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NspS, a Predicted Polyamine Sensor, Mediates Activation of *Vibrio cholerae* Biofilm Formation by Norspermidine

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Vibrio cholerae **is both an environmental bacterium and a human intestinal pathogen. The attachment of bacteria to surfaces in biofilms is thought to be an important feature of the survival of this bacterium both in the environment and within the human host. Biofilm formation occurs when cell-surface and cell-cell contacts are formed to make a three-dimensional structure characterized by pillars of bacteria interspersed with water channels. In monosaccharide-rich conditions, the formation of the *V. cholerae* biofilm requires synthesis of the VPS exopolysaccharide. MbaA (locus VC0703), an integral membrane protein containing a periplasmic domain as well as cytoplasmic GGDEF and EAL domains, has been previously identified as a repressor of *V. cholerae* biofilm formation. In this work, we have studied the role of the protein NspS (locus VC0704) in *V. cholerae* biofilm development. This protein is homologous to PotD, a periplasmic spermidine-binding protein of *Escherichia coli*. We show that the deletion of *nspS* decreases biofilm development and transcription of exopolysaccharide synthesis genes. Furthermore, we demonstrate that the polyamine norspermidine activates *V. cholerae* biofilm formation in an MbaA- and NspS-dependent manner. Based on these results, we propose that the interaction of the norspermidine-NspS complex with the periplasmic portion of MbaA diminishes the ability of MbaA to inhibit *V. cholerae* biofilm formation. Norspermidine has been detected in bacteria, archaea, plants, and bivalves. We suggest that norspermidine serves as an intercellular signaling molecule that mediates the attachment of *V. cholerae* to the biotic surfaces presented by one or more of these organisms.**

In their natural habitats, bacteria are often found in surface-attached sessile communities known as biofilms (11). The mature biofilm is a three-dimensional structure composed of pillars of bacteria encased in a self-produced exopolysaccharide matrix surrounded by water channels (12). Compared to their planktonic or free-swimming counterparts, biofilm-associated bacteria are less susceptible to UV irradiation, host defense, and the toxic effects of antimicrobial agents (16, 46). Thus, the ability to form a biofilm is hypothesized to confer a survival advantage both in the environment and within a host.

Genetic and microscopic analyses of many bacterial species have shown that biofilm development proceeds through several distinct stages (13, 52, 56, 71). In the free-swimming or free-floating planktonic stage, bacteria exist as single cells that are not associated with a surface. When a surface is encountered, bacteria form transient associations with the surface, which may lead to permanent attachment, thus initiating the monolayer stage. In the monolayer, only cell-surface interactions are present. Specific environmental signals activate the synthesis of exopolysaccharide, which heralds the passage into the biofilm stage (14, 28, 72, 77). Environmental signals identified to date include surfaces, osmolarity, quorum sensing, and nutritional cues (60).

Vibrio cholerae, the causative agent of the diarrheal disease cholera, is a natural inhabitant of diverse aquatic environments, including lakes, rivers, estuaries, and oceans (9, 10). In its natural habitats, *V. cholerae* is thought to exist primarily in the surface-attached state (31). It has been identified in association with phytoplankton, zooplankton, crustaceans, and aquatic plants (32, 33, 59, 65). *V. cholerae* biofilm development on abiotic surfaces proceeds through stages similar to those described above (52, 72, 73). Attachment to surfaces is accelerated by cell surface appendages such as the polar flagellum and the mannose-sensitive hemagglutinin type IV pilus. Exopolysaccharide synthesis and the formation of the mature *V. cholerae* biofilm are highly regulated. Exopolysaccharide synthesis and secretion are carried out by a number of proteins, including those encoded by the *vps* (*v*ibrio *p*olysaccharide) genes, which reside in two operons approximately 8.3 kb apart. These operons are comprised of genes *vpsA* to *vpsK* (*vpsA-vpsK*) and *vpsL-vpsQ*, respectively (69, 72, 77). Transcription of the *vps* genes is under the control of the σ^{54}-dependent two-component response regulator VpsR (75). As is the case for most two-component response regulators, VpsR is activated by phosphorylation (43). However, the signals that lead to the activation of VpsR are unknown, and its cognate phosphoryl group donor histidine kinase has not yet been identified. The transcription of the *vps* genes is also activated by VpsT, another two-component response regulator (4). VpsT and VpsR activate their own transcriptions, and each also activates that of the other (4). It is not known whether these regulators affect *vps* gene transcription directly or indirectly.

