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Repression by H-NS of genes required for the biosynthesis of the *Vibrio cholerae* biofilm matrix is modulated by the second messenger cyclic diguanylic acid

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Summary

Expression of *Vibrio cholerae* genes required for the biosynthesis of exopolysacchide (*vps*) and protein (*rbm*) components of the biofilm matrix is enhanced by cyclic diguanylate (c-di-GMP). In a previous study, we reported that the H-NS protein represses the transcription of *vpsA*, *vpsL* and *vpsT*. Here we demonstrate that the regulator VpsT can disrupt repressive H-NS nucleoprotein complexes at the *vpsA* and *vpsL* promoters in the presence of c-di-GMP while H-NS could disrupt the VpsT-promoter complexes in the absence of c-di-GMP. ChiP-Seq showed a remarkable trend for H-NS to cluster at loci involved in biofilm development such as the *rbmABCDEF* genes. We show that the antagonistic relationship between VpsT and H-NS regulates the expression of the *rbmABCDEF* cluster. Epistasis analysis demonstrated that VpsT functions as an antirepressor at the *rbmA/F*, *vpsU* and *vpsA/L* promoters. Deletion of *vpsT* increased H-NS occupancy at these promoters while increasing the c-di-GMP pool had the opposite effect and included the *vpsT* promoter. The negative effect of c-di-GMP on H-NS occupancy at the *vpsT* promoter required the regulator VpsR. These results demonstrate that c-di-GMP activates the transcription of genes required for the biosynthesis of the biofilm matrix by triggering a coordinated VpsR- and VpsT-dependent H-NS antirepression cascade.

Keywords

Vibrio cholerae; biofilm formation; H-NS; c-di-GMP

Introduction

Vibrio cholerae of serogroups O1 and O139 is the causative agent of the diarrheal disease cholera. A major obstacle to the eradication of cholera is the persistence of *V. cholerae* in

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the aquatic environment in the form of biofilm communities attached to chitinous surfaces (Pruzzo *et al.*, 2008). These biofilms are more resistant to multiple environmental stresses and to inactivation by the human gastric acid barrier (Zhu & Mekalanos, 2003). Further, *Vibrios* can form biofilms during infection (Faruque *et al.*, 2006) and these biofilms are in a stage of transient hyperinfectivity that can facilitate household transmission of cholera (Tamayo *et al.*, 2010).

The development of a mature biofilm requires the biosynthesis of an extracellular matrix consisting of *Vibrio* exopolysaccharide (VPS) and proteins (Yildiz & Schoolnik, 1999, Absalon *et al.*, 2011). The genes required for VPS biosynthesis are encoded by two operons located in chromosome I, in which *vpsA* and *vpsL* are the first genes of operons I and II, respectively (Fong *et al.*, 2010, Yildiz & Schoolnik, 1999). A third gene cluster termed *rbmABCDEF* encodes protein components of the biofilm matrix and is located between *vps* operons I and II (Fong & Yildiz, 2007). Transcription of *vps* and *rbm* genes is controlled by a complex regulatory network involving quorum sensing (Yang *et al.*, 2010, Rutherford *et al.*, 2011, Jobling & Holmes, 1997, Yildiz *et al.*, 2004) and the second messenger cyclic diguanylic acid (c-di-GMP) (Beyhan *et al.*, 2006, Lim *et al.*, 2007, Lim *et al.*, 2006, Tischler & Camilli, 2004). Quorum sensing controls biofilm formation through two master regulators: AphA and HapR (Rutherford *et al.*, 2011). At low cell density, AphA enhances the expression of the biofilm activator VpsT (Yang *et al.*, 2010). At high cell density, HapR diminishes biofilm formation by lowering the intracellular c-di-GMP pool (Waters *et al.*, 2008, Wang *et al.*, 2011) and repressing the transcription of *aphA* and *vpsT* (Srivastava *et al.*, 2011, Waters *et al.*, 2008).

Cyclic-di-GMP is synthesized from GTP by diguanylate cyclases (DGC) containing GGDEF domains. Degradation of c-di-GMP involve phosphodiesterases (PDE) exhibiting EAL or HD-GYP domains (Dow, 2007). The two major regulatory proteins that sense intracellular c-di-GMP levels to promote biofilm formation are the NtrC-like activator VpsR (Yildiz *et al.*, 2001) and the LuxR-type regulator VpsT (Casper-Lindley & Yildiz, 2004). VpsR binds c-di-GMP to activate the expression of *aphA* and *vpsT* (Srivastava *et al.*, 2011). VpsT has been shown to bind to the *vpsL* promoter in the presence of c-di-GMP (Krasteva *et al.*, 2010, Casper-Lindley & Yildiz, 2004). We recently showed that transcription of *vpsA*, *vpsL* and *vpsT* is repressed by the histone-like nucleoid structuring protein (H-NS) (Wang *et al.*, 2012b).

H-NS is a highly abundant protein that functions as a nucleoid organizer and a transcriptional silencer at promoters exhibiting AT-rich and highly curved DNA (Lang *et al.*, 2007). H-NS has been shown to preferentially silence the transcription of virulence factors acquired by horizontal gene transfer (Lucchini *et al.*, 2006, Dorman, 2007). In *E. coli*, H-NS can be observed associated with the nucleoid in the form of compact clusters that could bring broad regions of the genome into proximity (Dorman, 2013). Progress in this field has led to the emerging view that transcription regulation and nucleoid organization are interrelated functions of H-NS in which regulation of gene expression drives nucleoid architecture (Dorman, 2013). Similar studies have not been conducted in *V. cholerae*, which distinct from *E. coli* and *Salmonella*, contains two circular chromosomes (Heidelberg *et al.*, 2000). *E. coli* H-NS consists of an N-terminal domain, which promotes oligomerization

through hydrophobic coil-coil interactions, connected by a flexible linker to a nucleic acid binding domain (Atlung & Ingmer, 1997). Both domains are required for the biological activities of H-NS (Spurio *et al.*, 1997, Dame *et al.*, 2001). The *V. cholerae* H-NS protein shares 69 % similarity and 55 % amino acid identity with the *E. coli* protein and represses gene expression as an oligomeric protein (Nye & Taylor, 2003). However, the presence of an additional oligomerization domain in *V. cholerae* H-NS suggests that the *Vibrio* protein uses a different mechanism to self-associate compared to *E. coli* H-NS (Nye & Taylor, 2003). Repression by H-NS can be relieved in response to environmental cues that activate the expression of other regulators whose binding site overlaps that of H-NS (Dorman & Kane, 2009, Stoebe *et al.*, 2008). In *V. cholerae*, transcriptional silencing of the *tcpA* and *ctxA* promoters by H-NS (Nye *et al.*, 2000) is antagonized by the AraC-like transcriptional regulator ToxT and the integration host factor (IHF) (Yu & DiRita, 2002, Stonehouse *et al.*, 2008, Stonehouse *et al.*, 2011).

