

The Histone-Like Nucleoid Structuring Protein (H-NS) Is a Repressor of *Vibrio cholerae* Exopolysaccharide Biosynthesis (*vps*) Genes

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The capacity of *Vibrio cholerae* to form biofilms has been shown to enhance its survival in the aquatic environment and play important roles in pathogenesis and disease transmission. In this study, we demonstrated that the histone-like nucleoid structuring protein is a repressor of exopolysaccharide (*vps*) biosynthesis genes and biofilm formation.

Cholera is an acute waterborne diarrheal disease caused by ingestion of the bacterium *Vibrio cholerae* of serogroups O1 and O139. In nature, *Vibrio* can be found in the form of planktonic free-swimming cells or as sessile biofilm communities associated with phytoplankton and zooplankton (13, 14). The formation of such biofilm communities enhances the survival of *Vibrio* in the aquatic environment (8, 16, 23, 26, 32, 34). *V. cholerae* can also be found in the form of large biofilm aggregates in suspension of partially dormant cells that resist cultivation in conventional media but can be recovered as virulent bacteria by animal passage (8). These biofilm aggregates are more resistant to the initial low-pH stress encountered after ingestion by humans (54). As a result, mutations that block biofilm matrix exopolysaccharide biosynthesis impair colonization in the suckling mouse cholera model (9). *Vibrio* can associate into biofilm aggregates in the host late in infection (8). These biofilms, formed *in vivo*, are in a stage of transient hyperinfectivity (43) that enhances their dissemination through the fecal-oral route (24).

The development of a three-dimensional, mature biofilm involves a complex genetic program that includes the expression of motility and mannose-sensitive hemagglutinin for surface attachment and monolayer formation, as well as the biosynthesis of the exopolysaccharide matrix (*Vps*) (48). The genes responsible for *vps* biosynthesis are clustered in two operons, in which *vpsA* and *vpsL* are the first genes of operons I and II, respectively (50). These genes are regulated by several positive and negative transcription regulators. The positive regulators include the cyclic diguanylate (*c*-di-GMP) sensing protein *VpsT* (3) and the NtrC-type activator *VpsR* (51); the negative regulators include the master quorum sensing regulator, HapR (11, 15, 54), CytR (12), and the PhoBR two-component regulatory system (31, 41). The expression of *vps* genes is enhanced in response to an increase in the intracellular concentration of *c*-di-GMP (2, 21, 45).

The histone-like nucleoid structuring protein (H-NS) is a 15-kDa highly abundant nucleoid-associated protein that has two biological functions: DNA condensation and regulation of transcription (6). In regulation of transcription, H-NS most commonly negatively affects gene expression by binding to promoters exhibiting AT-rich, highly curved DNA regions that contain clusters of the more conserved 10-bp motif TCGATAAATT (18, 30). Binding of H-NS to a promoter apparently inhibits transcription by a bridging mechanism consisting of cross-linking DNA segments in a manner that traps the RNA polymerase within a repression loop (7). In *V. cholerae*, *hns* mutants form small colonies,

exhibit diminished motility and intestinal colonization capacity, and show altered responses to environmental stresses (10, 36, 44). H-NS also silences the transcription of virulence genes by acting at different levels of the ToxR regulatory cascade, which include the *toxT*, *tcpA*, and *ctxA* promoters (28, 39). In this study, we examined the role of H-NS in biofilm formation.

H-NS negatively affects biofilm formation in *V. cholerae*. In a previous study, we described the construction of an *hns* deletion mutant of the El Tor biotype using the standard pCVD442 suicide vector and sucrose selection procedure (see Table 2) (5, 36). The *hns* deletion mutant derived from strain C7258 Δ *lacZ* formed a large pellicle when grown in static cultures compared to findings for the wild type, suggesting the formation of enhanced biofilms. To further examine this phenotype, we compared biofilm formation between strain C7258 Δ *lacZ* and its isogenic Δ *hns* derivative, AJB80 (36), using confocal microscopy and crystal violet staining as previously described (19, 41). As shown in Fig. 1, the Δ *hns* mutant adhered more and formed a thicker biofilm than the wild type. Quantification using the crystal violet assay confirmed that deletion of *hns* results in enhanced biofilm formation. To further confirm that the enhanced biofilm phenotype is due to the *hns* deficiency, we used the primers HNS1 and HNS2 (Table 1) and the Advantage 2 PCR kit (Clontech) to amplify the *hns* (*vich*, VC1130) locus and its promoter. The PCR product was confirmed by DNA sequencing and inserted downstream of the *rrn* T₁T₂ transcription terminator (TT) in plasmid pTT3 (35). Then, the TT-*hns* cassette was transferred to pBR322 to yield plasmid pBRHNS (Table 1), which was introduced into strain AJB80 by electroporation. As shown in Fig. 1, strain AJB80 containing pBRHNS produced wild-type biofilm.

