented by providing the *hns* gene in *trans* using plasmid pHNS. The results discussed above suggest that, as documented for *hns* mutants of the classical biotype (45), H-NS acts to silence virulence gene expression in the El Tor biotype. In addition, the Δ*hns*::Km mutant exhibited additional phenotypes typical of *hns* mutants, such as reduced growth rate and motility (see below).

H-NS acts upstream of RpoS to positively regulate the production of HA/protease. We have shown that transcription of *hapA* occurs in the stationary phase and requires RpoS (49). Since in *E. coli* H-NS has been reported to repress the expression of multiple RpoS-dependent genes (19, 20), we examined the role of H-NS in HA/protease expression. It has been reported that overexpression of a truncated *hns* allele lacking the DNA-binding domain leads to phenotypes reminiscent of those observed for *hns* mutants (56). Thus, we first examined if depletion of H-NS by overexpression of the truncated allele hns^{90} affected the production of HA/protease. As shown in Fig. 1A, induction of hns^{90} with IPTG had a strong negative effect on HA/protease expression relative to that by an uninduced control. In agreement with this result, elimination of *hns* by mutation in strain AJB80 (Δ*hns*::Km) strongly diminished the production of HA/protease (Fig. 1B). This phenotype was complemented by providing the wild-type *hns* allele in *trans* (Fig. 1B). As shown in Fig. 1B, deletion of *rpoS* had a more severe effect on HA/protease production ($P < 0.05$ by the *t* test). In order to examine the relationship between H-NS and RpoS in the regulation of HA/protease production, we constructed the Δ*hns*::Km Δ*rpoS* double mutant AJB81 and performed an epistasis analysis. Production of HA/protease by the

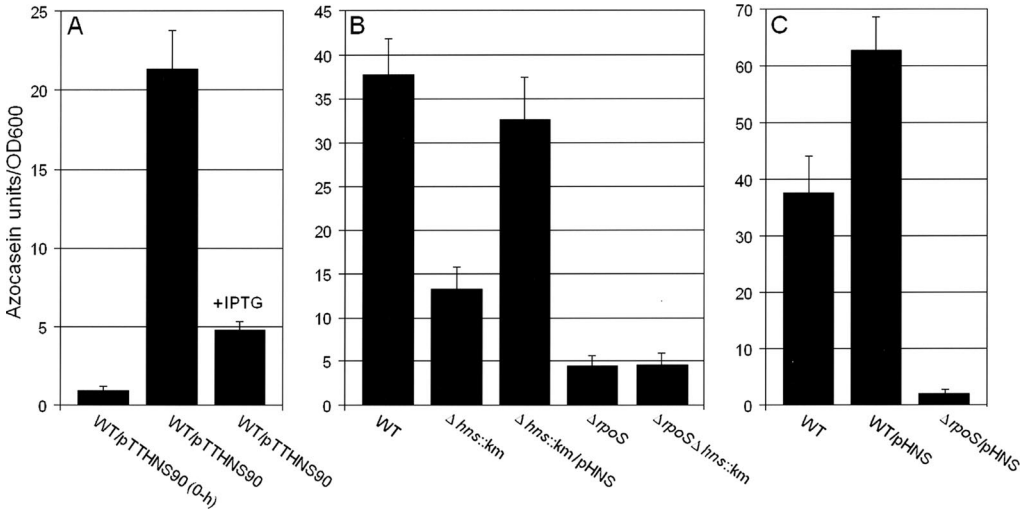

FIG. 1. Role of H-NS in HA/protease production. (A) Strain C7258Δ*lacZ* (wild type [WT]) containing pTTHNS90 was grown in TSB to an OD_{600} of 1, and the culture was divided in half. One half was induced by addition of IPTG, and the second half was used as a control. Cultures were further incubated for 3 h. (B) Strains C7258Δ*lacZ* (WT), AJB80 (C7258Δ*lacZ* Δ*hns*::Km), AJB50Δ*lacZ* (Δ*rpoS*), and AJB81 (Δ*lacZ* Δ*hns*::Km Δ*rpoS*) were grown to stationary phase (16 h) in TSB at 37°C. (C) Strain C7258Δ*lacZ* (WT) and strains C7258Δ*lacZ* and AJB50Δ*lacZ* (Δ*rpoS*) containing plasmid pHNS were grown as described above. Production of HA/protease (expressed in azocasein units) was measured as described in Materials and Methods. Each value is the average for three independent cultures. Error bars, standard deviations.

