Interaction of the Histone-Like Nucleoid Structuring Protein and the General Stress Response Regulator RpoS at *Vibrio cholerae* Promoters That Regulate Motility and Hemagglutinin/Protease Expression

Hongxia Wang, Julio C. Ayala, Jorge A. Benitez, and Anisia J. Silva

Southern Research Institute, Drug Discovery Division, Department of Biochemistry and Molecular Biology, Birmingham, Alabama, USA, and Morehouse School of Medicine, Department of Microbiology, Biochemistry and Immunology, Atlanta, Georgia, USA

The bacterium *Vibrio cholerae* colonizes the human small intestine and secretes cholera toxin (CT) to cause the rice-watery diarrhea characteristic of this illness. The ability of this pathogen to colonize the small bowel, express CT, and return to the aquatic environment is controlled by a complex network of regulatory proteins. Two global regulators that participate in this process are the histone-like nucleoid structuring protein (H-NS) and the general stress response regulator RpoS. In this study, we address the role of RpoS and H-NS in the coordinate regulation of motility and hemagglutinin (HA)/protease expression. In addition to initiating transcription of *hapA* encoding HA/protease, RpoS enhanced *flrA* and *rpoN* transcription to increase motility. In contrast, H-NS was found to bind to the *flrA*, *rpoN*, and *hapA* promoters and represses their expression. The strength of H-NS repression at the above-mentioned promoters was weaker for *hapA*, which exhibited the strongest RpoS dependency, suggesting that transcription initiation by RNA polymerase containing ρ could be more resistant to H-NS repression. Occupancy of the *flrA* and *hapA* promoters by H-NS was demonstrated by chromatin immunoprecipitation (ChIP). We show that the expression of RpoS in the stationary phase significantly diminished H-NS promoter occupancy. Furthermore, RpoS enhanced the transcription of integration host factor (IHF), which positively affected the expression of *flrA* and *rpoN* by diminishing the occupancy of H-NS at these promoters. Altogether, we propose a model for RpoS regulation of motility gene expression that involves (i) attenuation of H-NS repression by IHF and (ii) RpoS-dependent transcription initiation resistant to H-NS.

Cholera is an acute waterborne diarrheal disease caused by *Vibrio cholerae* of serogroups O1 and O139. This highly motile Gram-negative pathogen continues to be a major public health concern in areas of South Asia and Africa. Infecting *Vibrio* spp. that overcome the gastric acid barrier swim toward the intestinal mucosa and express two major virulence factors: the toxin coregulated pilus (TCP), required for intestinal colonization, and cholera toxin (CT), which is largely responsible for the profuse rice-watery diarrhea typical of this disease (16, 25). Later in infection, *V. cholerae* downregulates the expression of virulence factors and detaches to return to the environment (60). At this stage, the expression of motility and hemagglutinin (HA)/protease has been suggested to facilitate *V. cholerae* detachment from the intestinal mucosa (5, 17, 35, 47). Motility and HA/protease are positively regulated by the cyclic AMP (cAMP)-receptor protein (CRP), which acts by enhancing the quorum-sensing regulator HapR and the general stress response regulator RpoS (4, 29, 46). The expression of both phenotypes is diminished in response to an increase in the intracellular concentration of the second messenger cyclic diguanylate (c-di-GMP) (57).

The histone-like nucleoid structuring protein (H-NS) is a global regulator belonging to a family of small nucleoid-associated proteins that include the factor for inversion stimulation (FIS), the heat-unstable protein (HU), and integration host factor (IHF) (14, 15). Mutations that inactivate *hns* are highly pleiotropic and diminish bacterial growth, suggesting that H-NS influences a broad spectrum of physiological processes (1, 2, 23). H-NS consists of an N-terminal oligomerization domain connected by a flexible linker to a nucleic acid binding domain (2, 8, 14, 36). Both oligomerization and DNA binding are required for the biological activity of H-NS, which includes DNA condensation and the regulation of transcription (10, 50). In transcription regulation, H-NS has been shown to negatively affect gene expression by binding to promoters exhibiting AT-rich highly curved DNA regions that contain clusters of the more conserved 10-bp motif TC GATAAATT (28, 40, 55). In addition, H-NS can positively or negatively affect the expression of a broader spectrum of genes by acting indirectly or binding to mRNA to affect translation (7). A common theme in H-NS transcription regulation is the silencing of horizontally acquired genes (30, 34, 39). Consistent with this role, H-NS has been shown to silence virulence gene expression in *V. cholerae* by acting at different levels of the ToxR regulatory cascade, which includes the toxT, tcpA, and ctxA promoters (37). Further, *V. cholerae* hns mutants have been reported to exhibit diminished motility and intestinal colonization capacity (18, 27, 49, 53). There are numerous evidences indicating that repression by H-NS can be relieved in response to environmental stimuli that activate the expression of other regulators whose binding site overlaps with that of H-NS. For instance, transcriptional silencing of *V. cholerae* tcpA and ctxA promoters by H-NS is antagonized by the AraC-like transcriptional regulator ToxT and IHF (51, 52, 59).