A recent genetic analysis suggested a possible antagonistic relationship between H-NS and VpsT at the *vpsL* promoter (Zamorano-Sanchez *et al.*, 2015). In this study, we determine the mechanism by which H-NS and VpsT concertedly control the transcription of genes involved in the biosynthesis of the biofilm exopolysaccharide and protein matrix. Our data shows that c-di-GMP activation of biofilm formation involves the coordinated antirepression from H-NS of multiple genes that participate in biofilm development. We propose a model in which *vps* and *rbm* genes are transcriptionally silenced by H-NS at low cell density and are expressed or reset to silent depending on environmental-induced fluctuations in the c-di-GMP pool.

Results

H-NS and VpsT bind to overlapping DNA sequences at the *vpsA* and *vpsL* promoters

The *V. cholerae* LuxR-type regulator VpsT enhances the expression of *vps* and *rbm* genes by directly sensing the intracellular level of c-di-GMP (Shikuma *et al.*, 2012, Krasteva *et al.*, 2010, Beyhan *et al.*, 2006, Casper-Lindley & Yildiz, 2004, Fong & Yildiz, 2007). We hypothesized that VpsT could activate the transcription of both the *vpsA-K* and *vpsL-Q* operons by disrupting repressive H-NS nucleoprotein complexes formed at the corresponding promoters. To test this possibility, we determined the *vpsA* and *vpsL* transcription start sites (TSS) as well as the H-NS and VpsT binding sites (Fig. 1). The TSS for *vpsA* and *vpsL* were located 92 and 37 bp upstream of the *vpsA* and *vpsL* start codon, respectively (Fig. 1). These TSS were preceded by -10 and -35 regions separated by 18 and 16 bp spacers in the *vpsA* and *vpsL* promoters, respectively. DNase I footprinting showed that H-NS protected specific regions in both DNA strands of each promoter. In Fig. 1 we report the H-NS-protected sequences common to both DNA strands. We propose that these H-NS-protected regions could function as primary binding (nucleation) sites from which H-NS could oligomerize and spread along the *vpsA* and *vpsL* promoters. The DNase I protection analysis showed that H-NS occupies long stretches of DNA extending upstream and downstream the *vpsA* promoter elements, including the -35 and -10 positions (Fig. 1A). At the *vpsL* promoter H-NS protected a long DNA stretch starting at the -35 element and extending upstream the promoter (Fig. 1B). The VpsT binding pattern at the *vpsA* (Fig. 1A)

and *vpsL* promoters (Fig. 1B) differed from H-NS by being more sequence-specific and exhibiting minimal differences in protection between DNA strands. The VpsT binding sites overlapped some of the H-NS primary binding sites at both promoters further suggesting a possible antagonistic relationship between these regulators for binding to DNA. The electropherograms supporting the results summarized in Fig. 1AB are shown in supporting information Fig. S1–S5.

The *vpsA* and *vpsL* promoters exhibited a 20 bp inverted repeat sequence located within the VpsT-protected regions. We used the MEME application (multiple EM for motif elicitation) (Bailey & Elkan, 1994) to identify the VpsT binding motif. The resulting motif consisted of a sequence A[AT][AG]TAAACT[AT][AT]AGTTTA[TC][TA]T (Fig. 1C). We confirmed that VpsT binds *in vitro* to DNA fragments of the *vpsA* (–183 to –139) and *vpsL* (–262 to –208) promoters containing the VpsT motif. Binding was abolished when the inverted repeats were truncated (*vpsA* –160 to –98) or missing (*vpsL* –187 to –128) (Fig. S6).

VpsT can disrupt H-NS-DNA nucleoprotein complexes formed at the *vpsA* and *vpsL* promoters

We conducted competitive electrophoresis mobility shift assays (EMSA) to study the interaction between H-NS and VpsT at the *vpsA* and *vpsL* promoters. We incubated PCR fragments containing the overlapping H-NS and VpsT binding sites at the *vpsA* (Fig. 2A) and *vpsL* (Fig. 2B) promoters with H-NS protein for 15 min to allow the formation of the initial H-NS-DNA nucleoprotein complex (Fig. 2 A and B, lanes c and k). Then, we added increasing amounts of VpsT protein (Fig. 2 A and B lanes d through h) or VpsT plus c-di-GMP (Fig. 2A lanes l through m; Fig. 2B lanes l through o) and visualized the resulting protein-DNA complexes by polyacrylamide gel electrophoresis. These experiments showed that VpsT can disrupt the H-NS-DNA nucleoprotein complexes formed at the *vpsA* and *vpsL* promoters in a dose-dependent manner (Fig. 2AB). Further, a lower concentration of VpsT was required to displace H-NS from both promoters in the presence of c-di-GMP (see Fig. 2A lane l and Fig. 2B lane n). We further show that H-NS could displace VpsT from both promoters in the absence of c-di-GMP (Fig. 2C lanes f and l).

The finding that VpsT can effectively disrupt the interaction between the highly abundant H-NS protein and the *vpsA* and *vpsL* promoters predicted that this regulator should bind the above promoters with higher affinities compared to H-NS when c-di-GMP is present. We used the ImageQuant software and densitometry to measure VpsT and H-NS binding affinities for the *vpsA* and *vpsL* promoters and adjusted the experimental points to the Hill equation (Hill, 1910) by nonlinear regression. In the absence of c-di-GMP, H-NS and VpsT exhibited similar affinities for the *vpsA* and *vpsL* promoters (Fig. 3). In the presence of c-di-GMP, VpsT exhibited a higher affinity for the *vpsA* promoter ($K_d = 42.6 \pm 1.3$ nM) compared to that of H-NS ($K_d = 137.8 \pm 17.0$ nM) (Fig. 3). Similarly, in the presence of c-di-GMP, VpsT exhibited higher affinity for the *vpsL* promoter (35.4 ± 0.4 nM) compared to H-NS ($K_d = 204.0 \pm 9.0$ nM) (Fig. 3). We note that the VpsT-DNA complexes exhibited an aberrant V-shape migration pattern in the presence of c-di-GMP (see Fig. 2A and B lanes j). We investigated this phenomenon by conducting binding assays of VpsT to the *vpsA* promoter in the presence of decreasing concentrations of c-di-GMP. The result of this

analysis showed that the aberrant migration pattern is caused by dissociation of c-di-GMP from VpsT during electrophoresis resulting in the release of free and faster migrating DNA (Fig. S7).