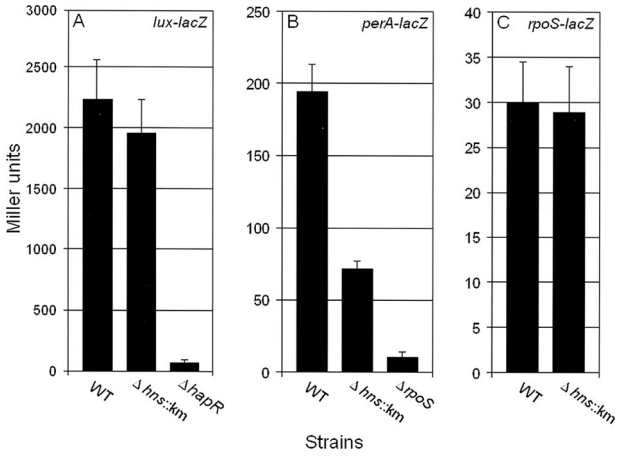

FIG. 2. Expression of HapR and RpoS reporter *lacZ* fusions in *V. cholerae hns* mutants. (A) Strains C7258Δ*lacZ* (wild type [WT]), AJB80 (C7258Δ*lacZ* Δ*hns*::Km), and AJB51Δ*lacZ* (Δ*hapR*) containing plasmid pLux-LacZ were grown to stationary phase (16 h) in TSB at 37°C. (B) Strains C7258Δ*lacZ* (WT), AJB80 (C7258Δ*lacZ* Δ*hns*::Km), and AJB50Δ*lacZ* (Δ*rpoS*) containing plasmid pPerA-LacZ were grown as described above. (C) Strains C7258Δ*lacZ* (WT) and AJB80 (C7258Δ*lacZ* Δ*hns*::Km) containing an *rpoS-lacZ* transcriptional fusion were grown as described above. β-Galactosidase activity was determined as described in Materials and Methods. Each value is the average for three independent cultures. Error bars, standard deviations.

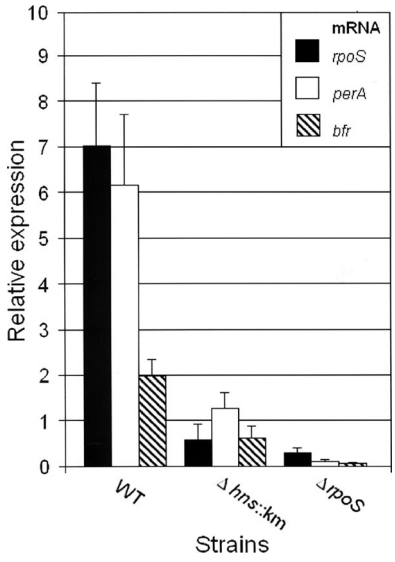

FIG. 3. qRT-PCR analysis of *rpoS* expression in an *hns* mutant. Strains C7258Δ*lacZ* (wild type [WT]), AJB80 (C7258Δ*lacZ* Δ*hns*::Km), and AJB50Δ*lacZ* (Δ*rpoS*) were grown to stationary phase (16 h) in TSB at 37°C. RNA was extracted, and *rpoS*, *perA*, and *bfr* mRNA abundances were determined by qRT-PCR as described in Materials and Methods. Results are averages for three independent cultures. Error bars, standard deviations.

Δ*hns*::Km Δ*rpoS* double mutant was similar to that by the Δ*rpoS* mutant, indicating that RpoS acts downstream of H-NS to activate HA/protease production (Fig. 1B). In Fig. 1C, we show that ectopic expression of H-NS in wild-type *V. cholerae* results in increased HA/protease production ($P < 0.05$ by the *t* test). Consistent with our epistasis analysis, overexpression of H-NS did not restore HA/protease expression in a Δ*rpoS* background. Taken together, we conclude that H-NS positively affects HA/protease expression by acting upstream of RpoS.

Since production of HA/protease requires both RpoS and the quorum-sensing regulator HapR, we examined if expression of these regulators was diminished in the Δ*hns*::Km mutant AJB80. For HapR expression, we constructed the reporter plasmid pLux-LacZ (Table 1). Expression of β-galactosidase from the *lux* promoter is dependent on the production of active HapR protein. As shown in Fig. 2A, the Δ*hns*::Km mutant produced a level of β-galactosidase activity similar to that of the wild type, while an isogenic Δ*hapR* mutant (AJB51Δ*lacZ*), used as a negative control, produced very little activity.