The alternative sigma factor RpoS (ρ) is a global regulator that controls the expression of more than 100 genes in response to
environmental stresses (20). *V. cholerae* *rpoS* mutants are more sensitive to starvation, high osmolarity, and oxidative stresses, are less motile than the wild type (WT), and do not express HA/protease (35, 49, 58). We recently reported that RpoS diminishes the cellular concentration of c-di-GMP, an inhibitor of flagellar motility (57). Consistently, microarray studies have shown that *rpoS* mutants express reduced levels of multiple motility and chemotaxis genes, suggesting that *rpoS* could act at an early stage of the motility regulatory cascade (35). In a previous study, we addressed the role of H-NS in the regulation of *V. cholerae* RpoS and RpoS-dependent genes that affect motility and HA/protease production (49). We found that H-NS posttranscriptionally affects RpoS expression in a positive manner, which in turn enhances motility by promoting transcription initiation resistant to H-NS. These results suggested a model in which H-NS can positively direct by promoting transcription initiation resistant to H-NS.

### MATERIALS AND METHODS

#### Strains and media.

The strains, plasmids, and oligonucleotide primers used in this study are listed and briefly described in Tables 1 and 2. Mutants and reporter strains were all derived from *V. cholerae* C7258 (El Tor biotype; Ogawa). *V. cholerae* strains were grown in tryptic soy broth (TSB) with agitation (225 rpm) at 37°C. For cloning purposes, *Escherichia coli* strains TOP10 (Invitrogen) and S17-1pir1 (11) were grown in LB medium at 37°C. When necessary, the culture medium was supplemented in higher expression levels of flaA, flaC, and motX mRNA (49).

### TABLE 1 Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Source or reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C7258</td>
<td>Wild type, El Tor biotype, Perú 1991</td>
<td>Clinical isolate</td>
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<tr>
<td>C7258Δhns-FLAG</td>
<td><em>hns</em>::FLAG cloning in pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>C7258ΔlacZ</td>
<td>lacZ deletion mutant</td>
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<tr>
<td>AJB80</td>
<td>C7258 ΔrpoS Δhns::Km</td>
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<td>AJB50</td>
<td>C7258 ΔrpoS</td>
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<tr>
<td>AJB50ΔlacZ</td>
<td>ΔlacZ deletion mutant</td>
<td>This study</td>
</tr>
<tr>
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<td>AJB81</td>
<td>C7258 ΔrpoS Δhns::Km</td>
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<td>ΔlacZ ΔrpoS cloning in pUC19</td>
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<td>AJB80ΔrpoS-FLAG</td>
<td>ΔrpoS cloning in pUC19</td>
<td>This study</td>
</tr>
<tr>
<td>HX120</td>
<td>C7258 ΔhpfA</td>
<td>This study</td>
</tr>
<tr>
<td>HX121</td>
<td>C7258 ΔhpfA</td>
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<td>HX121ΔHNS-FLAG</td>
<td>Δhns::FLAG cloning in pUC19</td>
<td>This study</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<td>pHNS-FLAG</td>
<td><em>hns</em> ORF cloned in pFLAG-CTC</td>
<td>Sigma-Aldrich</td>
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<td>pTT3</td>
<td><em>rrnB</em> T1T2</td>
<td>48</td>
</tr>
<tr>
<td>pTT3Δhns-FLAG</td>
<td>deleted <em>hns</em>::FLAG cloning in pTT3</td>
<td>This study</td>
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<tr>
<td>pCVDHNS-FLAG</td>
<td><em>hns</em>::FLAG cloning in pCVD442</td>
<td>This study</td>
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<tr>
<td>pKRS1</td>
<td>Plasmid carrying promoterless <em>lacZ</em> gene</td>
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<tr>
<td>pTT3ΔflaA</td>
<td>390-bp <em>flaA</em> promoter region cloned in pTT3</td>
<td>This study</td>
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<tr>
<td>pFlaA-LacZ</td>
<td>850-bp fragment containing <em>flaA</em> promoter in pKRS1</td>
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<tr>
<td>pTT3ΔflaA</td>
<td>450-bp DNA fragment carrying the flaA promoter in pTT3</td>
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<tr>
<td>placZ</td>
<td>Promoterless <em>lacZ</em> gene transferred from pKRS1 to pBR322</td>
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<td>pTT4</td>
<td>Transcription terminator <em>rrnB</em> T1T2, inserted in pUC19 as a KpnI-BamHI fragment</td>
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<td>pTT4ΔrpoN</td>
<td>410-bp DNA fragment carrying the rpoN promoter in pTT4</td>
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<tr>
<td>pRpoN-LacZ</td>
<td>DNA fragment containing <em>rrnB</em> T1T2 and rpoN promoter ligated to promoterless <em>lacZ</em> gene in pLacZ</td>
<td>This study</td>
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<tr>
<td>pHapLac11</td>
<td>Plasmid vector containing transcriptional <em>hapA-lacZ</em> fusions</td>
<td>46, 48</td>
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<td>pTXTB1</td>
<td>Expression vector for construction of in-frame fusions with the intein/chitin binding domain</td>
<td>New England BioLabs</td>
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<td>pTXTB1-RpoS</td>
<td><em>rpoS</em> ORF cloned in plasmid pTXTB1</td>
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<td>pCVDΔrpoS-FLAG</td>
<td>RpoS-FLAG fusion in suicide vector pCVD442</td>
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<tr>
<td>pUCΔIhpfA</td>
<td>0.3-kb SacI-BamHI and 0.3-kb BamHI-SphiI fragments sequentially cloned in pUC19</td>
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<tr>
<td>pUCΔIhpfA-Km</td>
<td>1.2-kb Km' gene in BamHI site of pUCΔIhpfA</td>
<td>This study</td>
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<tr>
<td>pCVDΔIhpfA-Km</td>
<td>SacI-SphiI ΔIhpfA::Km cassette in pCVD442</td>
<td>This study</td>
</tr>
</tbody>
</table>
with ampicillin (Amp; 100 μg/ml), kanamycin (Km; 25 μg/ml), rifampin (Rf; 150 μg/ml), polymixin B (PolB; 100 units/ml), isoperospol-β-d-thio-
galactopyranoside (IPTG; 20 μg/ml), or 5-bromo-4-chloro-3-indolyl-β-
galactopyranoside (X-Gal; 20 μg/ml).