H-NS negatively affects the expression of several regulators of exopolysaccharide biosynthesis. We used quantitative real-time RT-PCR (qRT-PCR) to compare the expression of *vps* genes and their regulators in strain C7258 Δ *lacZ* and AJB80 using the iScript two-step RT-PCR kit with SYBR green (Bio-Rad) and

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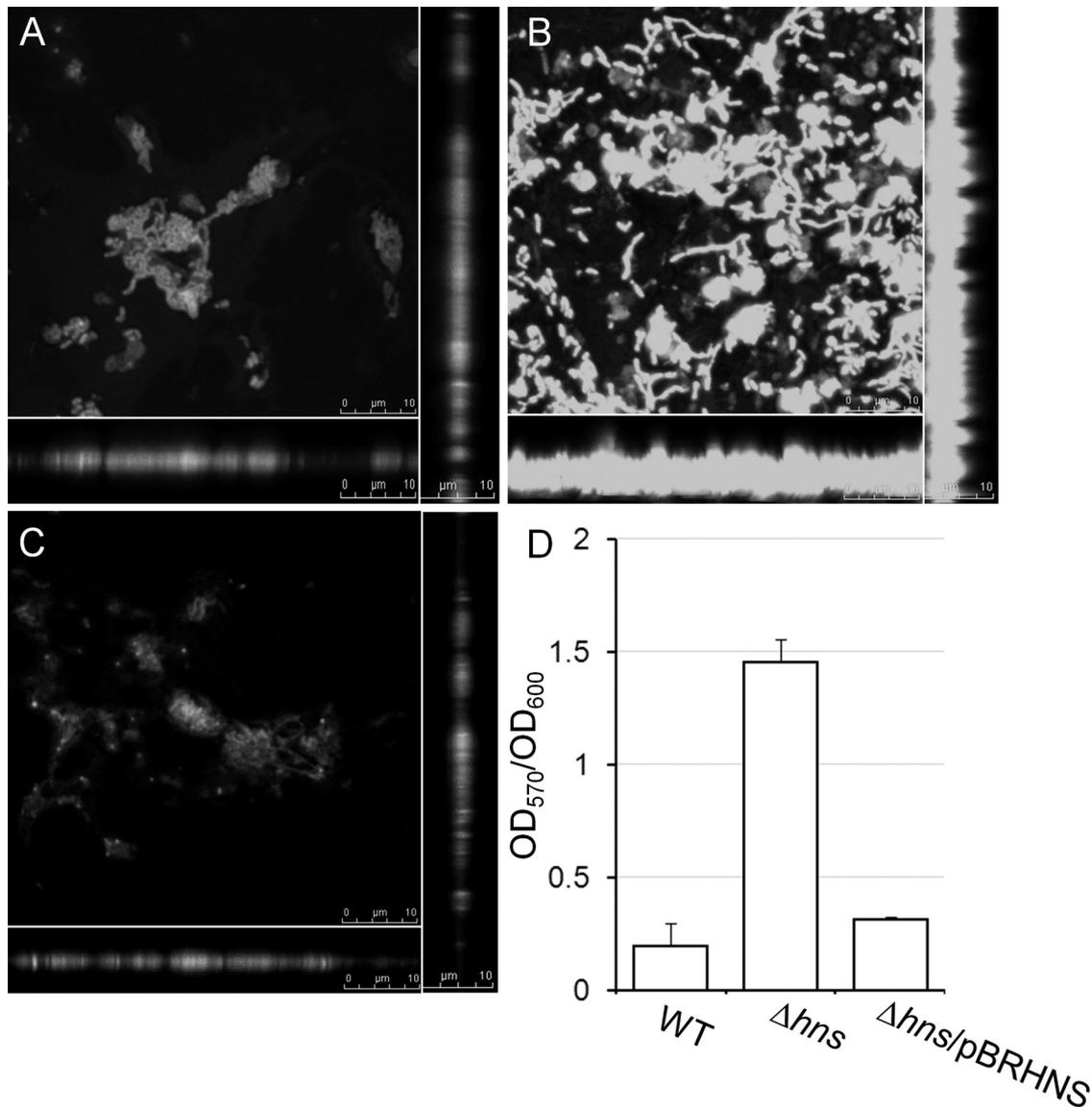