Preliminary gene expression profiling of a Δ*rpoS* mutant of strain C7258 revealed that the VC1560 gene, annotated as catalase-peroxidase (*perA*), and VC0365 (bacterioferritin; *bfr*) were strongly downregulated in the Δ*rpoS* mutant (data not shown). Using the same strategy as that for the HapR reporter plasmid, we constructed a reporter plasmid (pPerA-LacZ) containing a *perA-lacZ* transcriptional fusion to monitor the production of active RpoS protein. As shown in Fig. 2B, significantly less RpoS activity was detected in the Δ*hns*::Km mutant than in the wild type, while negligible activity was observed in an isogenic Δ*rpoS* mutant used as a negative control. These results indicate that the lower production of HA/protease by the Δ*hns*::Km mutant is due to diminished expression of active RpoS protein.

Positive regulation of RpoS by H-NS is posttranscriptional. To determine if the lower expression level of RpoS in the Δ*hns*::Km mutant (Fig. 2B) was due to reduced transcription, we introduced the *rpoS-lacZ* transcriptional fusion pRpoSLac5 (49) into the wild-type and Δ*hns*::Km mutant strains. As shown in Fig. 2C, the two strains produced similar β-galactosidase activities, suggesting that H-NS affects RpoS expression by a posttranscriptional mechanism. In *E. coli*, H-NS has been reported to affect *rpoS* expression at the levels of translation and protein stability (19, 64). Therefore, we used qRT-PCR to measure the abundances of *rpoS* mRNA and the RpoS-dependent genes *perA* and *bfr* in C7258Δ*lacZ* and the Δ*hns*::Km mutant. As shown in Fig. 3, the Δ*hns*::Km mutant produced a smaller amount of *rpoS* mRNA than the wild type, and this was reflected in diminished expression of the RpoS-dependent genes *perA* and *bfr*. These results suggest that H-NS has a strong positive effect on *rpoS* mRNA translation/stability. To further test the hypothesis that H-NS regulates *rpoS* expression posttranscriptionally, we constructed the pTTRpoS vector (Table 1), which expresses RpoS from the Tac promoter. In Fig. 4 we show that IPTG-induction of pTTRpoS in strain AJB50*lacZ* (Δ*rpoS*) complemented the RpoS defect, leading to HA/protease production. However, significantly less HA/protease could be detected upon induction of pTTRpoS in strain AJB81 (Δ*hns*::Km Δ*rpoS*). Taken together, our data suggest that in contrast to *E. coli*, in *V. cholerae* H-NS posttranscriptionally enhances the expression of RpoS and multiple RpoS-dependent genes, such as *hapA*, *perA*, and *bfr*. We further examined the stability of *rpoS* mRNA in the wild-type and Δ*hns*::Km mutant strains. As shown in Fig. 5, and consistent with a posttranscriptional regulatory mechanism, the *rpoS*

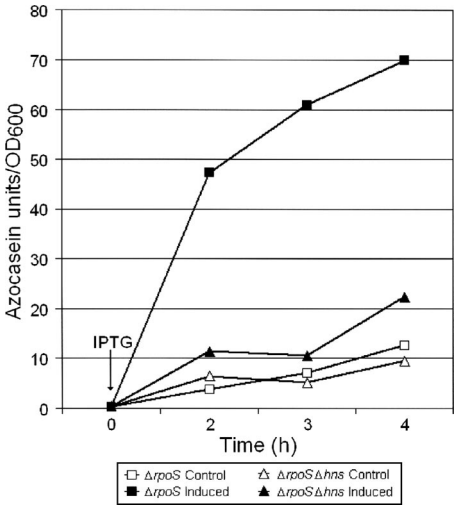

FIG. 4. Production of HA/protease as an indicator of ectopic expression of RpoS in Δ*rpoS* and Δ*rpoS* Δ*hns* mutants. Strains AJB50Δ*lacZ* (Δ*rpoS*) and AJB81 (Δ*hns*::Km Δ*rpoS*) containing plasmid pTTRpoS were grown in TSB medium to an OD_{600} of 1.0 and divided in half. One half was induced by the addition of IPTG, and the other half (uninduced) was used as a control. Samples were taken at different times to determine the production of HA/protease by using the azocasein assay described in Materials and Methods. Each data point is the mean for three independent cultures. Error bars, standard deviations.