**Construction of mutants and reporter strains expressing H-NS and**
**RpoS proteins tagged with the FLAG epitope.** To construct a strain
containing a deletion and Km insertion in *ihfA*, encoding the IHF A subunit,
DNA fragments flanking the *ihfA* locus (VC1222) were amplified using
primer combinations IHFB/R1 and IHFA/F1, and an Advantage 2 PCR kit (BD Biosciences Clontech). The PCR products were sequentially cloned in pUC19 to yield pUCAihFA. This plasmid was modif-
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sion number X06404) to generate pUCAihFA-Km, and the entire V. cholerae DNA harboring the Km-resistant gene replacing the *ihfA* open
reading frame (ORF) was transferred to pCVD442 (13) to yield
pCVD442-ihfA-Km. Finally, the above-mentioned suicide vector was transferred
to strains C7258 and AJB50, and mutants HX120 and HX121 (Ta-
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precipitation (ChIP). To this end, the *hns ORF* (vch, VC1130) lacking the
stop codon was amplified from C7258 genomic DNA using primers
VicH5 and VicH396. The amplification product was confirmed by DNA
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**RT-PCR.** V. cholerae cultures were treated with RNAprotect bacterial
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ovided by the manufacturer’s instructions. Quantitative RT-PCR (qRT-PCR)
was conducted using an iScript two-step RT-PCR kit with SYBR
green (Bio-Rad Laboratories). Relative expression values were calculated as
2^(-ΔΔCt), where ΔCt is the fractional threshold cycle for the
target gene and the reference is the *recA* mRNA. The following primer
combinations were used: FlrA723/FlrA884 for *flrA* mRNA, IFS/IF-
HA-R for *ihfA* mRNA, IHFB-F/IFH-B-R for *ihfb* mRNA, RecA578/RecA63 for
*recA* mRNA, and RpoNS6/RpoNS7 for *rpoN* mRNA.

**Transcriptional start mapping of the flrA and rpoN promoters by 5′ RACE analysis.** To locate the *rpoN* promoter region, cDNA was synthe-
sized using random hexamers and 20 ng of total RNA. Then, PCRs were
carried out using primer combinations consisting of a forward primer
annealing to each gene of the putative *rpoN* operon and a reverse primer
annealing to the adjacent 3′ open reading frame. A control using total
RNA as the template was run for each reaction to exclude chromosomal
dNA contamination. Once the *rpoN* promoter region was identified, 5′
rapid amplification of cDNA ends (5′ RACE) was used to determine the

**Table 2 Oligonucleotide primers**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′→3′)*</th>
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</thead>
<tbody>
<tr>
<td>HfaAp-F</td>
<td>GAACGAATGCCTTCAATGCTATGC</td>
</tr>
<tr>
<td>HfaAp-R</td>
<td>GCAGGTTGTGCATTTCCAGCAGAC</td>
</tr>
<tr>
<td>FlrA-R1</td>
<td>GAGTCTTCGAGGGATGAGG</td>
</tr>
<tr>
<td>FlrA-R2</td>
<td>CACCGAGCTAACACTTACCAAC</td>
</tr>
<tr>
<td>IHFB-R1</td>
<td>GATCGTGAGGATGTAATGTCGGAATCA</td>
</tr>
<tr>
<td>IHFB-R2</td>
<td>CTTTCATCGCTTGACCGTGTG</td>
</tr>
<tr>
<td>IHFB-R3</td>
<td>CCCTTTGCCATCACTATCGG</td>
</tr>
<tr>
<td>IHFB-R4</td>
<td>CGCAAACTCTCGGTCGAAGAC</td>
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<td>IHFB-R5</td>
<td>CCCTTTGCCATCACTATCGG</td>
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<td>IHFB-R6</td>
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<td>IHFB-R7</td>
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<td>IHFB-R8</td>
<td>AGGAGATCCAGGCAATCATTCC</td>
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<tr>
<td>IHFB-R9</td>
<td>GAGTTCTCCGACTCGTTCCTATAG</td>
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</tbody>
</table>

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* Restrictions sites used for directional cloning are underlined.
transcription initiation site. Similarly, 5′ RACE was conducted to deter-
mine the transcription initiation site of flrA. To this end, first-strand
DNA synthesis was conducted using a second-generation 5′/3′ RACE kit
(Roche Applied Sciences). Briefly, cDNA was synthesized from 5 μg of
total RNA using primer FlrA-R1 for flrA and primer VC2522-R2 for rpoN
as gene-specific primers. The cDNA was treated with RNase H and RNase
T1, followed by purification with a High Pure PCR product purification kit
(Roche Applied Sciences). Next, cDNA was incubated with terminal
transferase for 30 min and the deoxyribosyladenylase (da)‐tailed cDNA
was amplified with FastStart Tag DNA polymerase (Roche Applied Sci-
ences) according to the 5′/3′ RACE kit protocol and by using primers
FlrA-R2 and VC2522-R2 for flrA and rpoN, respectively. Finally, nested
PCRs were conducted using each amplified tailed cDNA as a substrate and
primers FlrA-R3 and VC2522-R3 for flrA and rpoN, respectively. The
nested PCR products were ligated into SalI- and EcoRI-digested pUC19,
and 10 positive clones were sequenced for each 5′ RACE experiment.