FIG 1 Repression of biofilm formation by H-NS. Strains C7258Δ*lacZ* (A), AJB80 (Δ*hns*) (B), and AJB80 complemented with plasmid pBRHNS (C) were allowed to form static biofilm in LB medium, stained with Syto-9, and examined by confocal microscopy. The center of each panel is an XY section through the biofilm. Vertical sections through the biofilm are shown to the right and bottom. (D) Quantification of biofilm formation using the crystal violet stain.

gene-specific primers previously described (19, 20). Relative expression values were calculated as $2^{C_{t_{\text{Target}}} - C_{t_{\text{Reference}}}}$, where $C_{t_{\text{Target}}}$ is the fractional threshold cycle (C_T) for the target gene and the reference was the *recA* mRNA. As shown in Table 2, the *hns* mutant AJB80 expressed significantly higher levels of *vpsA*, *vpsL*, *vpsT*, and to a lesser extent *vpsR* mRNAs than the wild type. Deletion of *hns* did not significantly affect the expression of the *vps* repressors *hapR* and *cytR*. The qRT-PCR analysis was followed by construction of *vpsA*-, *vpsL*-, *vpsR*-, and *vpsT*-*lacZ* fusions. Primer combinations VpsA1/VpsA2, VpsL1/VpsL2, VpsR1/VpsR2, and VpsT1/VpsT2 (Table 1) were used to amplify *vpsA*, *vpsL*, *vpsR*, and *vpsT* DNA fragments containing each promoter region, respectively. The PCR products were confirmed by DNA sequencing and subcloned downstream of the TT in pTT3 (35). Finally, the TT promoter fragments were inserted upstream of a promoterless *lacZ* gene in plasmid pKRZ1 (33) to generate pVpsA-*LacZ*,

pVpsL-*LacZ*, pVpsR-*LacZ*, and pVpsT-*LacZ* (Table 1). The resulting *lacZ* fusions were introduced into C7258Δ*lacZ* and AJB80 by electroporation, and β-galactosidase activity was measured as an indicator of promoter activity as described by Miller (25). As shown in Fig. 2, significantly elevated β-galactosidase activities were detected in the *hns* mutant compared to those for the wild type ($P < 0.01$, one-tailed *t* test) for all *vps*-*lacZ* fusions, with *vpsT*-*lacZ* exhibiting the strongest effect.

To investigate if H-NS repressed the above promoters directly, we purified H-NS and conducted DNA binding assays. To purify H-NS, the *hns* open reading frame (ORF) was amplified from C7258Δ*lacZ* genomic DNA using the primers HNS-F31 and HNS-R32 (Table 1). The amplified fragment was subcloned into similarly digested pTXB-1 (New England BioLabs) to generate pTXB1-HNS (Table 1). H-NS was then expressed and purified using the Impact kit (New England BioLabs), following the man-