H-NS silences the transcription of multiple genes involved in biofilm development

We conducted a ChIP-Seq experiment to investigate the role of H-NS in the expression of additional genes affecting biofilm development. Fig. 4A shows the H-NS binding and transcriptome profiles for the *V. cholerae* large and small chromosomes. The transcriptome data was retrieved from RNA-Seq data generated using the same strains and growth conditions (Wang *et al.*, 2015) (GEO accession number GSE62785). The ChIP-Seq analysis showed that H-NS associates with 6.3 % of the genome in clusters with medians of ≈ 2.7 and 2.1 kb in chromosomes I and II, respectively. The complete ChIP-Seq peak dataset and statistics is provided as supporting information in Table S1 and Fig. S8. We found H-NS to predominantly associate with clusters of functionally-related genes and operons. Examples of gene clusters occupied by H-NS are the 41.2 kb *Vibrio* pathogenicity island (VPI) (Karaolis *et al.*, 1998); the *vps* and *rbm* clusters; the cluster encoding LPS biosynthetic enzymes and O-antigen assembly in chromosome I; the mega-integron located on chromosome II, (Mazel *et al.*, 1998), and the *vas* (virulence-associated secretion) operon (Mueller *et al.*, 2009) (Table S3). We used sequences within H-NS ChIP-Seq peaks as input for motif elicitation with the MEME algorithm. The resulting *V. cholerae* H-NS binding motif is shown in Fig. 4B.

ChIP-Seq and RNA-Seq analyses revealed new loci transcriptionally silenced by H-NS that coordinate biofilm development in *V. cholerae*. These loci include *vpsU*, encoding a phosphotyrosine protein phosphatase required for VPS production (Fong *et al.*, 2010); the *rbm* genes (Fig. 4C) encoding proteins present in the extracellular matrix (Fong & Yildiz, 2007); *hlyA* (hemolysin), a component of the biofilm protein matrix (Absalon *et al.*, 2011); the chitin utilization program (chitoporin ChiP, extracellular chitinase VC1073, the cellobiose PTS transporter CelAB, the chitin-regulated pilus ChiRP) (Meibom *et al.*, 2004)), and the tryptophanase operon (*tna*) (Mueller *et al.*, 2009) (Table S1 and Fig. 4C).

We examined if VpsT could antagonize H-NS transcriptional silencing at loci affecting biofilm development other than *vpsA* and *vpsL*. To this end, we used the FIMO application (Find Individual Motif Occurrences) (Grant *et al.*, 2011) to identify genes exhibiting overlapping VpsT and H-NS motifs. A compilation of genes exhibiting H-NS motif is provided in supportive information Table S2. A list of genes exhibiting overlapping motifs is shown in Table 1. These genes included *vpsU* (Fong *et al.*, 2010); *rbmA*, encoding a matrix protein proposed to participate in early cell-to-cell contact (Giglio *et al.*, 2013, Absalon *et al.*, 2011, Fong & Yildiz, 2007, Berk *et al.*, 2012); *rbmF*, a gene transcribed divergently from *vpsL* encoding an hypothetical protein, and *hlyA* (hemolysin). We selected *rbmA* and *vpsU* to validate the occurrence of the predicted H-NS and VpsT binding sites. To this end, we amplified fragments of the *rbmA* and *vpsU* promoters containing the overlapping H-NS and VpsT motifs by PCR and conducted an EMSA to confirm that both regulators bind to these promoters (Fig. 5). Binding of VpsT to the *vpsU* and *rbmA* was enhanced in the presence of c-di-GMP (see Fig. 5CD, lanes c-d).

Effect of VpsT and c-di-GMP on H-NS occupancy at *vps* and *rbm* promoters in the cell

The above results indicated that H-NS coordinately silences multiple genes involved in biofilm development and that VpsT could antagonize H-NS repression at these loci. We conducted a ChIP assay to examine the role of VpsT in H-NS occupancy at the *vps* and *rbm* promoters in the cell. To this end, a *vpsT* deletion was introduced in strain C7258 H-NS-FLAG and the resulting mutant was transformed with a vector encoding an IPTG-inducible *vpsT* allele (pVpsT). As shown in Fig. 6A, deletion of *vpsT* resulted in higher H-NS promoter occupancy at the *rbmA*, *vpsU*, *vpsA*, *vpsL/rbmF* promoters compared to the wild type background. This effect could be complemented *in trans* by plasmid pVpsT upon induction of VpsT expression with IPTG (Fig. 6A). We note that, although VpsT has been reported to activate its own transcription (Srivastava *et al.*, 2011, Beyhan *et al.*, 2007), the *vpsT* mutant did not exhibit enhanced H-NS occupancy at its promoter (Fig. 6A). Deletion of *vpsT* had no effect on H-NS occupancy at the *tcpA* promoter that lacks a VpsT binding motif. Western blot analysis revealed that expression of H-NS-FLAG was not affected in the different genetic backgrounds tested (data not shown). These results indicate that the VpsT protein could directly antagonizes H-NS occupancy at the *vps* and *rbm* promoters under physiological conditions.

Since c-di-GMP enhanced the capacity of VpsT to displace H-NS from the *vpsA* and *vpsL* promoters *in vitro* (Fig. 2), we predicted that H-NS occupancy at the promoters tested in Fig. 6A should diminish under conditions in which the c-di-GMP pool is elevated. To test this prediction, we transformed strain C7258 H-NS-FLAG with vectors pCMW75 encoding *V. harveyi* diguanylate cyclase QrgB under the control of the *tac* promoter or pCMW98 encoding a catalytic inactive QrgB protein (QrgB*) as a control (Waters *et al.*, 2008). As expected, the reporter strain expressing the active QrgB enzyme exhibited reduced swarming motility and elevated biofilm when treated with IPTG while no effect could be detected in the strain expressing QrgB* (data not shown). As shown in Fig. 6B, induction of QrgB expression resulted in a significant decrease in H-NS occupancy at the *rbmA*, *vpsU*, *vpsA*, *vpsL/rbmF*, and *vpsT* promoters while over expression of the inactive QrgB* protein had no effect. As above, manipulation of the c-di-GMP pool did not affect H-NS occupancy at the *tcpA* promoter. Western blot analysis of H-NS-FLAG expression showed that manipulation of the c-di-GMP pool did not affect H-NS levels (data not shown).

The above data demonstrated the occurrence of an antagonistic relationship between VpsT and H-NS occupancy at the *vps* and *rbm* promoters. The data, however, does not exclude VpsT having additional regulatory functions at these promoters. To determine if VpsT acts at the *rbmA*, *vpsU*, *vpsA*, *vpsL* and *rbmF* promoters solely as an antirepressor, we conducted an epistasis analysis. In Table 2 we show that VpsT was not required for the expression of the above promoters in the absence of H-NS.