Construction of flrA-, flaA-, and rpoN-lacZ promoter fusions. To construct an
flrA-lacZ promoter fusion, we amplified a 390-bp fragment containing
the flrA promoter (as defined by 5′ RACE analysis) with primers
FlrA-F and FlrA-R. For the flaA-lacZ promoter fusion, a 450-bp fragment
containing the flaA promoter (26) was amplified with primers
FlaA-F and FlaA-R. In both cases, the promoter fragments were in-
duced with IPTG (0.4 mM) and the culture was incubated for 3 h at
30°C. Finally, protein-DNA complexes were separated by electropho-
resis in 5% Tris-borate-EDTA (TBE) polyacrylamide gels and transferred
to nylon membranes, and DNA was visualized using an anti-DIG Fab
fragment-AP conjugate, followed by chemiluminescence detection. The
following primer combinations were used to amplify the promoter
regions under study: FlrA-F41 and FlrA-R42 for flrA, HapA-F29 and HapA-
R259 for hapA, RpoF21 and RpoF22 for rpoN, and TcpA-F1 and
TcpA-R2 for tcpA. The promoter of VC1922, an ortholog of Salmonella
enterica serovar Typhimurium STM1033 not regulated by H-NS (34), was
amplified using primers VC1922-F61 and VC1922-F62.

ChIP. For H-NS promoter occupancy, strains expressing H-NS-FLAG
were grown to the stationary phase in TSB medium (16 h). Then, 40 ml of
culture was sequentially treated with R (20 min, 37°C), 1% formaldehyde
(cross-linking, 10 min, 30°C), and 227 mM glycine (30 min, 4°C). Cells
were collected by centrifugation, washed twice with phosphate-buffered
saline (PBS) supplemented with protease inhibitor cocktail (PIC) and
methylthioursulfonyl fluoride (PMSF; Roche Applied Science), and di-
vided into aliquots equivalent to 1/(culture OD600 reading) ml, and the
cell pellets were maintained at −80°C if not processed immediately. Next,
the cells were lysed by suspending the frozen pellets in 500 μl of 10 mM
Tris-HCl, pH 8.0, 50 mM NaCl containing 20 μg/ml of RNase A, and 105
kU of Ready-Lyse lysozyme (Epicentre Biotechnologies), followed by
30-min incubation at 37°C. One volume of double-strength immunopre-
cipitation (IP) buffer (200 mM Tris-HCl, pH 7.5, 600 mM NaCl, 4% Triton X-100) containing PIC and PMSF was added to each lysate, and
DNA was broken down to a range of 150 to 1,000 bp by sonication.
The cell debris was removed by centrifugation, and the lysate was diluted 10-
fold in IP buffer. At this stage, a 10-μl input sample was saved as a refer-
cence and PCR efficacy control. Protein-DNA complexes were immuno-
precipitated by overnight incubation at 4°C with 8 μg of anti-FLAG M2
monoclonal antibody (Sigma-Aldrich) or 8 μg of an unrelated anti-
Xpress monoclonal antibody (Invitrogen) for a mock ChIP. The
antibody–protein–DNA complexes were pulled down with salmon sperm
dNA-treated protein A agarose beads (Imgenex, San Diego, CA) for 1 h
at 4°C. The beads were washed twice with 100 mM Tris-HCl, pH 7.5, 250
mM LiCl, and 2% Triton X-100, collected in Spin-X centrifuge tube filters
(Costar), and washed three times with IP buffer containing 600 mM NaCl,
IP buffer, and TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The
immunoprecipitated complexes were eluted from the beads by incubation
at 65°C for 30 min in TE buffer containing 1% SDS. After reversal of
cross-linking (4 h, 65°C), proteins were removed by incubation with
20 μg of proteinase K (1 h, 45°C). Then, immunoprecipitated DNA was
purified using a MiniElute PCR purification kit (Qiagen). At least three
ChIP assays were conducted for each strain.

Detection of immunoprecipitated DNA. Immunoprecipitated DNA
was qualitatively detected by PCR and agarose gel electrophoresis using
the primer combinations to amplify the promoter fragments described for
the EMSA. Real-time quantitative PCR (qPCR) was used to quantitate
promoter occupancy by H-NS. To this end, PCR was conducted using
iTag SYBR green supermix with ROX (Bio-Rad). The quantity of immu-
noprecipitated DNA was calculated as the percentage of the DNA present
in the input sample using the formula IP = 2(2^CtInput CADT), where Ct is the
fractional threshold cycle of the input and IP samples. The relative IP
was calculated by standardizing the IP of each sample by the IP of the corre-

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sponding mock ChIP. Finally, a region within the rpsM ORF to which
H-NS does not bind acting as a transcriptional repressor was amplified
with primers RpsM-F51 and RpsM-R52 and used as a negative control.