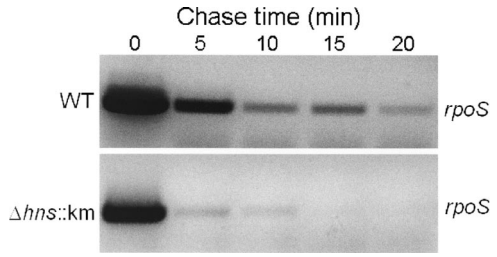

FIG. 5. Analysis of *rpoS* mRNA longevity in a *V. cholerae* Δ*hns* mutant. Strains C7258Δ*lacZ* (wild type [WT]) and AJB80 (C7258Δ *lacZ* Δ*hns*::Km) were grown to an OD_{600} of 1.5 at 37°C; rifampin was added to block transcription; and samples were collected at different time points for RNA extraction and *rpoS* mRNA analysis as described in Materials and Methods.

mRNA signal was found to disappear more rapidly in the Δ*hns*::Km mutant than in the wild-type after transcription had been blocked with rifampin.

H-NS affects the *V. cholerae* stress response. In *V. cholerae*, RpoS has been shown to mediate a general stress response (62). The results described above prompted us to investigate the role of H-NS in the *V. cholerae* response to environmental stressors. The Δ*hns*::Km and Δ*rpoS* mutants were both more sensitive to 2.4 M NaCl than the wild type (Fig. 6). This result is in agreement with the lower expression of RpoS in the Δ*hns*:: Km mutant. However, the Δ*hns*::Km mutant was more resistant than the wild-type strain and the Δ*rpoS* mutant to 3 mM hydrogen peroxide (Fig. 6). In order to explain this result, we used qRT-PCR to determine the relative expression of other catalase/peroxidase genes present in the *V. cholerae* genome. We observed that the Δ*hns*::Km mutant expressed higher levels of VC0089, encoding c551 cytochrome/peroxidase, than the wild type (0.64 for the wild type and 4.7 for the Δ*hns*::Km mutant). In addition, elevated expression of VC1585, encoding KatB catalase, was observed in the Δ*hns*::Km mutant (levels were 0.030 for the wild type and 0.3 for the Δ*hns*::Km mutant). These results suggest that H-NS is a repressor of c551 and *katB*. Elevated expression of c551 and *katB* in the Δ*hns*::Km mutant explains its resistance to hydrogen peroxide in spite of expressing lower levels of the RpoS-dependent *perA* gene. The Δ*hns*::Km mutant also exhibited greater resistance than the wild type to a pH of 4.5 (Fig. 6). We have not observed elevated expression of lysine decarboxylase, known to mediate the inorganic acid tolerance response (36–38), in the Δ*hns*::Km mutant. However, we did observe that the Δ*hns*::Km mutant expressed higher *toxR* mRNA levels than the wild type and the Δ*rpoS* mutant (*toxR* mRNA levels were 0.31 for the wild type, 0.25 for the Δ*rpoS* mutant, and 0.64 for the Δ*hns*::Km mutant). Expression of ToxR has been reported to enhance resistance to organic acid shock (35).

Role of H-NS in the expression of motility and chemotaxis. Gene expression profiling of *V. cholerae* C7258Δ*rpoS* revealed that several motility and chemotaxis genes were downregulated in the mutant background (data not shown). We found that both Δ*rpoS* and Δ*hns*::Km mutants were less motile than the wild type by using a swarm assay in semisolid agar medium (Fig. 7A). These results prompted us to examine the relationship between H-NS and RpoS in the regulation of motility and chemotaxis gene expression. To investigate the reduced motility of the Δ*hns*::Km mutant, we used qRT-PCR to measure the relative expression levels of the regulator genes *flrA*, *flrB*, *flrC*, *fliA*, and *rpoN*; the flagellin structural genes *flaA* and *flaC*; the motor genes *motX* and *motY*; and the chemotaxis genes *cheA-2* (VC2063), *cheR*-2 (VC2201), and *cheY-3* (VC2065). Inactivation of these genes, except *flaC*, impairs the ability of *V. cholerae* to swarm away from the inoculation site in semisolid agar (5, 17, 22, 47, 51). The Δ*hns*::Km mutant expressed lower *flaA*, *flaC*, *motX*, and *cheR-2* mRNA levels than the wild type (Fig. 7B). Compared to the deletion of *rpoS*, the deletion of *hns* had a smaller but still very significant negative effect on the expression of these genes. *cheR-2* was found to be the most strongly RpoS dependent gene under the experimental conditions used. An interesting pattern was observed for *flaA*, *flaC*, and *motX* expression in the Δ*rpoS* Δ*hns* double mutant. Although expression of these genes was lower in the Δ*rpoS* and Δ*hns*::Km single mutants than in the wild type, deletion of *hns* in the Δ*rpoS* background significantly enhanced their expression (*t* tests for *flaA*, *flaC*, and *motX* expression yielded *P* values of <0.05) (Fig. 7B).