VpsR is required for c-di-GMP to diminish H-NS occupancy at the *vpsT* promoter

The fact that increasing the c-di-GMP pool also diminished H-NS occupancy at the *vpsT* promoter suggested that another protein capable of sensing c-di-GMP could antagonize H-NS repression of *vpsT* transcription. The transcription factors AphA and VpsR have been shown to bind to the *vpsT* promoter and activate its transcription (Yang *et al.*, 2010,

Srivastava *et al.*, 2011). Of these factors, VpsR has been shown to bind c-di-GMP (Srivastava *et al.*, 2011). To determine whether any of these transcription factors could antagonize the transcriptional silencing of *vpsT* by H-NS, we constructed *aphA*, *vpsR* and *vpsT* mutants expressing H-NS-FLAG and conducted ChIP. Consistent with Fig. 6A, deletion of *vpsT* did not alter H-NS occupancy at its own promoter (Fig. 7A). In contrast, deletion of *vpsR* and *aphA* resulted in a significant increase in H-NS occupancy at the *vpsT* promoter (Fig. 7A). This result suggests that both VpsR and AphA could antagonize H-NS binding to the *vpsT* promoter when the c-di-GMP pool is elevated. Western blot analysis showed that deletion of *vpsR*, *vpsT*, or *aphA* did not affect the level of H-NS-FLAG expression (data not shown). To determine the signaling pathway responsible for the negative effect of c-di-GMP on H-NS occupancy at the *vpsT* promoter, we artificially increased the c-di-GMP pool in *vpsR* and *aphA* genetic backgrounds. As shown in Fig. 7B, though AphA could contribute to lessen the amount of H-NS associated to DNA sequences upstream *vpsT* (Fig. 7C), the effect of c-di-GMP on H-NS occupancy required the regulator VpsR. The location of the VpsR motif (Zamorano-Sanchez *et al.*, 2015) at this promoter relative to H-NS motifs is shown in Fig. 7D.

Discussion

In *V. cholerae*, VpsT and VpsT-activated genes are expressed at low cell density and repressed at high cell density by quorum sensing. This knowledge, however, does not explain how c-di-GMP regulates the transition between alternative lifestyles at low cell density. We previously showed that H-NS directly represses the transcription of exopolysaccharide biosynthetic genes *vpsA*, *vpsL* and *vpsT* at low cell density (Wang *et al.*, 2012b). The present study supports the notion that *vps* and *rbm* genes are transcriptionally silenced by H-NS and are expressed as a result of environmentally-induced fluctuations in the c-di-GMP pool that activate VpsR and VpsT to trigger a coordinated and multilocus antirepression cascade. Analysis of the *vpsA* and *vpsL* promoters provided a mechanism for H-NS repression and antirepression. The H-NS binding profile at the *vpsL* promoter indicated that H-NS could hinder the access of RNA polymerase to promoter elements required for transcription initiation as previously observed in other genes (Schlax *et al.*, 1995, Hawley *et al.*, 1985). The H-NS binding pattern identified for the *vpsA* promoter resembled the one observed in promoters that are repressed through a DNA looping mechanism where H-NS associates with regions extending upstream and downstream from the promoter elements to trap RNA polymerase inside the promoter (Schroder & Wagner, 2000, Dame *et al.*, 2002).

We demonstrate that the c-di-GMP-binding protein VpsT binds to a 20 bp inverted repeat motif at the *vpsA* and *vpsL* promoters at positions that overlap H-NS binding sites. The 20 bp VpsT binding site identified in this study was identical to a recently reported 22 bp VpsT motif except for its first and last nucleotides (Zamorano-Sanchez *et al.*, 2015). A competitive EMSA showed that VpsT can efficiently disrupt the H-NS-DNA repressive nucleoprotein complexes formed at the *vpsA* and *vpsL* promoters *in vitro* in the presence of c-di-GMP. Accordingly, VpsT bound to the *vpsA* and *vpsL* promoters with higher affinity compared to H-NS in the presence of c-di-GMP. Conversely, in the absence of c-di-GMP

VpsT bound these promoters with lower affinity and H-NS could disrupt the VpsT-DNA complexes to potentially reset transcription of *vpsA* and *vpsL* back to silent.

We used ChIP-Seq to determine if the antagonistic effect between H-NS and VpsT could regulate the expression of other genes involved in biofilm development in addition to *vpsA-K* and *vpsL-Q*. ChIP-Seq identified additional loci repressed by H-NS affecting biofilm development such as *vpsU* and the *rbmBCDEF* cluster. The *V. cholerae* H-NS motif identified through ChIP-Seq was found to be structurally similar to the one previously reported in *E. coli* (Lang *et al.*, 2007). The similarity in both H-NS binding motifs is consistent with the H-NS C-terminal DNA binding domains of *V. cholerae* and *E. coli* K-12 exhibiting 71% of sequence identity. Having identified the VpsT and H-NS binding motifs, we examined if the antagonistic relationship between these regulators at the *vpsA* and *vpsL* promoters could occur at other loci. We identified 22 loci that contained overlapping H-NS and VpsT binding motifs. Among these loci, we identified *vpsU*, *rbmA* and *rbmF*. This finding indicates that H-NS and VpsT coordinately regulate the expression of both the exopolysaccharide and protein components of the biofilm matrix.

In the cell, binding of H-NS to DNA could be affected by the presence of proteins other than VpsT and by changes in DNA topology (Stoebel *et al.*, 2008). Thus, we used ChIP to examine the interplay between VpsT and H-NS occupancy under physiological conditions. Although the ChIP assay is less precise with regard to the DNA sequence bound to H-NS, deletion of VpsT increased H-NS occupancy at all *vps* and *rbm* promoters examined except *vpsT*.

We conducted an epistasis analysis to determine if VpsT functions at these promoter solely as an antirepressor or has an additional activating function as recently suggested for the *vpsL* promoter (Zamorano-Sanchez *et al.*, 2015). Our data showed that VpsT acts solely as an antirepressor. Several features, in addition to strains differences, could explain the slightly distinct results between our study and (Zamorano-Sanchez *et al.*, 2015). We measured *vps* expression at low cell density based on the knowledge that expression of HapR at high cell density acts to terminate *vpsT* transcription (Waters *et al.*, 2008). In the preceding study, expression of *vpsL* was determined in stationary phase (Zamorano-Sanchez *et al.*, 2015). Further, we directly measured the amount of mRNA transcribed from each chromosomal loci while the previous report used a *vpsL-luxCDABE* promoter fusion on a plasmid (Zamorano-Sanchez *et al.*, 2015).

Artificially increasing the c-di-GMP pool diminished H-NS occupancy at the *vpsT* promoter. We note that the *vpsT* promoter is responsive to c-di-GMP and this effect requires the biofilm activator VpsR (Srivastava *et al.*, 2011). A second activator, the PadR-like regulator AphA, also activates the *vpsT* promoter (Yang *et al.*, 2010). We found that deletion of *vpsR* or *aphA* increased H-NS occupancy at the *vpsT* promoter. Analysis of the effect of artificially increasing the c-di-GMP pool in *aphA* and *vpsR* mutants showed that c-di-GMP acts through VpsR to lessen H-NS occupancy at the *vpsT* promoter. An H-NS binding site identified by ChIP-Seq located 151 bp upstream the *vpsT* TSS (Srivastava *et al.*, 2011) and 16 bp upstream from the VpsR binding site (Zamorano-Sanchez *et al.*, 2015). H-NS could spread by oligomerization from this site to contact the polymerase and repress

transcription similar to the *E. coli* LEE5 promoter (Shin *et al.*, 2012). The close proximity of the H-NS and VpsR binding motifs suggests that binding of VpsR could either weaken the interaction between H-NS and the *vpsT* promoter or prevent H-NS from spreading downstream from its primary binding site.