**Western blot detection of H-NS and RpoS.** For detection of native
H-NS and RpoS proteins, serum exhibiting high anti-H-NS and anti-
RpoS titers was first preabsorbed with crude extracts of *V. cholerae* Δ*hns*
and Δ*rpoS* mutants, respectively. To this end, cells were collected by cen-
trifugation at 4°C and resuspended in Tris-buffered saline containing
0.1% Tween 20 (TBST) supplemented with PIC. The cells were disrupted
by sonication, and the debris was removed by centrifugation. Then, a
1:1,000 dilution of anti-H-NS or anti-RpoS serum was incubated over-
night at 4°C with crude extracts of strain AJB80 (Δ*hns*) or AJB50 (Δ*rpoS*)
containing 2 mg/ml protein, respectively. For Western blot analysis, a
volume of cells corresponding to 1.0 OD*600* units was centrifuged and the
pellet was resuspended in 0.1 ml of Laemmli’s sample buffer (Bio-Rad
Laboratories). The cell suspension was placed in a boiling water bath for
10 min, and the cell debris was removed by centrifugation. Proteins were
separated using Criterion Precast 10% gels (Bio-Rad) and transferred to
polyvinylidene difluoride (PVDF) membranes. The expression of H-NS
and RpoS was determined using the corresponding preabsorbed antise-
rum and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG
(Thermo Fisher Scientific, Rockford, Ill.). A similar procedure was used to
detect RpoS-FLAG and H-NS-FLAG but by using monoclonal anti-FLAG
M2-peroxidase (Sigma-Aldrich). Membranes were developed using a BM
bioluminescence Western blotting kit (Roche Applied Science). To esti-
mate the concentration of H-NS and RpoS in cell lysates, known quanti-
ties of each protein were analyzed by Western blotting. Then, a standard
curve was constructed for each regulator by plotting band intensities de-
termed using TotalLab Quant software (TotalLab Ltd., Newcastle upon
Tyne, United Kingdom) versus concentration.

**Enzyme assays.** β-Galactosidase activity was measured as described
by Miller (33) using the substrate o-nitrophenyl-β-n-galactopyranoside.
Specific activities are given in Miller units [1,000 × (OD*540* × t × OD*600*)],
where *t* is the reaction time and *v* is the volume of enzyme extract per
reaction.

**TEM.** For transmission electron microscopy (TEM), the cells were
pelleted and fixed by reconstitution in 2.5% glutaraldehyde sodium caco-
dylic buffer, pH 7.3. Samples were adhered to a carbon-coated grid and
stained with 1% uranyl acetate before microscopy.

**RESULTS**

**Deletion of rpoS partially suppresses the slow-growth pheno-
type of the hns mutant.** In our previous study, we observed that an
Δ*rpoS* Δ*hns* double mutant exhibited a slightly larger swarm di-
ameter than the Δ*hns* single mutant (49). Since differences in
growth rate could affect the swarm diameter observed in semisolid
agar, we examined the growth patterns of the WT, Δ*rpoS*, Δ*hns*,
and Δ*rpoS* Δ*hns* strains. As shown in Fig. 1A, the Δ*hns* mutant
exhibited diminished growth compared to the WT and Δ*rpoS*
strains. Interestingly, deletion of *rpoS* in the *hns* mutant led to a
partial suppression of its slow-growth phenotype. Transmission
electron microscopy showed an abundance of elongated cells in the
Δ*hns* mutant (Fig. 1B). However, the presence of elongated
cells was not suppressed by deletion of *rpoS* (Fig. 1B). It is note-
worthy that both the Δ*hns* and Δ*rpoS* Δ*hns* mutants were flagel-
lated but still appeared similarly depressed for motility by
hanging-drop bright-field microscopy examination.

The finding that deletion of *rpoS* partially suppressed the slow-
growth phenotype of an Δ*hns* mutant suggested that, as observed in
*E. coli*, H-NS acts as a repressor of many RpoS-dependent genes
(3). Thus, we used specific H-NS and RpoS antisera to measure the
expression of these regulators at different stages of the bacterial
growth curve. As shown in Fig. 2, H-NS can be detected in both
exponentially growing and stationary-phase cultures, while RpoS
was induced in the late logarithmic phase (OD*500* ≥ 2). Thus, we
decided to use cells in the stationary phase (expressing both pro-
teins) for subsequent studies on the interaction between these
global regulators.

**Regulation of the flaA, rpoN, hapA, and flaA promoters by
RpoS and H-NS.** Microarray studies have shown that deletion of
*rpoS* results in reduced expression of motility and chemotaxis genes
belonging to the class II, III, and IV hierarchies (35). Therefore, we
decided to examine the role of RpoS and H-NS in the transcription of
the upstream *rpoN* and *flaA* regulator genes. To specifically address
the effect of RpoS and H-NS on the *rpoN* and *flaA* promoters, we
determined the transcription initiation sites of both genes and con-
structed *lacZ* promoter fusions.