Several negative regulators of *vps* gene transcription have also been identified. CytR, first identified as a nucleoside-responsive transcriptional repressor of genes responsible for nucleoside uptake and catabolism, is also a repressor of *vps* gene transcription and biofilm formation in *V. cholerae* (25). HapR, which was initially identified as a transcriptional activator of the *hap* gene encoding hemagglutinin/protease,

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TABLE 1. Bacterial strains and plasmids

Strain/plasmid	Genotype	Reference/source
E. coli strain SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2 Tc::*MuλpirR6K*; Km^r	51
V. cholerae strains		
PW249	MO10, clinical isolate of *V. cholerae* O139 from India, Sm^r	70
PW357	MO10*lacZ*::*vpsLp*→*lacZ*, Sm^r	25
PW396	MO10, Δ*vpsA-vpsK*, Sm^r	40
PW444	MO10*lacZ*::*vpsLp*→*lacZ*, Δ*mbaA*, Sm^r	This study
PW512	MO10*lacZ*::*vpsLp*→*lacZ*, ΔVC0702, Sm^r	This study
PW514	MO10*lacZ*::*vpsLp*→*lacZ*, ΔVC0704, Sm^r	This study
PW522	MO10*lacZ*::*vpsLp*→*lacZ*, ΔVC0704Δ*mbaA*, Sm^r	This study
Plasmids		
pWM91	*oriR6K mobRP4 lacI Ptac tnp* mini-Tn*10*Km; Km^r, Ap^r	49
pACYC184	Km^r Cm^r	6
pCR2.1-TOPO	Plasmid for TOPO cloning, Ap^r	Invitrogen
PK105	pWM91 carrying an internal in-frame deletion of VC0704	This study
PK104	pWM91 carrying an internal in-frame deletion of VC0702	This study
pNB4	pWM91 carrying an internal in-frame deletion of *mbaA*	3

represses *vps* gene transcription and biofilm formation in response to high cell density in some strains of *V. cholerae* (24, 36, 43, 76, 79). Although the roles of nucleosides and quorum-sensing signals in the regulation of *vps* gene transcription and biofilm formation have been established, the signal transduction pathways that underlie this regulation have not yet been elucidated.

Genes encoding proteins belonging to the GGDEF and EAL families are highly abundant in the genomes of gram-negative organisms. Some of the functions regulated by GGDEF and EAL family proteins include cellulose production in *Gluconacetobacter xylinus* and *Salmonella enterica* serovar Typhimurium, autoaggregation and the wrinkled colony morphology in *Pseudomonas aeruginosa*, swarmer-to-stalked cell differentiation in *Caulobacter crescentus*, and biofilm formation in *Yersinia pestis* (15, 26, 41, 64). These domains are involved in nucleotide cyclization, specifically in the synthesis and degradation of bis-(2′,5′)-cyclic diguanylic acid (c-di-GMP).

It has been shown that GGDEF domains can function as diguanylate cyclases, enzymes that catalyze the conversion of GTP to c-di-GMP, while some EAL domains have been demonstrated to be phosphodiesterases, enzymes that degrade c-di-GMP (2, 57). However, there are also a number of diguanylate cyclases and phosphodiesterases that contain both of these domains (64). c-di-GMP has been shown to affect the function of some enzymes by an allosteric mechanism, and changes in the intracellular levels of c-di-GMP modulate gene transcription within the cell (35).

Recently, several proteins belonging to the GGDEF and EAL families have been shown to affect biofilm formation in *V. cholerae* (3, 58, 67). In a study by Tischler and Camilli, the overexpression of the putative diguanylate cyclase encoded by VCA956, a GGDEF family protein of unknown function, or the deletion of VieA, a putative phosphodiesterase containing an EAL domain, resulted in increased intracellular levels of the secondary messenger c-di-GMP, *vps* gene transcription, and biofilm formation in *V. cholerae* (67). Furthermore, MbaA, a putative integral membrane protein containing GGDEF and EAL domains, has also been identified as a repressor of biofilm formation (3). Because decreased biofilm formation has been correlated with the degradation of c-di-GMP, it is likely that MbaA functions as a phosphodiesterase. MbaA is encoded by the second gene in a putative three-gene operon that also includes genes coding for a predicted periplasmic protein with similarity to polyamine-binding proteins (locus VC0704) and a predicted cytoplasmic protein (locus VC0702).