At the genome level, ChiP-Seq revealed a striking correlation between regions in the genome exhibiting high H-NS occupancy and clusters of genes required for surface attachment and biofilm formation. Recent studies in *E. coli* have suggested that H-NS organizes the bacterial chromosome in foci to bring into proximity functionally-related regions facilitating communication between a regulatory gene and its target operon(s) (Dorman, 2013). Our study supports such a link between gene regulation and nucleoid organization with the caveat that, distinct from *E. coli*, *V. cholerae* contains two circular chromosomes (Heidelberg *et al.*, 2000). One example supporting this principle is the clustering of H-NS at the TCP VPI and the *vpsABCDEFGHIJK-rbmABDFEF-vpsLMNOPQ* region. The VPI in chromosome I encodes the toxin-coregulated pilus (TCP), a type-4 pilus required for attachment to intestinal cells (Krebs & Taylor, 2011) and chitinous surfaces (Reguera & Kolter, 2005). The cellular processes encoded by the TCP VPI and the *vps-rbm* cluster are functionally-related and negatively co-regulated by quorum sensing (Skorupski & Taylor, 1999, Yang *et al.*, 2010, Waters *et al.*, 2008, Rutherford *et al.*, 2011). Another example is the clustering of H-NS at the indole-producing tryptophanase operon and the indole-activated *vas* operon in chromosome II (Mueller *et al.*, 2009). Indole has been reported to act as a signaling molecule that modulates biofilm development in *V. cholerae* (Mueller *et al.*, 2009). In addition, expression of the type VI secretion system encoded by the *vas* operon is induced upon bacterial attachment to chitinous surfaces and is co-regulated with the TCP and *vps-rbm* gene clusters by quorum sensing (Borgeaud *et al.*, 2015). H-NS also silenced the transcription of *chiP*, *chiRP* and VC1073, which are required for *V. cholerae* attachment and growth on chitinous surfaces (Meibom *et al.*, 2004). Clustering of H-NS at sites of the chromatin involved in surface attachment and biofilm development could bring these regions into close proximity rendering their coordinate regulation more effective. H-NS clustering could also function to synchronize the expression of gene clusters involved in biofilm formation in responses to environmental conditions that affect their negative superhelical density (Hatfield & Benham, 2002). To our knowledge, our data provides the first evidence of a link between nucleoid architecture and biofilm development.

In summary, our results suggest a regulatory model in which an increase in the c-di-GMP pool induces a coordinated and multilocus H-NS antirepression cascade. For instance, under conditions in which the c-di-GMP pool is low, the highly abundant H-NS protein silences the transcription *vpsT* and downstream *vps* and *rbm* genes and *V. cholerae* preferentially adopts the planktonic lifestyle. An environmentally-induced up-shift in the c-di-GMP pool results in release of *vpsT* from H-NS repression facilitated by VpsR and in allosteric activation of VpsT. Active VpsT acts to diminish H-NS occupancy at downstream *vps* and *rbm* promoters resulting in the activation of biofilm exopolysaccharide and protein matrix biosynthesis. Conversely, an environmentally-induced down-shift in the c-di-GMP pool results in cessation of *vpsT* expression followed by displacement of bound VpsT from promoters by H-NS to reset the system back to silent.

Experimental procedures

Strains and media

V. cholerae mutants used in this study were derived from El Tor biotype strain C7258 and are described in Table S3. *Escherichia coli* TOP10 (Life Technologies) and S17-1 λ pir (de Lorenzo *et al.*, 1993) were used for cloning purposes. *V. cholerae* strains were grown in LB medium at 37°C with agitation (225 rpm). When necessary, culture media were supplemented with ampicillin (Amp) (100 μ g/ml), chloramphenicol (Cm) (10 μ g/ml), kanamycin (Km) (25 μ g/ml), rifampin (Rf) (150 μ g/ml), polymyxin B (PolB) (100 units/ml), isopropyl- β -D-thiogalactopyranoside (IPTG) (0.05 to 1.0 mM as indicated), or 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) (20 μ g/ml). Plasmids and oligonucleotide primers used throughout this work are described in Tables S3 and S4, respectively.

Construction of *aphA*, *vpsT*, *vpsR* mutants expressing H-NS tagged with the FLAG epitope

To construct an *aphA* deletion mutant, the suicide vector pCVD AphA (Silva & Benitez, 2004) was transferred by conjugation from *E. coli* S17-1 λ pir to strain C7258. *V. cholerae* exconjugants were selected in LB agar containing Amp and PolB and strain JCA1 (C7258 *aphA*) was isolated by sucrose selection as described previously (Liang *et al.*, 2007). Deletion of the *aphA* allele was confirmed by PCR with primer pairs AphA1/AphA2 and DNA sequencing. To express the epitope-tagged H-NS-FLAG protein from native *hns* transcription and translation initiation signals in wild type and mutant backgrounds, the suicide vector pCVDHNS-FLAG (Wang *et al.*, 2012a) was transferred from S17-1 λ pir to strain HX17 (C7258 *vpsT*) (Wang *et al.*, 2014), HX13 (C7258 *vpsR*) (Wang *et al.*, 2014) and JCA1 (C7258 *aphA*) by conjugation and the corresponding exconjugants were selected on LB plates containing Amp and PolB (Table S3). Integration of the *hns-flag* allele by homologous recombination within the *hns* locus was confirmed by PCR using primer VicH567, which anneals to DNA sequences upstream of *hns* (not present in pCVDHNS-FLAG), and FlagR, which anneals to DNA encoding the FLAG epitope. The resulting PCR products were further confirmed by DNA sequencing and the expression of H-NS-FLAG was established by Western blotting using anti-FLAG M2-peroxidase monoclonal antibody (Sigma-Aldrich) and chemiluminescence detection as described previously (Wang *et al.*, 2012a, Wang *et al.*, 2014). To conduct epistasis analysis a *vpsT* gene deletion/insertion was introduced in strains C7258 *lacZ* and AJB80 to generate *vpsT* and *hns vpsT* mutants, respectively. Briefly, suicide vector pCVD VpsT-Km (Wang *et al.*, 2011) was transferred by conjugation from *E. coli* S17-1 λ pir to strains C7258 *lacZ* and AJB80 to create strains JCA5 (C7258 *lacZ vpsT*) and JCA6 (C7258 *lacZ hns vpsT*) as described above.

For genetic complementation of *vpsT* mutants we modified the vector pTTQ18 (Stark, 1987). A blunt-ended 1129 bp DNA fragment encoding chloramphenicol acetyl transferase expression cassette was ligated into pTTQ18 digested with *ScaI* to generate pTTQ18cm. The entire *vpsT* ORF was amplified from the C7258 genome using primer pairs VpsT-1F/VpsT-1R and ligated into pTTQ18cm digested with *EcoRI* and *HindIII* to generate pVpsT. VpsT is expressed in this vector from the *tac* IPTG-inducible promoter.