The *rpoN* gene is predicted to be part of a large operon, sug-
suggesting that it might lack its own promoter (26). By using RT-PCR
and different primer combinations, we found that the *rpoN*
promoter is located in the intergenic region between VC2520 and
VC2522 (data not shown). We then used 5′ RACE analysis to
determine the transcription initiation site of *rpoN* and its cognate
\sigma^{54}\text{-dependent activator} \text{flrA. Sequencing of the 5’ RACE 500-bp nested PCR product amplified using primer VC2522-R3 indicated that} rpoN \text{transcription is initiated at a cysteine located 277 bp upstream of the start codon of VC2522. Further, sequencing of the 5’ RACE 250-bp nested PCR product amplified using primer FlrA-R3 indicated that} flrA \text{transcription is initiated at a cysteine located 146 bp upstream of the} flrA \text{start codon. Based on this information, we constructed} lacZ \text{fusions consisting of the} rpoN \text{and} flrA \text{promoters linked to a promoterless} lacZ \text{gene. A similar fusion consisting of the} rpoS \text{and} flrA \text{promoters linked to a promoterless} lacZ \text{gene has been described previously (48). Expression of the above-noted} lacZ \text{fusions were examined in the WT,}\ \Delta rpoS, \Delta hns, \text{and} \Delta rpoS \Delta hns \text{genetic backgrounds. As shown in Fig. 3, transcription of} rpoN, flrA, \text{and} hapA \text{was diminished in the}\ \Delta rpoS \text{mutant while deletion of} hns \text{enhanced their transcription. However,} flrA \text{and} rpoN \text{differed from} hapA \text{in that the first two promoters were less dependent on} RpoS \text{but more strongly repressed by} H-NS. \text{The expression of} flrA \text{and} rpoN \text{in the absence of both} H-NS \text{and} RpoS \text{reflects the level of unpressed transcription initiation by RNA polymerase (RNAP) containing}\ \sigma^9, \text{which was negligible in the case of} hapA. \text{We also examined the effect of the regulation on the expression of} flaA, \text{a gene belonging to the downstream class III transcription hierarchy encoding the major} V. \text{cholerae flagellin (Fig. 3). As predicted, regulation of} flaA \text{in stationary-phase cultures followed the same pattern as that of its upstream regulators} RpoN \text{and} FlrA.}

\text{H-NS binds to the} flrA, rpoN, \text{and} hapA \text{promoters in vitro.} \text{Our results suggested that} H-NS \text{negatively affects the transcription of} rpoN, flrA, \text{and} hapA. \text{Therefore, we decided to examine whether pure} H-NS \text{can bind to these promoters.} H-NS \text{is known to bind DNA with a more relaxed sequence specificity than that of other regulators that have more-stringent binding requirements. Thus, we used the} tcpA \text{promoter, a gene silenced by} H-NS \text{(37), and the region upstream of} VC1922 \text{as positive and negative controls, respectively.} VC1922 \text{is an ortholog of} Salmonella \text{STM1033 located in a chromosomal region not occupied by} H-NS, \text{as described previously in a ChIP-on-chip study (34). We used qRT-PCR to confirm that this} \text{gene is also not affected by} H-NS \text{in}\ V. \text{cholerae} \text{(data not shown). As shown in Fig. 4,} H-NS \text{was found to bind to the} tcpA, flrA, rpoN, \text{and} hapA \text{promoters while very little binding to the} VC1922 \text{promoter could be detected.}

\text{RpoS negatively affects} H-NS \text{promoter occupancy.} \text{To determine} H-NS \text{occupancy at the} flrA, rpoN, \text{and} hapA \text{promoters at the cellular level, we constructed strains} C7258HNS-FLAG \text{and} AJB50HNS-FLAG \text{(Table 1) and conducted ChIP. In these experiments, we used two negative controls: (i) a DNA sequence located within an ORF of the housekeeping gene} rpsM \text{and (ii) the} hapA \text{promoter region of} VC1922, \text{to which} H-NS \text{failed to exhibit signifi-}

\text{FIG 2 Detection of} H-NS (A) \text{and} RpoS (B) \text{expression levels. The} V. \text{cholerae} C7258lacZ \text{strain was grown in TSB medium, and samples were withdrawn for analysis in the log phase (OD}_{600} 0.5) \text{(lane 1), late log phase (OD}_{600} 2.0) \text{(lane 2), and stationary phase (4 h after the culture reached an OD}_{600} \text{of 2.0) (lane 3).} H-NS \text{and} RpoS \text{protein concentrations are shown below the bands.}