CRP and H-NS act on the same pathway to regulate RpoS expression. In a previous study, we showed that CRP positively modulates the expression of RpoS (49). Since we have shown above that H-NS also enhances the expression of RpoS, we asked if CRP and H-NS act along the same pathway to enhance RpoS. To investigate this question, we constructed the Δ*hns*::Km Δ*crp* double mutant AJB82 and performed epistasis analysis of RpoS-dependent *perA* expression. We did not observe significant differences between the levels of *perA* expression in the Δ*hns*::Km, Δ*crp*, and Δ*crp* Δ*hns*::Km mutants (Fig. 8), suggesting that CRP and H-NS act along the same pathway to enhance RpoS expression. Next, we performed qRT-PCR to

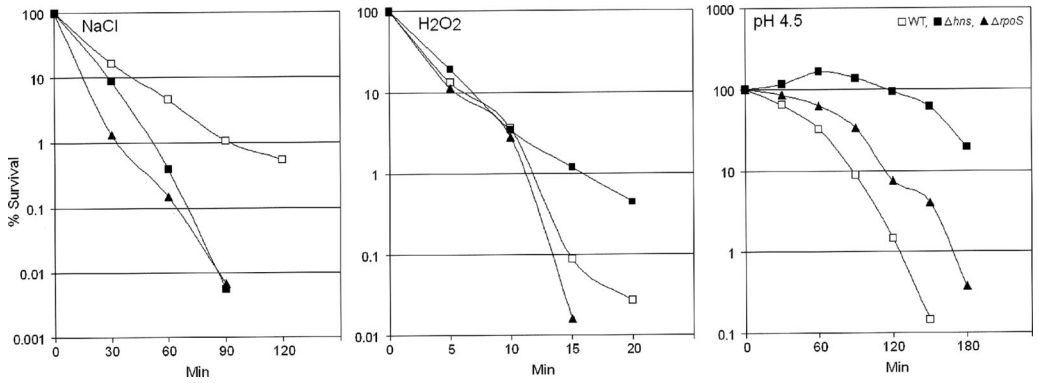

FIG. 6. Responses of a *V. cholerae* El Tor biotype *hns* mutant to environmental stresses. *V. cholerae* strains were grown and prepared as described in Materials and Methods. They were then subjected to either 2.4 M NaCl, 3 mM hydrogen peroxide, or a pH of 4.5 in LB medium. At different time points, samples were withdrawn and the viable count determined by dilution plating. Each point is the average for three experiments. WT, wild type.

measure *hns* mRNA in wild-type and Δ*crp* strains. Strain WL7258Δ*lacZ* (Δ*crp*) was found to produce less *hns* mRNA than its wild-type precursor (0.49 for the wild type and 0.21 for the Δ*crp* mutant).

DISCUSSION

The experiments described above increase our understanding of the complex regulatory interactions that control the expression of HA/protease, a factor proposed to enhance *V. cholerae* enterotoxicity and the reactogenicity of live vaccine candidates (4, 15, 51). By using HA/protease as an RpoS reporter activity, we show that in contrast to its action in *E. coli*, H-NS positively affects the expression of RpoS and multiple RpoS-dependent genes in *V. cholerae*. Recent studies have revealed important differences in the regulation of RpoS expression between *E. coli* and *V. cholerae*. For instance, we have shown that expression of RpoS in *V. cholerae* is positively affected by CRP (49), but the *V. cholerae rpoS* promoter does not contain the CRP boxes present in the *E. coli* promoter (see below). Also, *V. cholerae* mutants lacking *relA* and polyphosphate kinase (*ppk*), which are responsible for ppGpp and polyphosphate synthesis, respectively, produced wild-type levels of RpoS and HA/protease (24, 50). Moreover, deletion of Hfq, a factor that enhances *rpoS* translation in *E. coli*, did not affect RpoS expression in *V. cholerae* (10). These results clearly suggest that the regulation of RpoS expression in *E. coli* and *V. cholerae* 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, *V. cholerae* can persist for longer periods in aquatic ecosystems.