TABLE 1 Strains, plasmids, and oligonucleotide primers

Strain, plasmid, or primer	Description or sequence (5' → 3') ^a	Reference
Strains		
C7258	Wild type (El Tor, Ogawa)	Peru isolate, 1991
C7258Δ <i>lacZ</i>	C7258 <i>lacZ</i> deletion mutant	36
AJB80	C7258 Δ <i>lacZ</i> Δ <i>hns</i> ::Km	36
Plasmids		
pTT3	Transcription terminator <i>rrnB</i> T ₁ T ₂ cloned in pUC19	35
pKRZ1	Plasmid containing promoterless <i>lacZ</i> gene	33
pTT3VpsA	0.6-kb SphI-HindIII <i>vpsA</i> promoter inserted downstream of transcription terminator in pTT3	This study
pVpsA-LacZ	1.0-kb KpnI-HindIII terminator- <i>vpsA</i> promoter fragment inserted upstream of promoterless <i>lacZ</i> gene in pKRZ1	This study
pTT3VpsL	0.6-kb SphI-HindIII <i>vpsL</i> promoter fragment in pTT3	This study
pVpsL-LacZ	1.0-kb KpnI-HindIII terminator- <i>vpsL</i> fragment in pKRZ1	This study
pTT3VpsR	span lang=DE>0.6-kb SphI-HindIII <i>vpsR</i> promoter fragment in pTT3	This study
pVpsR-LacZ	1.0-kb KpnI-HindIII terminator- <i>vpsR</i> fragment in pKRZ1	This study
pTT3VpsT	0.7-kb SphI-HindIII <i>vpsT</i> promoter fragment in pTT3	This study
pVpsT-LacZ	1.1-kb KpnI-HindIII terminator- <i>vpsT</i> fragment in pKRZ1	This study
pBRHNS	Transcription terminator <i>rrnB</i> T ₁ T ₂ and the <i>hns</i> locus were sequentially cloned in pBR322	This study
Primers		
HNS1	<u>CGCGGATCCGTGAGAAAAACAAGTGCCAC</u>	
HNS2	<u>GATGCATGCTTCCTGAGCGAGAAGATAGC</u>	
HNS-F31	<u>GATCGCATATGGTAATGTGCGAAATCACTAAG</u>	
HNS-R32	<u>GATCGGCTCTTCAGCACAGAGCGAATTCTCCAGAGA</u>	
TcpA-F1	<u>GTTAATAATTCGATCTCCACTCCG</u>	
TcpA-R2	<u>GTTAACCACACAAAGTCACCTGCAA</u>	
VC1922-F61	<u>TAGAAGGTTGACGAAACAAGCAATCA</u>	
VC1922-R62	<u>GGTTCAACCACCATAGGTACGAGT</u>	
VicH5	<u>GGAAGCTTGTAATGTGCGAAATCA</u>	
VicH396	<u>AGGAGATCTCAGAGCGAATTCTTCC</u>	
VicH7	<u>GTTGCATGCATGTGCGAAATCACTA</u>	
RpoS1020	<u>CTTCTGCAGTTACTTGTGTCGTCATCG</u>	
RpsM-F51	<u>GCAACTGCGTGATGGTGTAGCTAA</u>	
RpsM-R52	<u>GCTTGATCGGCTTACGCGGACC</u>	
VpsA1	<u>GAAGCATGCGCGTAGTAGTGAATTTTTTCC</u>	
VpsA2	<u>GCGAAGCTTTACCTGATCTAACATCTCAC</u>	
VpsAF	<u>CCTTTATATCGCGCTTAAACTATATGT</u>	
VpsAR	<u>ATCCTCTTTAAAAAAGAAATTTCAAAAAT</u>	
VpsL1	<u>GAAGCATGCGTAATGGTACGGGTTTTTATA</u>	
VpsL2	<u>GCGAAGCTTCCGTGATAGTTAGTAATGCG</u>	
VpsLF1	<u>CTTTTCGATAAACGATACATATTATTCT</u>	
VpsLR1	<u>GCATGAAAATAAACTTTAGTTTACTTTTAT</u>	
VpsR1	<u>GAAGCATGCGAGTCTGGTGTATGATGAAGC</u>	
VpsR2	<u>GCGAAGCTTAATCTGCTACTTGAGTACAG</u>	
VpsRF	<u>TGGGGCTTTTTTTATTTTCTCAAGAAA</u>	
VpsRR	<u>TCGATATTCCTTATTTTTATTGTTACGAT</u>	
VpsT1	<u>GAAGCATGCGTTGGCATGTGGTTAAAGTG</u>	
VpsT2	<u>GCGAAGCTTGCAAACATCAGAAAGCATTC</u>	
VpsTF2	<u>AGGCTATGAATAAATTGAGTGGTAAGT</u>	
VpsTR2	<u>GAAGACAAACGTTAATCAAGCGGAA</u>	

^a Restriction sites used for cloning are underlined.