Polyamines are organic polycations that are positively charged at physiological pH due to their protonated amine groups (63). They are involved in numerous cellular processes, including the modulation of DNA and RNA functions, protection against oxidative damage, and the regulation of bacterial porins and eukaryotic ion channels (34, 37, 45, 53, 78). In this work, we have studied the function of the predicted polyamine-binding protein encoded at locus VC0704. We show that this protein activates biofilm formation in response to increased environmental concentrations of the polyamine norspermidine. Thus, we have named this protein NspS (for *n*or*sp*ermidine *s*ensor). To our knowledge, this is the first report of the modulation of bacterial biofilms by environmental polyamines. Although studies are limited, norspermidine has been identified in many types of bacteria and in eukaryotes. Because signaling through NspS most likely does not require the entry of norspermidine into the cell, we suggest that we have identified a novel intercellular signaling pathway that enables *V. cholerae* to communicate with both its prokaryotic and its eukaryotic neighbors.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and reagents. The bacterial strains and plasmids used in this study are listed in Table 1. pCR2.1-TOPO was propagated in the DH5α strain, and pWM91 was propagated in the DH5αλpir strain. All experiments were done in Luria-Bertani (LB) broth except where otherwise indicated. Norspermidine and spermidine were purchased from Sigma (St. Louis, MO). Restriction endonucleases were from New England Biolabs (Beverly, MA). *Taq* and *Pfx* polymerases and salmon sperm DNA were from Invitrogen (Carlsbad, CA). Primers used in this study are listed in Table 2. Primer synthesis and DNA sequencing were done by Tufts University Core Facility (Boston, MA).

Construction of the deletion mutants. In-frame deletions that removed 957 nucleotides from the 1,080-bp coding sequence at locus VC0704 and 469 nucleotides from the 552-bp coding sequence at locus VC0702 were constructed as follows. For the VC0704 deletion, a 392-bp region of chromosome I between positions 753277 and 753669 and a 404-bp region between positions 754624 and

TABLE 2. Primers

Construct	Primer	Description	Sequence
V. cholerae VC0702 deletion	P200	Forward primer for upstream fragment 1	5′-GGAACTCTCGCGTCTTGGCTATG-3′
	P201	Reverse primer for upstream fragment	5′-GCCGCAGCGGCCGCAGGTGCTAAAGGCACTTCTAACG-3′
	P202	Forward primer for downstream fragment	5′-TGCGGCCGCTCGGGCGCTCTGATCCCCTTTATTAATCCA-3′
	P203	Reverse primer for downstream fragment 1	5′-TCCTTCTTTTAGTGAGTGGCAGCAAG-3′
	P208	Forward primer for upstream fragment 2	5′-TCGAGGAACTCTCGCGTCTTGGCTATG-3′
	P209	Reverse primer for downstream fragment 2	5′-CTAGTCCTTCTTTTAGTGAGTGGCAGCAAG-3′
V. cholerae VC0704 deletion	P204	Forward primer for upstream fragment 1	5′-GATATGACTAAGCTGGAATCGCGTC-3′
	P205	Reverse primer for upstream fragment	5′-GCCCGAGCGGCCGCACAGCGACAGAAAGCGTTTGATC-3′
	P206	Forward primer for downstream fragment	5′-TGCGGCCGCTCGGGCCTCAGTCTGCGTGCCAAAATCATC-3′
	P207	Reverse primer for downstream fragment 1	5′-TTGGCCATTGAGGATCGATAACG-3′
	P210	Forward primer for upstream fragment 2	5′-TCGAGATATGACTAAGCTGGAATCGCGTC-3′
	P211	Reverse primer for downstream fragment 2	5′-CTAGTTGGCCATTGAGGATCGATAACG-3′

755028 were amplified by PCR. For the deletion at locus VC0702, a 421-bp region of chromosome I between positions 750399 and 750820 and a 411-bp region between positions 751232 and 751643 were amplified by PCR. In both cases, the internal primers contained a 15-bp complementary region, which allowed the amplified fragments to be spliced together by overlap extension PCR (29). The spliced fragments were then amplified in two separate reactions for each gene by using primer pairs P200/P209 (reaction 1) and P203/P208 (reaction 2) for the VC0702 deletion and primer pairs P204/P211 (reaction 3) and P207/P210 (reaction 4) for the VC0704 deletion. The amplified products were heated to 100°C for 5 min for denaturation; mixtures for reactions 1 and 2 were combined and mixtures for reactions 3 and 4 were combined. They were then left to anneal by slow cooling to room temperature to yield sticky ends as described elsewhere (68). These were cloned directly into the suicide plasmid pWM91 digested with XhoI and SpeI to create pK104 and pK105. The correct assembly of the fragments was confirmed by sequencing. These plasmids were transformed into SM10λpir and conjugated into the relevant strains. The deletion mutants were created by double homologous recombination and sucrose selection as described previously (49). Similarly, pNB4 was used to create the Δ*mbaA* strain (3). All of the deletions were confirmed by PCR.