\text{FIG 3 Expression levels of} flrA-, rpoN-, flaA-, \text{and} hapA-lacZ \text{promoter fusions in} V. \text{cholerae}\ \Delta rpoS \text{and} \Delta hns \text{mutants.} C7258\Delta lacZ \text{(WT), AJB50\Delta lacZ (\Delta rpoS), AJB80 (\Delta hns), and AJB81 (\Delta rpoS \Delta hns) strains containing} flrA-, rpoN-, flaA-, \text{and} hapA-lacZ \text{promoter fusions were grown to the stationary phase in TSB at 37°C. Detection of}\ \beta\text{-galactosidase activity was measured as described in Materials and Methods and expressed in Miller units. Each value is the mean for six independent cultures. The error bars indicate standard deviations (+, significantly different from that of the wild type [P < 0.01 by a one-tailed t test]). The spans of the promoter fragments used relative to the start codon were as follows: positions –536 to –142 for} flrA, \text{positions –685 to –273 relative to} VC2522 \text{for} rpoN, \text{positions –407 to + 43 for} flaA, \text{and positions –410 to + 3 for} hapA.
Integration host factor positively affects the expression of flrA and rpoN. Analysis of the flrA and rpoN promoters using Virtual Footprint software (http://www.prodoric.de/vfp/vfp_promoter.php) showed the presence of IHF binding sites with scores higher than those found at the tcpA promoter, which is enhanced by this regulator (52). Thus, we introduced an ihfA deletion in strains C7258 and AJB50 (∆rpoS) and used qRT-PCR to assess the effect of RpoS and IHF on the expression of flrA and rpoN. As shown in Fig. 6, significantly less flrA and rpoN mRNA was detected in the rpoS and ihfA mutants. For both genes, the ∆rpoS ΔihfA double mutant exhibited the lowest level of expression. To examine the role of IHF in H-NS occupancy at the tcpA, flrA, and rpoN promoters, the allele encoding H-NS–FLAG was introduced in strains containing flrA, tcpA, and rpoN promoters (Fig. 5B). Western blot analysis did not reveal differences in H-NS–FLAG expression between the wild-type and ∆rpoS strains, indicating that the observed increase in promoter occupancy is not due to an increase in H-NS–FLAG expression in the ∆rpoS mutant (Fig. 5C).
wild type. The ΔrpoS ΔihfA double mutant exhibited the highest H-NS occupancies for tcpA, flrA, and rpoN, suggesting the occurrence of more than one mechanism involved in this process.

H-NS can negatively affect its occupancy of target promoters by enhancing RpoS. The data noted above revealed a regulatory pathway by which the expression of RpoS in the stationary phase could diminish H-NS occupancy of target promoters at which σS could initiate transcription (Fig. 5). In a previous study, we showed that H-NS positively affected the expression of rpoS by stabilizing its mRNA (49). To demonstrate that H-NS enhances RpoS protein expression, we compared the production levels of a chromosomally integrated RpoS protein expression, we compared the production levels of a stabilizing its mRNA (49). To demonstrate that H-NS enhances RpoS protein expression, we compared the production levels of a chromosomally integrated RpoS-FLAG allele from its native transcription and translation signals in WT and Δhns strains. As shown in Fig. 8, the Δhns mutant expressed reduced RpoS-FLAG expression compared to the wild-type strain.

DISCUSSION

The general stress response regulator RpoS and the nucleoid structuring protein H-NS are global regulators known to affect intestinal colonization, virulence gene expression, and mucosal escape in the cholera bacterium (18, 27, 32, 35, 49). Here, we focus on the role of these proteins in the transcription of motility regulators rpoN and flrA as well as hapA, encoding HA/protease. Like other Gram-negative bacteria, V. cholerae hns mutants exhibit a slow-growth phenotype. It has been suggested that the inadequate overexpression of numerous cellular proteins and defective chromosome replication contribute to slow growth of hns mutants (1, 20, 24). In E. coli, RpoS and entire sets of RpoS-dependent genes are repressed by H-NS, and it has been shown that deletion of hns leads to deleterious overexpression of these genes in the exponential phase (3, 6, 19, 20). Our finding that deletion of rpoS partially suppressed the slow-growth phenotype of a V. cholerae hns mutant is consistent with the presence of an important set of genes in the cholaer bacterium’s genome under dual regulation by RpoS and H-NS. The fact that deletion of rpoS did not fully suppress the Δhns slow-growth phenotype indicates that other cellular processes are compromised in the hns mutant. For instance, we observed that V. cholerae hns mutant cells appear elongated compared to wild-type cells, and their morphology was not suppressed by deletion of rpoS. This result is consistent with data showing that defective chromosome replication in hns mutants is independent of RpoS (1). Microscopic examination revealed that the Δhns and ΔrpoS Δhns mutants are both flagellated and similarly depressed for swimming. Thus, we suggest that the increase in swarm diameter observed in the ΔrpoS Δhns double mutant compared to that of its Δhns precursor (49) is a consequence of partial suppression of the Δhns slow-growth phenotype.

In V. cholerae, RpoS activates hapA and enhances the expression of motility genes belonging to the class II, III, and IV hierarchies (35, 46, 57). We examined the role of RpoS and H-NS in the expression of rpoN and flrA, which control the expression of the class II, III, and IV hierarchy motility genes (42). In this study, we demonstrate that RpoS can enhance motility by positively affecting the expression of rpoN and flrA while H-NS acts as a repressor of these genes. These results suggest that RNAP containing σS can contribute to the transcription of flrA and rpoN in the stationary phase. Repression by H-NS was stronger at the flrA and rpoN promoters than at the hapA promoter. The difference between flrA, rpoN, and hapA is that while the first two promoters are transcribed by RNAP containing σ70 in exponentially growing cultures, the expression of hapA is tightly RpoS dependent and restricted to the stationary phase. The behavior of these promoters appears to be in agreement with studies suggesting that H-NS exhibits higher selectivity for inhibition of transcription initiated by RNAP containing σ70 (19–21, 54). Thus, we propose that participation of RNAP-S in the transcription of flrA and rpoN in the stationary phase could render these promoters more resistant to repression by H-NS. As expected, a similar interplay between RpoS and H-NS was observed in the regulation of the downstream gene flaA, encoding the major V. cholerae flagellin. The observed regulation raises the question of why the hns mutant is less motile in spite of being flagellated and having elevated flaA expression. One possibility is that the altered cell morphology of this mutant
could indirectly hamper flagellum rotation and/or bacterial swimming speed. A second explanation, suggested by analogy to E. coli, is that H-NS could participate directly in torque generation by interacting with the switch complex proteins FliG and MotA (12). In this case, the lack of H-NS leads to a paralyzed flagellum.