Analysis of *rpoS-lacZ* transcriptional fusions and ectopic expression of *rpoS* in Δ*rpoS* and Δ*rpoS* Δ*hns* mutants suggested that H-NS acts by a posttranscriptional mechanism. Our data suggest that positive regulation of *rpoS* expression by H-NS involves *rpoS* coding sequences cloned into the expression vector pTTRpoS (Fig. 4). In concurrence with the above data, *rpoS* mRNA was found to decay more rapidly in the Δ*hns*::Km than in the wild-type background (Fig. 5). These findings are in agreement with multiple observations indicating that positive regulation by H-NS is by and large posttranscriptional (1, 12, 26). The reduced abundance of *rpoS* mRNA in the Δ*hns* mutant suggests that H-NS could bind to *rpoS* mRNA, as reported for *E. coli* (6), or act indirectly to enhance *rpoS* transcript stability. However, our data do not rule out the possibility of H-NS additionally acting at the level of RpoS protein stability. Consistent with the positive effect of H-NS on RpoS expression, our *hns* mutant was more sensitive than the wild type to high osmolarity (Fig. 6). The surprising resistance of the Δ*hns* mutant to hydrogen peroxide could be explained by elevated expression of other catalase/peroxidase enzymes encoded by *V. cholerae*. We do not, at the moment, have a clear explanation for the enhanced resistance of the Δ*hns* mutant to a pH of 4.5. Possibly, acidification of LB medium could generate free organic acids to which the Δ*hns* mutant could be more resistant due to elevated expression of ToxR (35). Alternatively, as reported for *E. coli* (21), H-NS could affect the expression of other amino acid decarboxylases, antiporter systems, or porins that could attenuate the deleterious effect of a low pH.

The Δ*hns*:Km mutant was found to be less motile than the wild type and exhibited reduced expression of *flaA*, *flaC*, *motX*, and *cheR-2*. In contrast to a previous study using a classical biotype *V. cholerae hns* mutant (16), we did not observe reduced expression of the regulator *flrA* in our mutant. Deletion of *rpoS* negatively affected the expression of *flaA*, *flaC*, *motX*, and *cheR-2*. This result is in agreement with the finding that RpoS enhances motility in vitro and in vivo to facilitate mucosal escape (42). Analysis of the expression of *flaA*, *flaC*, and *motX* in Δ*hns*, Δ*rpoS*, and Δ*hns* Δ*rpoS* mutants revealed an interesting regulatory pattern. While elimination of *rpoS* had the strongest negative effect on *flaA*, *flaC*, and *motX* expression, elimination of *hns* was epistatic to *rpoS*. This result suggests that in the absence of σ^S, H-NS could act as a repressor of these genes. It has been proposed that H-NS can contribute to σ^S promoter specificity (20). For instance, initiation of transcription at some RpoS-dependent promoters in the stationary phase by RNAP containing σ^S can be more resistant to H-NS repression than transcription by the σ^{70}-containing holoenzyme (20). Transcription of *flaA* and *motX* requires σ^{54} (RpoN), while that of *flaC* requires a σ^{28} analog (28, 47). Our studies suggest that transcription of *flaA*, *motX*, and *flaC* by RNAP containing σ^{54} or σ^{28} could be repressed by H-NS,