TABLE 2 Effect of H-NS on expression of *V. cholerae* exopolysaccharide biosynthesis genes and their regulators

Gene ^a	Relative expression		Mutant/WT ratio
	WT	Δ <i>hns</i>	
<i>vpsA</i> *	0.009 ± 0.0030	0.397 ± 0.2402	44
<i>vpsL</i> *	0.006 ± 0.0001	0.689 ± 0.2500	115
<i>vpsR</i> *	0.688 ± 0.1727	1.321 ± 0.1900	1.9
<i>vpsT</i> *	0.058 ± 0.0030	1.338 ± 0.3683	23
<i>hapR</i>	4.724 ± 1.4778	6.566 ± 0.5593	1.4
<i>cytR</i>	0.830 ± 0.1690	1.087 ± 0.1077	1.3

^a *, mutant significantly different from wild type ($P < 0.01$, one-tailed t test; $n = 3$).

ufacturer's instructions. The purity of recombinant H-NS determined by SDS-PAGE was found to be higher than 90%. Electrophoresis mobility shift assays (EMSA) were conducted using the DIG gel shift kit, 2nd generation (Roche Applied Sciences). Binding reaction mixtures contained 3 fmol of digoxigenin (DIG)-labeled promoter DNA and 0 to 20 ng of pure H-NS in a total volume of 20 μl. After incubation (20 min, 30°C), protein-DNA complexes were separated by electrophoresis in 5% Tris-borate-EDTA (TBE) polyacrylamide gels, followed by transfer to nylon membranes, and DNA was visualized using an anti-DIG Fab frag-

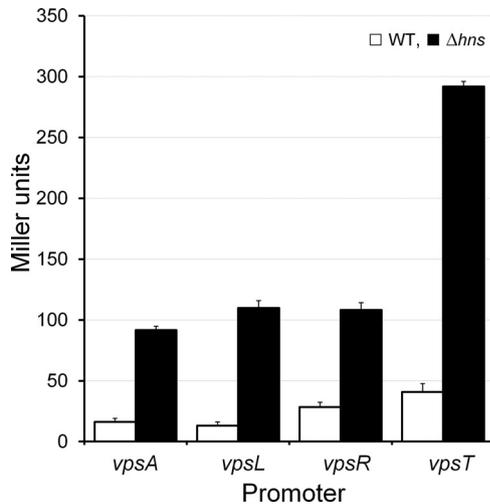


FIG 2 Effect of H-NS on the expression of *vpsA*-, *vpsL*-, *vpsR*-, and *vpsT*-*lacZ* fusions. Strains C7258 Δ *lacZ* (WT) and AJB80 (Δ *hns*; filled bar) containing *vpsA*-, *vpsL*-, *vpsR*-, and *vpsT*-*lacZ* fusions were grown in LB at 37°C, and β -galactosidase activities were expressed in Miller units. Each value is the mean for six independent cultures. The error bars indicate the standard deviations.

ment-AP conjugate followed by chemiluminescence detection. As a positive control, the *tcpA* promoter was amplified using the primers *TcpA*-F1 and *TcpA*-R2. The promoter of VC1922, a gene not regulated by H-NS, was amplified using the primers VC1922-F61 and VC1922-F62 and used as a negative control. Similar to *tcpA*, a promoter subject to H-NS transcriptional silencing (28), H-NS exhibited strong binding to the *vpsA*, *vpsL*, and *vpsT* promoter (Fig. 3). H-NS binding to the *vpsR* promoter was weaker than that for *vpsA*, *vpsL*, and *vpsT*.

To determine H-NS occupancies at the *vpsA*, *vpsL*, *vpsR*, and *vpsT* promoters at the cellular level where other regulators could compete with H-NS for promoter access, we constructed strain C7258HNS-FLAG (Table 1) and conducted chromatin immunoprecipitation (ChIP) assays. In these experiments, we used two negative controls: (i) a DNA sequence located within an ORF of the housekeeping gene *rpsM* and (ii) the promoter region of VC1922, to which H-NS failed to exhibit significant binding affinity in the EMSA (Fig. 3). The rationale for the *rpsM* ORF control was that though H-NS could bind to this sequence, such binding would not be relevant to transcription regulation and could provide a cutoff for distinguishing between H-NS binding as a repressor versus nucleoid organization. The internal *rpsM* probe was

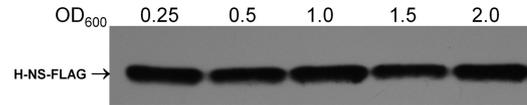


FIG 4 Expression of H-NS-FLAG in the bacterial growth curve. Strain C7258HNS-FLAG was grown in LB medium at 37°C, and samples were taken at the indicated optical densities for Western blot analysis as described in the text.