Primer extension and DNase I footprint analysis

The *vpsA* and *vpsL* transcriptional start sites were identified by primer extension using 5' HEX (6-carboxy-2',4,4',5',7,7'-hexachlorofluorescein)-labeled primers and analysis on an automated capillary electrophoresis instrument as described previously (Wang *et al.*, 2014). Briefly, 17 µg of total RNA from *V. cholerae* C7258 grown in LB medium to mid-exponential phase was annealed with primers HEX-*vpsA* or HEX-*vpsL*, complementary to the 5' untranslated region (5'UTR) of *vpsA* and *vpsL*, respectively. Then, the primers were extended with AMV Reverse Transcriptase (Promega). For DNase I footprinting, DNA fragments encoding the promoter region of *vpsA* and *vpsL* were amplified by PCR using 6-carboxyfluorescein (FAM)- and HEX-labeled primers pairs FAM-*vpsA*-1F/HEX-*vpsA*-1R and FAM-*vpsL*-1F/HEX-*vpsL*-1R, respectively. Protein binding and DNase I digestion was performed as described previously (Wang *et al.*, 2014). Concisely, 200 ng of fluorescently-labeled DNA was incubated with 0.125 to 2.0 µg of purified H-NS protein (Wang *et al.*, 2012a) or 15 µg of purified VpsT protein (Wang *et al.*, 2014) (with or without 100 µM c-di-GMP as indicated) prior to digestion with 0.11 U of RQ1 RNase-Free DNase I (Promega) for 5 min at 26°C. Fluorescently-labeled cDNA primer extension and DNase I digestion products were detected with a 3730 capillary DNA analyzer (Applied Biosystems). To accurately assign a nucleotide base to the peaks detected in the primer extension and DNase I footprint assays, a sequence ladder was generated using the Thermo Sequenase Dye Primer Manual Cycle sequencing kit (USB Corporation). Template DNA fragments encoding the *vpsA* and *vpsL* promoters were generated by PCR using primer pairs *vpsA*-1F/*vpsA*-1R and *vpsL*-1F/*vpsL*-1R, respectively. The fragments were ligated into vector pCR2.1 (Table S3) to generate the vectors pCR-*vpsAP* and pCR-*vpsLP*. Sequencing reactions were conducted according to the manufacturer's instructions with 200 fmol of DNA template and 2 pmol of the corresponding fluorescently-labeled primer as indicated in the legend of supporting figures (Fig. S1–S5). The electropherograms corresponding to the primer extension and DNase I footprint reactions were aligned to the manually-generated nucleotide sequence by using GeneMapper software v. 4.0 (Applied Biosystems).

Electrophoresis mobility shift assays

Electrophoresis mobility shift assays (EMSAs) were conducted using the second-generation digoxigenin (DIG) gel shift kit (Roche Applied Sciences) as previously described (Wang *et al.*, 2012b, Wang *et al.*, 2012a, Wang *et al.*, 2014). DNA fragments encoding the *vpsA*, *vpsL*, *vpsU* and *rbmA* promoter regions were generated by PCR from *V. cholerae* C7258 chromosomal DNA with the primer pairs *vpsA*-2F/*VpsA*-2R, *VpsA*-3F/*VpsA*-2R, *vpsA*-4F/*vpsA*-3R for *vpsA*, *vpsL*-2F/*vpsL*-2R, *vpsL*-3F/*vpsL*-3R, *vpsL*-4F/*vpsL*-4R for *vpsL*, *vpsU*-1F/*vpsU*-1R for *vpsU* and *rbmA*-1F/*rbmA*-1R for *rbmA*. DNA binding was quantitated by densitometry using the ImageQuant TL software (GE Healthcare). Protein-DNA equilibrium dissociation constants (K_d) were estimated by fitting the data to the Hill equation (Hill, 1910) by nonlinear regression with the aid of GraphPad Prism 5.0 (GraphPad, San Diego, CA).

A competitive EMSA was performed to recreate the interaction of H-NS and VpsT at the *vpsA* and *vpsL* promoters *in vitro*. DIG-labeled promoter DNA fragments were pre-incubated with a fixed amount of H-NS to form the initial nucleoprotein complex. After 15

min at 30°C, increasing amounts of VpsT or VpsT plus c-di-GMP was added and incubation continued for an additional 15 min. The final nucleoprotein complexes were resolved and detected as described above. Similar experiments were carried but incubating first with VpsT and then with H-NS in the absence of c-di-GMP.

Chromatin immunoprecipitation

H-NS promoter occupancy was determined by chromatin immunoprecipitation (ChIP) analysis as described previously (Wang *et al.*, 2012a). Promoter occupancy by H-NS-FLAG was quantitated using the iTaq Universal SYBR Green Supermix kit and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The quantity of immunoprecipitated (IP) DNA was calculated as a percentage of the input DNA (10 µl sample taken prior to IP) using the formula $IP = 2^{(Ct_{input} - Ct_{IP})}$ where CT is the fractional threshold cycle of the input and IP samples. The relative IP was calculated by normalizing the IP of each sample by the IP of a mock ChIP using the mouse monoclonal antibody G3A1 IgG1 isotype control (Cell Signaling Technology). To study the effect of VpsT on H-NS promoter occupancy, strain C7258H-NS-FLAG (wild type) and JCA2 (*vpsT*/HNS-FLAG) harboring the empty vector pTTQ18cm (control) or pVpsT were grown in LB medium containing Amp and Cm at 37°C to optical density at 600 nm (OD₆₀₀) 0.4. Expression of *vpsT* in strains containing pVpsT was induced with IPTG (1 mM).

To study the effect of artificially increasing the cellular c-di-GMP pool on H-NS promoter occupancies, strains C7258HNS-FLAG, JCA3 (*vpsR hns::hns-flag*) and JCA4 (*aphA hns::hns-flag*) were transformed with plasmids pCMW75 or pCMW98 expressing *V. harveyi* DGC QrgB or the catalytically inactive mutant QrgB* (Waters *et al.*, 2008), respectively. The transformants were grown in LB medium supplemented with Amp and Km or the same medium containing IPTG (1 mM) to increase the c-di-GMP pool. Cultures were incubated at 37°C and collected at OD₆₀₀ 0.5.

Promoter regions containing the H-NS binding sites were amplified and quantitated with primer pairs *rbmA*-1F/*rbmA*-1R for *rbmA*, *vpsU*-1F/*vpsU*-1R for *vpsU*, *vpsA*-4F/*vpsA*-3R or *vpsA*-5F/*vpsA*-4R for *vpsA*, *vpsL*-2F/*vpsL*-5R for *vpsL/rbmF*, *vpsT*-2F/*vpsT*-2R or *vpsT*-3F/*vpsT*-3R for *vpsT*. The *tcpA* and VC1922 promoters were used as H-NS occupancy positive and negative controls, respectively (Wang *et al.*, 2012a). The *tcpA* and VC1922 promoters were amplified with primer pairs *tcpA*1F/*tcpA*2R and *vc1922*-F/*vc1922*-R, respectively.