Fluorescence microscopy. For fluorescence microscopy, biofilms were formed on borosilicate coverslips inserted into 50-ml Falcon tubes containing 7 ml of LB broth inoculated with *V. cholerae* to yield a starting optical density at 655 nm (OD_{655}) of 0.02, as measured in a 96-well flat-bottomed plate by using a Bio-Rad model 680 microplate reader (Hercules, CA). After an 18-h incubation, the media and the planktonic cells were discarded, and biofilms were incubated in fresh media containing 2 μg of the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) for 30 min at room temperature, washed with fresh LB, and placed on a concave microscope slide filled with sterile medium. An Eclipse TE-200 inverted fluorescence microscope (Nikon) equipped with an Orca digital charge-coupled-device camera (Hamamatsu) and a focus motor were used to collect a stack of transverse sections through the biofilm. The image stacks were subjected to nearest-neighbor deconvolution and three-dimensional image reconstruction using the Metamorph Imaging software (Universal Imaging).

Biofilm assays. Borosilicate tubes were filled with 300 μl of growth medium. These tubes were inoculated with the strains of interest to yield an OD_{655} of 0.02. These cultures were incubated for 18 h at 27°C. Planktonic cells were removed, the remaining biofilm was washed with 300 μl of LB, and the biofilm was dispersed by vortexing for 10 s in the presence of 1-mm-diameter borosilicate glass beads (Biospec, Bartlesville, OK). The final planktonic and biofilm cell densities were measured by using 150 μl of the relevant cell suspension. All experiments were performed in triplicate and repeated multiple times to confirm reproducibility.

β-Galactosidase measurements. β-Galactosidase assays were performed as previously described with the following modifications (25). Briefly, Erlenmeyer flasks were filled with 20 ml of LB broth with supplements as noted and inoculated with an overnight culture of the relevant strain at a 1:100 dilution. The culture was grown to logarithmic phase (OD_{655} of 0.3 to 0.4) at 27°C with shaking. One-ml aliquots were used for β-galactosidase measurements. Cells were washed twice and resuspended in 200 μl of Z buffer (50). Cells were lysed by three freeze-thaw cycles and incubated for 18 h at 37°C with *O*-nitrophenyl-β-D-galactopyranoside (Sigma). β-Galactosidase activity was determined by measuring the A_{415}. Values were normalized using cell density measurements. All experiments were performed in triplicate and repeated multiple times.

RNA extraction. RNA for use in microarray hybridizations was prepared as follows. Wild-type and Δ*mbaA* mutant cultures were grown as described above for β-galactosidase assays and grown to logarithmic phase. Half of the culture was pelleted by centrifugation at 5,000 rpm for 5 min at 4°C. RNA was extracted by lysing the cells in TRIzol reagent (Invitrogen), a procedure followed by chloroform extraction and isopropanol precipitation. The RNA was dissolved in 300 μl of RNase-free water, treated with DNase (Ambion, Austin, TX), and further purified for use in the microarray experiments by use of RNeasy columns following the manufacturer's instructions (QIAGEN, Valencia, CA).

cDNA synthesis. Ten μg of purified RNA was reverse transcribed using random hexamers, a mixture of dNTPs and aminoallyl-labeled dUTP, and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. After reverse transcription, 10-μl portions of 0.5 M EDTA and 1 N NaOH were added to inactivate the enzyme and hydrolyze the RNA, respectively, and the mixture was incubated at 65°C for 15 min. After the addition of 10 μl 1N HCl and 40 μl water, cDNA was purified using a QIAGEN MinElute kit as described by the manufacturer with the following modification: cDNA bound to the column was washed with 75% ethanol rather than manufacturer-supplied PE buffer and eluted with 10 μl of water. PE buffer may contain free amines that would compete with the coupling of the Cy5 and Cy3 dyes to the aminoallyl-labeled nucleotides. The purified cDNA was quantified by measuring absorbance at 260 nm. We routinely obtained approximately 1 to 2 μg of cDNA by this method.