amplified with the primers *RpsM*-F51 and *RpsM*-R52 (Table 1). To construct C7258HNS-FLAG, the *hns* ORF lacking the stop codon was amplified using the primers *VicH5* and *VicH396*. The amplification product was confirmed by DNA sequencing and cloned as a *Bgl*II-*Hind*III fragment in pFLAG-CTC (Sigma-Aldrich) in frame with the FLAG epitope to yield pHNS-FLAG (Table 1). The H-NS-FLAG fusion was retrieved from pHNS-FLAG by PCR with primers *VicH7* and *RpoS1020*, containing *Sph*I and *Pst*I overhangs, and cloned into pTT3 (35) upstream of the TT to yield pTT3HNS-FLAG. The *hns*-FLAG-TT cassette was transferred to the suicide vector pCVD442 (5) to yield pCVDHNS-FLAG. Finally, pCVDHNS-FLAG was introduced into strain C7258 by conjugal transfer from S17-1 λ *pir* (4), exconjugants were selected in LB agar containing ampicillin (100 μ g/ml) and polymyxin B (100 units/ml), and correct integration into the *hns* locus was confirmed by PCR using primers *VicH567*, which anneals to DNA sequences upstream of *hns* not present in pCVDHNS-FLAG, and *RpoS1020*, which anneals to DNA encoding the FLAG epitope (Table 1). The expression of H-NS-FLAG was ascertained by Western blot analysis. To this end, strain C7258HNS-FLAG was grown in LB medium at 37°C. Samples of a volume corresponding to 1.0 OD₆₀₀ (optical density at 600 nm) unit were taken at different time points and centrifuged, and the pellet was resuspended in 0.1 ml of Laemmli sample buffer. Proteins were separated using Criterion precast 10% gels (Bio-Rad) and transferred to polyvinylidene difluoride (PVDF) membranes, and H-NS-FLAG was detected with anti-FLAG M2-peroxidase (Sigma-Aldrich) and the BM Bioluminescence Western blotting kit (Roche Applied Science). As shown in Fig. 4, H-NS was expressed at similar levels at the different time points evaluated. For H-NS promoter occupancy, 40 ml of C7258HNS-FLAG grown in LB medium to an OD₆₀₀ of 0.5 at 37°C was sequentially treated with rifampin (150 μ g/ml, 20 min, 37°C), 1% formaldehyde (cross-linking, 10 min, 30°C), and 227 mM glycine (30 min, 4°C). The cells were collected by centrifugation, washed with phosphate-buffered saline (PBS) containing protease inhibitor cocktail (PIC)

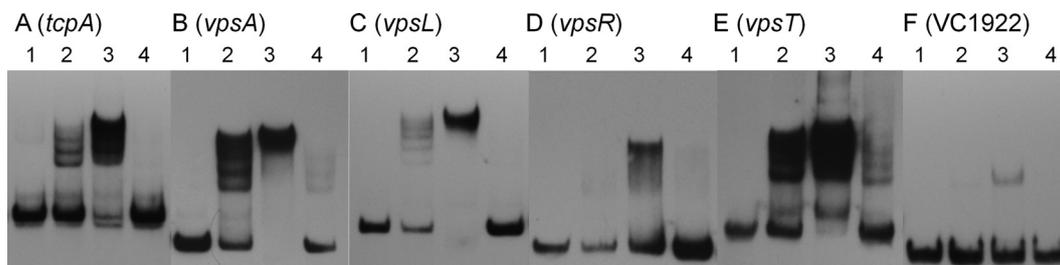


FIG 3 Binding of H-NS to *vps* promoters. DIG-labeled DNA fragments containing the *tcpA* (A), *vpsA* (B), *vpsL* (C), *vpsR* (D), *vpsT* (E), and VC1922 (F) promoters were incubated with pure H-NS protein. Each labeled DNA was incubated with 0 (lanes 1), 10 (lanes 2), and 20 (lanes 3) ng of H-NS and 20 ng of H-NS plus a 62.5-fold excess of unlabeled competitor DNA (lanes 4). The spans of the promoter fragments used relative to the start codon were as follows: -306 to -83 for *tcpA*; -257 to -9 for *vpsA*; -240 to -13 for *vpsL*; -237 to -7 for *vpsR*; -224 to +3 for *vpsT*; and -113 to +43 for VC1922.