ChIP-Seq

For ChIP-Seq, the ChIP part of the protocol was conducted as described previously (Wang *et al.*, 2012a, Wang *et al.*, 2012b) with minor modifications. Briefly, nine cell lysate samples of 100 µl were combined, immunoprecipitated and the final purified IP DNA was resuspended in 30 µl of distilled water. The DNA concentration of the anti-FLAG (7.2 ng/µl) and input (23.0 ng/µl) samples were determined using the Qubit® Fluorometric Quantitation System (Life Technologies). The anti-FLAG IP DNA and input DNA were used for library preparation for Illumina sequencing using the TruSeq ChIP kit (Illumina, San Diego, CA) following manufacturer's protocol. Briefly, 20 ng of ChIP DNA samples were end repaired-

ligated to Illumina adaptors and selected for a fragment size of approximately 300 bp by gel extraction. Multiplex Illumina primers were used to amplify gel-extracted products. The amplified ChIP-Seq libraries were quantitated by qPCR and loaded to a concentration of 2.5 pM per lane in the Illumina HiSeq2000 platform. A standard paired-end sequencing reaction was performed to generate 50 bp of sequence in each direction. The raw data was converted from .bcl file format to .fastq format for downstream analysis. This was done using CASAVA v1.8.2 software from Illumina.

ChIP-Seq bioinformatics analysis

The raw fastq data files were aligned to the *V. cholerae* El Tor N16961 reference genome (NC_002505.1 and NC_002506.1) using Bowtie, an ultrafast memory-efficient short read aligner (Langmead *et al.*, 2009) available on the Galaxy web-based platform (Blankenberg *et al.*, 2010). The generated sam data files were filtered to remove unmapped reads and converted to bam data files using the Galaxy Next Generation Sequencing (NGS): SAM Tools. SeqMonk v0.27.0 was used for peak calling, quantitation and annotation. The MACS (Model- Based Analysis for ChIP-Seq) algorithm (Zhang *et al.*, 2008) within SeqMonk was used for peak calling with p-value 1.0E-05, a sonicated fragment size of 300 bp and input sample as control. The Read Count Quantitation algorithm within SeqMonk was used for peak quantitation. The ChIP-Seq raw and processed data files have been deposited in NCBI's Gene Expression Omnibus (GEO) (Edgar *et al.*, 2002) and are accessible through the GEO series accession number GSE64249.

qRT-PCR

Total RNA extraction and quantitative real-time reverse-transcription-PCR (qRT-PCR) was conducted using the iScript two-step RT-PCR kit with SYBR green (Bio-Rad Laboratories) as described previously (Liang *et al.*, 2007, Silva *et al.*, 2008). Relative expression values were calculated as $2^{(CT_{\text{target}} - CT_{\text{reference}})}$, where CT is the fractional threshold cycle. The level of *recA* mRNA was used as an internal reference. The following primer pairs were used: vpsU-2F/vpsU-2R for *vpsU* mRNA, rbmA-2F/rbmA-2R for *rbmA* mRNA, rbmF-F/rbmF-R for *rbmF* mRNA, vpsAqFw/vpsAqRv for *vpsA* mRNA, vpsLqFw/vpsLqRv for *vpsL* mRNA and RecA578/RecA863 for *recA* mRNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Architecture of the *vpsA* (A) and *vpsL* (B) promoters

The transcription start sites (TSS) of the *vpsA* and *vpsL* promoters were determined by primer extension as described in methods. Contact regions between H-NS and VpsT and each promoter were determined by DNase I footprinting. The H-NS contact regions are indicated by a red line above the sequence. The VpsT contact regions are indicated with a green line below the sequence. Promoter -35 and -10 elements, TSS and Shine-Dalgarno (SD) sequences are underlined. A 20 bp inverted repeat within VpsT-protected regions is shown in bold font. (C) VpsT binding motif generated by the MEME server application using VpsT protected regions as input sequences. The electropherograms corresponding to the results summarized in panels A and B are shown in supporting information Fig. S1–S5.

Fig. 2. Disruption of H-NS and VpsT nucleoprotein complexes

Competitive EMSA were conducted using DIG-labeled DNA fragments containing H-NS and VpsT DNase I protected regions at the *vpsA* (−223 to −139) (**A**) and *vpsL* (−262 to −185) (**B**) promoters. DNA fragments were pre-incubated with H-NS for 15 min (lanes c and k) and subsequently treated with increasing concentrations of VpsT or VpsT plus 50 μ M c-di-GMP for an additional 15 min. The mobility of free DNA (lanes a and i) and each nucleoprotein complex are indicated at the right of each gel. The aberrant V-shaped band is due to dissociation of c-di-GMP from VpsT and release of a faster migrating free DNA from the VpsT nucleoprotein complex during electrophoresis. In panel C, the *vpsA* (lanes a–f) and *vpsL* (lane g–l) promoter fragments were pre-incubated with VpsT for 15 min (lanes c and i) and subsequently treated with increasing concentrations of H-NS.

Fig. 3. Comparison of H-NS and VpsT binding affinities to the *vpsA* (A) and *vpsL* (B) promoters Dissociation constants were determined by EMSA and densitometry as described in methods. The promoter fragments used were as indicated in Fig. 2. When indicated, c-di-GMP was added at concentration of 50 μ M.

Fig. 4. Genome-wide H-NS binding and transcriptome profiles during mid-exponential growth phase

(A) Schematic diagram of *V. cholerae* chromosome I and II showing the H-NS binding regions determined by ChIP-Seq and H-NS-regulated genes determined by RNA-Seq (GEO accession numbers GSE64249 and GSE62785 respectively). *V. cholerae* cells were grown in LB medium at 37°C and collected at OD₆₀₀ 0.5 for the ChIP-Seq and RNA-Seq as described in methods. The numbering indicated within the inner circle in both chromosomes represents the positions in kb pairs. (B) *V. cholerae* H-NS motif. (C) H-NS binding profile and transcription regulation at the *vps* and *rbm* loci. BAM files containing the sequence reads for the Wt and *hns* cells transcriptome and the H-NS ChIP-Seq binding profiles were aligned to the reference genome and visualized using IGV software (Thorvaldsdottir *et al.*, 2013). Numbers in parenthesis indicate the maximum read count in the (Y) axis. The complete ChIP-Seq dataset and statistics is provided in supporting information Table S1 and Fig. S8.

Fig. 5. Binding of H-NS and VpsT to the *rbmA* and *vpsU* promoters

DIG-labeled DNA fragments spanning nucleotides -237 to -98 of the *rbmA* promoter (**A** and **C**) or nucleotides -199 to -51 of the *vpsU* promoter (**B** and **D**) with reference to the start codons were incubated with increasing concentrations of purified H-NS (panels **A** and **B**) or VpsT plus c-di-GMP (panels **C** and **D**).

Fig. 6.