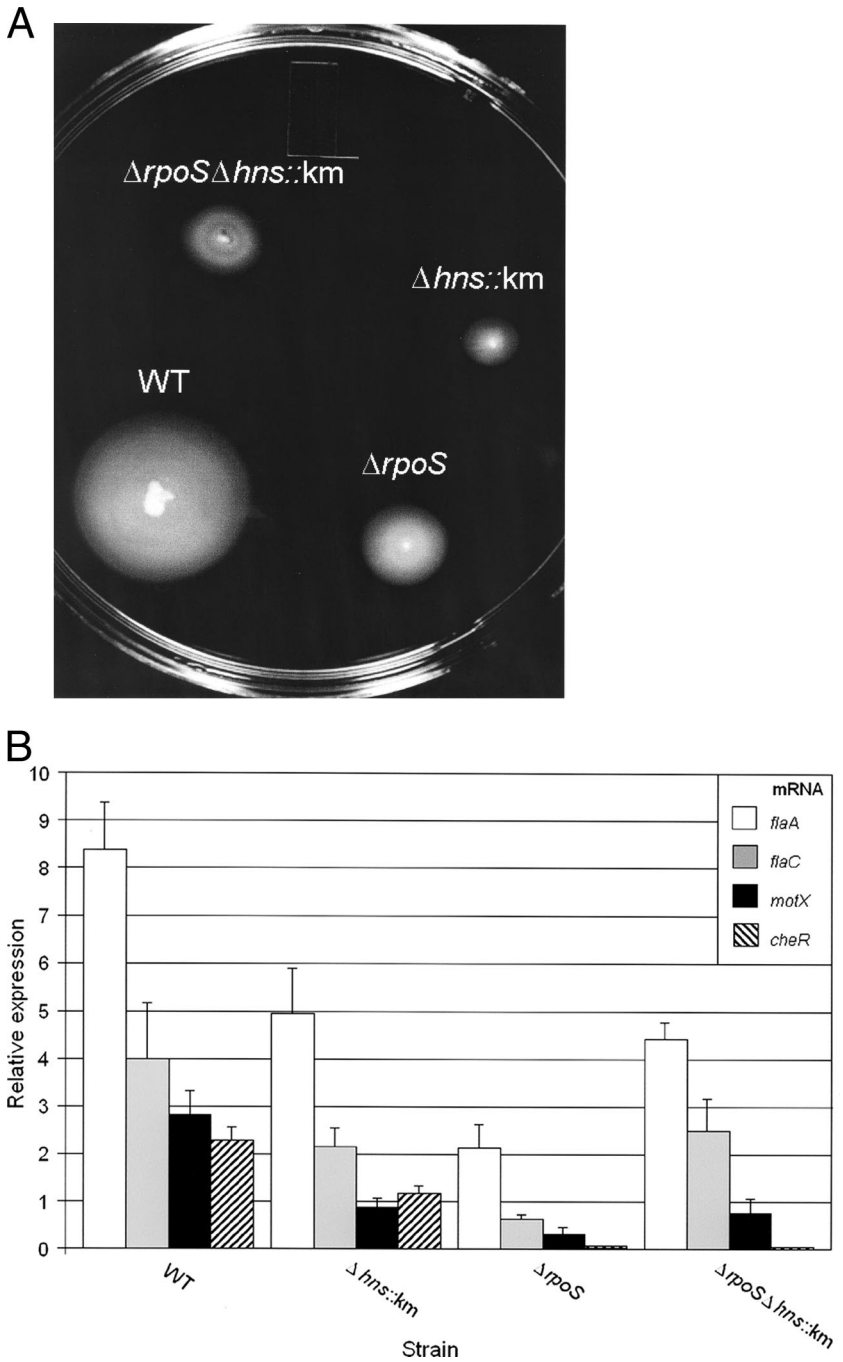

FIG. 7. Role of H-NS in the expression of motility and chemotaxis. (A) Strains C7258Δ*lacZ* (wild type [WT]) and isogenic mutants AJB80 (C7258Δ*lacZ* Δ*hns*::Km), AJB50Δ*lacZ* (Δ*rpoS*), and AJB81 (Δ*hns*::Km Δ*rpoS*) were stabbed into LB medium containing 0.3% agar and incubated for 8 h at 30°C. (B) Strains C7258Δ*lacZ* (WT), AJB80 (C7258Δ*lacZ* Δ*hns*::Km), AJB50Δ*lacZ* (Δ*rpoS*), and AJB81 (Δ*lacZ* Δ*hns*::Km Δ*rpoS*) were grown in LB medium to an OD_{600} of 1.5 at 37°C. RNA was extracted, and the relative expression levels of *flaA*, *flaC*, *motX*, and *cheR* were determined by qRT-PCR as described in Materials and Methods, with *recA* mRNA as a reference. Results are averages for three independent cultures. Error bars, standard deviations.

while in the stationary phase RpoS could partially override H-NS repression. To further examine this possibility, we used the DNA Curvature Analysis program (http://www.lfd.uci.edu/~gohlke/curve/) to predict the occurrence of curved AT-rich DNA sequences within the 5′ noncoding DNA preceding *flaA*, *flaC*, and *motX*. In all cases, we have found regions to which H-NS could potentially bind, with curvature indices similar to or higher than those calculated for the *ctxA* and *toxT* promoters, known to be repressed by H-NS (45, 63). Moreover, use of the Virtual Footprint program (http://www.prodoric.de/vfp/) identified high-scoring putative Fis sites within the 5′ noncoding sequences preceding *flaA*, *flaC*, and *motX*. Fis sites occur in