and phenylmethylsulfonyl fluoride (PMSF) (Roche Applied Science), and lysed by incubation in 10 mM Tris-HCl (pH 8.0), 50 mM NaCl containing 20 ng/ μ l of RNase A, and 10^5 kU of ready-lyse lysozyme (Epicentre Biotechnologies) (30 min, 37°C). The lysate was mixed with 1 volume of double-strength IP buffer (200 mM Tris-HCl [pH 7.5], 600 mM NaCl, and 4% Triton X-100) containing PIC and PMSF, and DNA was broken down by sonication to a molecular size range of 150 to 1,000 bp. The cell debris was removed by centrifugation, and the lysate was diluted 10-fold in IP buffer. At this stage, a 10- μ l input sample was saved as a reference and PCR efficacy control. Protein-DNA complexes were immunoprecipitated by overnight incubation at 4°C with 8 μ g of anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) or 8 μ g of the 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/G agarose beads (Imgenex, San Diego, CA) for 1 h at 4°C. The beads were washed 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 sequentially with IP buffer containing 600 mM NaCl; IP buffer and TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA). The immunoprecipitated (IP) 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 treatment with 20 μ g of proteinase K (1 h, 45°C). Then, IP DNA was purified using the MiniElute PCR purification kit (Qiagen) and detected by PCR and agarose gel electrophoresis using the same primer combinations designed to amplify the promoter fragments described for the EMSA. To quantitate promoter occupancies, quantitative PCR (qPCR) was conducted using the iTaq SYBR green supermix with ROX (Bio-Rad). The quantity of IP DNA was calculated as the percentage of the DNA present in the input sample using the following formula: $IP = 2^{Ct_{input} - Ct_{IP}}$, where Ct is the fractional threshold cycles (C_T) of the input and IP samples. The relative IP was calculated by standardizing the IP of each sample by the IP of the corresponding mock ChIP. ChIP analysis demonstrated significant H-NS occupancies at the *tcpA*, *vpsA*, *vpsL*, and *vpsT* promoters compared to results for the negative controls ($P < 0.01$) (Fig. 5). Occupancies at the *vpsT* and *vpsL* promoters were similar to or higher than those at the *tcpA* promoter, respectively (Fig. 5). We could not demonstrate H-NS promoter occupancy at the *vpsR* promoter, suggesting that H-NS does not directly control the transcription of this regulator. This result is consistent with the lower H-NS binding activity detected for this promoter in the EMSA. The above-described experiments conclusively establish H-NS as a transcriptional repressor of *V. cholerae* exopolysaccharide biosynthesis.

Discussion. The formation of quorum sensing-regulated biofilm communities has been suggested to play a critical role in cholera ecology, pathogenesis, and disease transmission. In this study, we have provided conclusive evidences that H-NS is a transcriptional silencer of exopolysaccharide biosynthesis genes and biofilm development. Based on the above results, we conclude that H-NS directly binds to the *vpsA*, *vpsL*, and *vpsT* promoters to repress their expression, while its effect on *vpsR* transcription could be indirect. Our results add a new variable to the complex regulatory network involved in biofilm development. In transcription regulation, H-NS commonly acts as a promiscuous repressor affecting a large number of genes, particularly foreign genes acquired through horizontal gene transfer (22, 27, 29).