Gel retardation assays indicated that H-NS can bind the flrA, rpoN, and hapA promoters with an affinity comparable to that of tcpA, a promoter known to be silenced by H-NS (37). H-NS is known to exhibit a broad specificity window for DNA binding that could be affected by the presence of other regulators. Thus, we conducted ChIP to determine H-NS occupancy at the tcpA, flrA, rpoN, and hapA promoters, using the promoter region of VC1922 and a sequence within the rpsM ORF as negative controls. According to our data, the highest H-NS occupancy was found for tcpA, followed by flrA and hapA. Very little H-NS occupancy was found at the rpoN promoter, suggesting that, at the time cells were collected for ChIP, other regulatory factors could prevent H-NS binding to this promoter. Deletion of rpoS significantly enhanced H-NS occupancy at the tcpA, flrA, and hapA promoters. The effect of RpoS on H-NS occupancy suggests a second mechanism by which the expression of α5 can partially counteract H-NS repression. Sigma S has less affinity for the core polymerase than α70, with which it needs to compete for binding before it can access promoters (9). In addition, RNAP containing either α70 or α5 does not compete with H-NS for binding to promoter DNAs (45). Thus, we suggest that RpoS could diminish H-NS occupancy indirectly by inducing the expression of other trans-acting transcriptional regulators. IHF is known to alleviate H-NS silencing of S. enterica hilA (43), E. coli cgD (38), Shigella flexneri vir genes (41), and the bacteriophage Mu early promoter (56). In V. cholerae, IHF positively affects tcpA expression by binding to its promoter at a position that overlaps the H-NS binding site (52). Here, we show that the expression of RpoS in the stationary phase led to a significant increase in IHF expression. Moreover, we found that, similar to tcpA (52), IHF enhanced the expression of flrA and rpoN. The finding that deletion of both ihfA and rpoS diminished the expression of flrA and rpoN in an additive manner is consistent with the occurrence of more than one mechanism affecting the expression of these genes in the double mutant. Furthermore, H-NS occupancies at the tcpA and flrA promoters were significantly enhanced in the ihfA mutant compared to those in the wild-type strain.

It has been suggested that the open initiation complex formed with the promoter in RNAP-α5 differs in architecture from that in RNAP-α70. The complex formed by RNAP-α70 facilitates lateral oligomerization of H-NS by cooperative recruitment of H-NS molecules, leading to the formation of a repression loop (45). In contrast, the RNAP-α5 open initiation complex does not promote H-NS oligomerization and exhibits H-NS-resistant transcription initiation (45). Consistent with this mechanism, the elevated promoter occupancies found in the ΔrpoS ΔihfA double mutant could result from the absence of IHF, required to displace H-NS from its binding site, and the formation of only RNAP-α70 open initiation complexes that favor H-NS oligomerization along DNA. In addition, deletion of rpoS could lead to enhanced activity of α70 (19–21), favoring a higher association between H-NS and promoter DNA. Also, our finding that H-NS exerts weaker repression at the hapA promoter is in agreement with the above-mentioned model for H-NS promoter selectivity.

Consistent with our previous finding that H-NS enhances rpoS mRNA stability (49), here we show that H-NS positively affected the expression of RpoS at the protein level. This regulation could generate a negative regulatory loop in which elevated RpoS expression could diminish H-NS occupancy at target promoters that could be transcribed using RNAP-α5 by activating the expression of IHF or other trans-acting regulatory factors.
FIG 9 Model for RpoS regulation of motility in V. cholerae. The expression of RpoS in the stationary phase enhances rpoN and fliA transcription by two parallel mechanisms. In mechanism I, RNAP-σ^S can initiate transcription at the rpoN and fliA promoters. Transcription initiation by RNAP-σ^S is more resistant to H-NS repression. In mechanism II, RpoS diminishes H-NS occupancy at the rpoN and fliA promoters indirectly by enhancing the expression of IHF, which could compete with H-NS for binding to DNA.

Based on our results, we propose a model for the interplay between RpoS and H-NS at V. cholerae promoters that control motility (Fig. 9). Briefly, H-NS binds to the fliA and rpoN promoters to repress transcription. RpoS is expressed in the stationary phase, and RNAP-σ^S contributes to the transcription of fliA and rpoN, rendering these promoters more resistant to H-NS repression (Fig. 9, I). In addition, the expression of RpoS can indirectly attenuate H-NS-mediated repression of fliA and rpoN by inducing the expression of IHF (Fig. 9, II). IHF acts by displacing H-NS from its binding site to enhance transcription of fliA and rpoN, as described for the tcpA promoter (52). Our data provide an explanation for RpoS regulation of motility and mucosal escape (35). Moreover, RpoS was shown to be required for intestinal colonization in an El Tor biotype strain (32). Our results also suggest that RpoS could favor colonization by diminishing H-NS repression of motility and tcpA expression, which are required to establish infection (22, 47).

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