(A) Effect of VpsT on H-NS occupancy at *vps* and *rbm* promoters. ChIP analysis of H-NS occupancy at *vps* and *rbm* promoters in *V. cholerae* C7258/H-NS-FLAG (Wt) and the isogenic *vpsT* mutant JCA2 containing pTTQ18cm (empty vector) or plasmid pVpsT encoding *vpsT* expressed from the IPTG-inducible *tac* promoter. Strains were grown in LB cultures and cells were collected at OD₆₀₀ 0.4. The *tcpA* and VC1922 promoters were used as positive and negative controls for H-NS binding, respectively. **(B) Effect of increasing the intracellular c-di-GMP pool on H-NS occupancy at *vps* and *rbm* promoters.** ChIP analysis of H-NS occupancy at the indicated promoters in *V. cholerae* C7258/H-NS-FLAG (Wt) containing the *V. harveyi* DGC QrgB or the active site mutant QrgB* expressed from the IPTG-inducible *tac* promoter. Cells were grown in LB medium to mid-exponential phase (OD₆₀₀ 0.5) with or without IPTG induction. Data are represented as the mean (bars) plus the standard error of the mean (SEM) (error bars). Statistical differences between Wt and mutants or between IPTG-induced and uninduced cultures (* at $p < 0.01$) were determined by an ANOVA test and a Tukey's Multiple Comparison Posttest ($n=4$).

Fig. 7. Effect of VpsR and AphA on H-NS occupancy at the *vpsT* promoter

(A) ChIP analysis of H-NS occupancy at the *vpsT* promoter in *V. cholerae* C7258 H-NS-FLAG (Wt) and isogenic *vpsT* (JCA2), *vpsR* (JCA3) and *aphA* (JCA4) mutants expressing *hns*-flag. Cells were grown in LB medium to OD₆₀₀ 0.3. (B) Effect of artificially increasing the c-di-GMP pool by overexpression of DGC QrgB on H-NS occupancy of the *vpsT* promoter in mutants lacking VpsR or AphA. (C) H-NS occupancy upstream *vpsT* and transcriptome assay as determined by ChIP-Seq and RNA-Seq. (D) Sequence of *vpsT* promoter. The H-NS motifs present in the coding strand are underlined; H-NS motifs in the template strand are indicated by a line above the sequence; the VpsR binding motif (Zamorano-Sanchez *et al.*, 2015) is boxed; * indicates the TSS (+1 base) (Srivastava *et al.*, 2011); the Shine-Dalgarno sequence and start codon are shown in bold font. ChIP data are represented as the mean plus the SEM. Statistical differences from the Wt (* at $p < 0.01$) or between IPTG-induced and uninduced cultures (* at $p < 0.05$) were determined by an ANOVA test and a Tukey's Multiple Comparison Posttest ($n=3$).

Table 1

Genes repressed by H-NS showing overlapping VpsT and H-NS motifs

Gene	Function	VpsT motif	H-NS motif	H-NS Log ₂ Enrichment
VC0176	Transcription regulator	AAATTAAC TAAGATTTCTT	AAAGGTAAAC	17.40
VC0177	Hypothetical protein	AAATTAAC TAAGATTTCTT	AACAATAAAC	17.40
VC0490	Conserved hypothetical protein	TAATTAAG TTGACTTTATTT	AAACATAAAC	18.79
<i>vpsU</i>	VPS biosynthesis	AAATGAAAG TAGTTTATTT	ATAAATAAAA	17.27
<i>vpsA-K</i>	Exopolysaccharide biosynthesis	AAATAAACT AAAGTTATAT	GAAAATAAAC	17.27
<i>rbmA</i>	Biofilm matrix protein	AAATAAACT TTGGTTTATTT	AAAAATAAAC	19.14
<i>rbmF-D</i>	Hypothetical proteins	AAATAAACT TTAGTTTACTT	AAAAGTAAAA	18.07
<i>vpsL-Q</i>	Exopolysaccharide export	AAGTAAACT AAAGTTTATTT	GAAAATAAAC	18.07
VC1329	Opacity-related protein	TAAATACCT AAAGTTTATTT	AAAAATAACC	17.13
VC1448	RTX toxin transporter	CATTAAATT AGAGTATATAT	AAAAATAAAA	18.05
VC1449	Hypothetical protein	CATTAAATT AGAGTATATAT	AAAGAGAAAA	18.05
<i>ankB</i>	Ankyrin-like protein detoxification	ATTTAAACG AAAGTTTATGA	GAGAATGAAG	16.94
<i>katB</i>	Hydrogen peroxide- inducible catalase	ATTTAAACG AAAGTTTATGA	GAGAATGAAG	16.94
VC1947	LysR-type transcription regulator	AAAAAATCG TTTGGTTATAT	GAAAATAGAA	17.77
VCA0201	hypothetical protein	ACGGAAACT ATAGTTTTCAT	CAAGTTAAAA	19.99
VCA0218	Thermolabile hemolysin	AACTAGACT TGATCTTACTT	AAAAATAACA	18.08
<i>hlyA</i>	Hemolysin	AACTAGACT TGATCTTACTT	AAAAATAACA	18.08
VCA0363	Hypothetical protein	TTATAAACT TAAGGCTTTCT	AAAAATAAGT	17.34
VCA0448	Hypothetical protein	CAATACTCT TGATTTTACTT	AAAAATAAAG	15.16
VCA0451	Hypothetical protein	ATATTAAAT ACATTTTATTT	ATAAATAAAA	17.78
VCA0691	Acetoacetyl-CoA reductase	AAATTCAG TTTTGTGTATT	AAAAATACAC	17.13
<i>dsdC</i>	D-serine deaminase activator	ATATTATCCT TAGTGACAT	AAAGCTGAAA	17.24
VCA0965	DGC GGDEF family protein	AAGTTAGCT AAAGTTTAGAT	AAAAATAACG	14.98
VCA1065	Conserved hypothetical protein	ATATATATTT TAGATTTATT	AAATCTAAAA	18.09

Table 2

Epistasis analysis of the expression of *vps* and *rbm* genes in *hms* and *vpsT* mutants.

Strain	Relative mRNA abundance*				
	<i>rbmA</i>	<i>vpsU</i>	<i>vpsA</i>	<i>vpsL</i>	<i>rbmF</i>
Wt	0.186 ± 0.087	0.179 ± 0.046	0.085 ± 0.023	0.020 ± 0.013	0.009 ± 0.003
<i>vpsT</i>	0.114 ± 0.024	0.086 ± 0.030	0.037 ± 0.015	0.007 ± 0.004	0.006 ± 0.003
<i>hms</i>	2.467 ± 0.120	2.295 ± 0.195	1.480 ± 0.232	0.865 ± 0.041	0.500 ± 0.044
<i>hms vpsT</i>	2.608 ± 0.382	2.787 ± 0.143	1.724 ± 0.294	0.959 ± 0.077	0.484 ± 0.033

* mRNA abundance was determined by qRT-PCR. The data is presented as the mean of three independent experiments ± standard deviation.