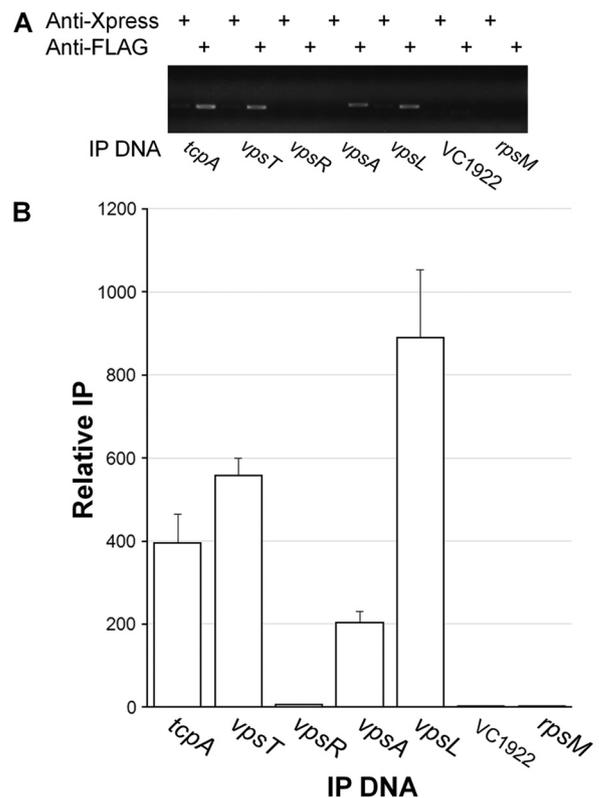


FIG 5 ChIP analysis of H-NS binding to *vps* promoters. (A) Agarose gel electrophoresis of the PCR products obtained after ChIP using anti-FLAG and anti-Xpress (mock reaction) monoclonal antibodies. (B) Quantification by qPCR of H-NS occupancies at the corresponding promoter. Each value represents the mean for three experiments, and error bars indicate the standard deviations.

There are no evidences that the *V. cholerae* *vps* clusters are the result of horizontal gene transfer events. However, binding of H-NS to the *vpsA*, *vpsL*, and *vpsT* promoters *in vitro* and its occupancy at these chromosomal sites were comparable to the *tcpA* promoter located within a pathogenicity island. Overexpression of *vps* genes has been shown to favor the adoption of a rugose colonial morphology (46, 50). We note that unlike *hapR* mutants of strain C7258, which exhibit a rugose colonial morphology (20), *hms* mutants show a smooth colonial morphology (data not shown). HapR is expressed at high cell density (1, 11, 15, 47, 54), while the Western blot experiment shown in Fig. 4 shows that H-NS is expressed at similar levels at low and high cell densities. Therefore, we suggest that deletion of *hms* enhances the expression of *vps* genes until the cultures enter quorum sensing mode and their expression is turned off by HapR (1, 47, 52). On the contrary, deletion of *hapR* allows the cell to over express *vps* at low and high cell densities, leading to higher exopolysaccharide accumulation and colonial rugosity. Further, deletion of *hapR* could also diminish H-NS levels, since HapR was found to bind to the *hms* promoter and enhance the expression of an *hms-lux* fusion (42).

It is well established that H-NS repression can be attenuated by other small nucleoid proteins, such as Fis and integration host factor (IHF) (7, 38, 40), and by non-histone-like proteins, such as the AraC-like transcriptional regulator ToxT and *Shigella flexneri* ParB-type virulence regulator VirB (38, 53). The regulators AphA and VpsR have been shown to bind to the *vpsT* promoter and

enhance its expression (37, 49). It is possible that binding of AphA or VpsR to the *vpsT* promoter could attenuate H-NS repression at low cell density by either displacing H-NS from its binding site or preventing H-NS oligomerization along DNA to form a repression loop. Elevated expression of VpsR and VpsT in the *hns* mutant led to enhanced *vpsA* and *vpsL* transcription. Unfortunately, the architecture of these promoters is less understood. In the case of *vpsL*, a putative VpsR binding site (52) is located downstream from a high-scoring H-NS site detected using the Virtual Footprint software program (http://www.prodoric.de/vfp/vfp_promoter.php). In addition, VpsT has been shown to bind to the *vpsL* promoter (17). Therefore, it is possible that binding of VpsR and/or VpsT to the *vpsL* promoter could attenuate H-NS repression. Taken together, the involvement of H-NS in the regulation of *vpsT*, *vpsL*, and *vpsA* suggests the possibility of an antirepression cascade mediated by VpsR and/or VpsT to enhance exopolysaccharide expression. A more detailed characterization of these promoters is required to address the mechanism of H-NS silencing and antisilencing at these locations. Finally, it is noteworthy that VpsT and VpsR have been shown to directly sense cellular *c*-di-GMP (17, 37). The expression of both regulators is enhanced when the *c*-di-GMP pool is artificially increased by inducing the expression of a diguanylate cyclase (1). Thus, we speculate that attenuation of H-NS repression of VpsT and VpsR could be required to increase the cellular level of these receptors and potentiate *c*-di-GMP signal transmission.

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