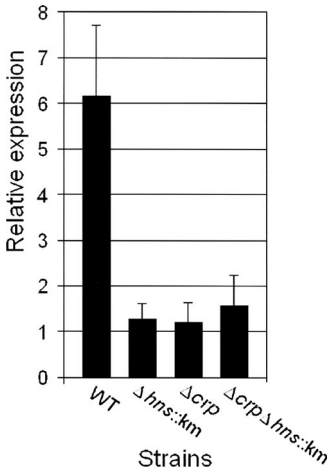

FIG. 8. Analysis of *perA* transcription as a measure of RpoS expression in *V. cholerae* Δ*hns* and Δ*crp* mutants. Strains C7258Δ*lacZ*, AJB80 (C7258Δ*lacZ* Δ*hns*::Km), WL7258Δ*lacZ* (Δ*crp*), and AJB82 (Δ*lacZ* Δ*hns*::Km Δ*crp*) were grown to stationary phase (16 h) in TSB at 37°C. Total RNA was extracted, and the abundance of *perA* mRNA was determined by qRT-PCR. Each value is the average for three independent cultures. Error bars, standard deviations.

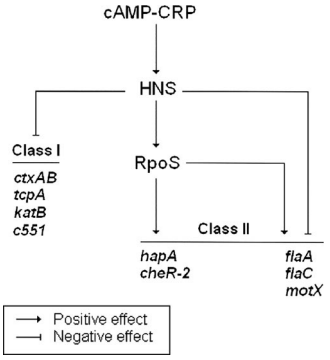

FIG. 9. Model for the regulatory input of H-NS in virulence, the general stress response, and motility. We propose that CRP acts through H-NS to silence virulence gene expression and enhance RpoS expression. RpoS, in turn, activates genes involved in protease production, stress response, motility, and chemotaxis. For some RpoS-dependent genes (*flaA*, *motX*, and *flaC*), H-NS can also act as a repressor in the absence of an active *rpoS* gene.

many promoters repressed by H-NS, in which binding of Fis has been reported to hinder the interaction of H-NS with DNA and to antagonize repression (1, 12). Our results raise the intriguing question of how the different σ factors and H-NS interact to regulate the temporal expression of motility and chemotaxis. Knowledge of the temporal expression of RpoS and H-NS in *V. cholerae* is required to fully clarify their regulatory input on motility.

In a previous study, we demonstrated that CRP positively affects RpoS expression (49). Use of the Virtual Footprint program did not reveal high-scoring putative CRP binding sites, suggesting that CRP acts indirectly. Therefore, we analyzed whether or not CRP and H-NS act through the same pathway to positively regulate RpoS expression. To this end, we constructed an Δ*hns* Δ*crp* double mutant and performed an epistasis analysis for the expression of RpoS-dependent *perA* mRNA. Simultaneous deletion of *hns* and *crp* did not significantly diminish *perA* expression more than deletion of *hns* or *crp* alone. Since CRP was found by qRT-PCR to positively affect the expression of H-NS, we propose that CRP positively affects RpoS expression in *V. cholerae* by enhancing H-NS expression (Fig. 9). The positive effect of CRP on H-NS expression is also consistent with the finding that *crp* mutants are less motile, express reduced *flaA* and *flaC* levels (31), and make more CT (31, 53) than the wild type. In fact, CRP can diminish CT expression by parallel mechanisms, which include enhancing H-NS expression, activating quorum sensing (31), and antagonizing the positive regulators AphA and AphB at the *tcpPH* promoter (29). As for the expression of HA/proteases, our data suggest that CRP impacts *hapA* transcription by activating quorum sensing (31) and by increasing the expression of H-NS to enhance RpoS biosynthesis.

In Fig. 9, we summarize the regulatory interactions between CRP, H-NS, and RpoS that could affect virulence, stress response, motility, and chemotaxis. The genes found to be affected by H-NS in this study can be divided into at least two categories. In class I genes, H-NS appears to affect gene expression by the well-documented mechanism of transcriptional silencing. These genes include *ctxA*, *tcpA* (45), *katB*, and *c551*. Expression of class II genes is positively affected by H-NS, which acts indirectly by enhancing the expression of RpoS. This class includes the RpoS-dependent genes *hapA*, *cheR-2*, *flaA*, *flaC*, and *motX*. Among these genes, *flaA*, *flaC*, and *motX* appear to constitute a subclass in which H-NS could also act as a transcriptional silencer in the absence of *rpoS*.

The ability of *V. cholerae* to respond to environmental changes is crucial to both intestinal colonization and survival outside the human host. This adaptation requires the concerted activity of multiple global regulators. A highly complex regulatory network controls the expression of the general stress response, motility, and chemotaxis to enhance *V. cholerae* environmental fitness. In this study, we provide evidence that H-NS regulates the stress response and motility by RpoS-dependent and -independent mechanisms.

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