Coupling of Cy3 and Cy5 to cDNA. All reactions with dyes were maintained in the dark when possible. cDNA was coupled to monoreactive Cy3 or Cy5 dye (Amersham) in the presence of 0.05 M $NaHCO_3$ for 1 h at room temperature. The reaction was quenched by incubation with 1.2 M hydroxylamine, and the labeled cDNA was purified by use of Qiaquick columns (QIAGEN). The efficiencies of incorporation were determined by measuring absorbances at 260 and 650 nm for Cy5 and at 260 and 550 nm for Cy3. The Cy3- and Cy5-labeled

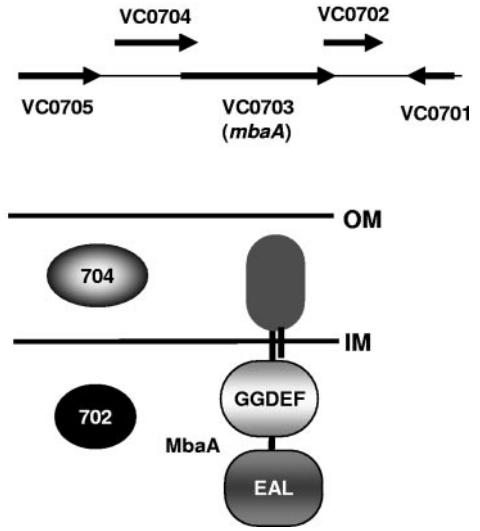

FIG. 1. Genomic arrangement of VC0702, *mbaA*, and VC0704 and predicted cellular locations of the proteins these genes encode. Horizontal lines represent the bacterial inner membrane (IM) and outer membrane (OM).

cDNAs were combined such that they contained equal amounts of incorporated dyes. The mixture was dried using a SpeedVac system.

Array printing. Microarrays were printed on UltraGAPS-coated slides (Corning, Acton, MA) using a Biorobotics MicroGrid II system. Each array consisted of 4,000 spots containing 70-mers designed to correspond to unique sequences in the open reading frames (ORFs) of the *V. cholerae* genome as well as to positive and negative controls (Illumina, San Diego, CA). Each slide contained two microarrays.

Preparation of cDNA for hybridization. The labeled and dried cDNA was dissolved in 27 μl of hybridization solution (250 μl formamide, 250 μl 20× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 10 μl 10% sodium dodecyl sulfate [SDS], 490 μl water). After the addition of 3 μl of blocking solution (10 μl 10-mg/ml salmon sperm DNA, 20 μl 10-mg/ml tRNA, 20 μl water), the mixture was incubated in boiling water for 3 min, snap-chilled on ice for 30 s, and kept at room temperature. Before it was applied to the microarray for hybridization, it was centrifuged for 5 min at 13,000 rpm to remove any debris.

Microarray hybridization. Arrays were prepared for hybridization by incubation in 50 ml prehybridization solution (12.5 ml 20× SSC, 500 μl 10% SDS, 1 g bovine serum albumin, and 37 ml water filtered through 0.22-μm syringe filters; Fisher) at 42°C for 45 min. The slides were rinsed with water and subsequently with isopropanol. Labeled cDNAs were applied to the microarrays and covered with a lifter slip. The microarrays were then incubated with the labeled cDNAs in hybridization chambers for 48 h in 42°C in a water bath. After hybridization, the slides were successively washed at room temperature for 5 min in solution 1 (2× SSC, 0.2% SDS), 10 min in solution 2 (0.1× SSC, 0.2% SDS), and three times for 1 min each in solution 3 (0.1× SSC) and scanned.

Image quantitation and data analysis. Microarrays were scanned for Cy5 and Cy3 fluorescence intensities using a Packard ScanArray 4000 system. cDNAs synthesized from wild-type *V. cholerae* and the Δ*mbaA* mutant were labeled twice with Cy3 and twice with Cy5 each, so as not to introduce biases due to any possible differences between the dyes. Spot intensities and qualities were calculated using Imagene version 5.6.1 (BioDiscovery, El Segundo, CA). Gene transcription levels were calculated from background-subtracted fluorescence intensities. These data were normalized using GeneSpring version 6.1 (Silicon Genetics, Redwood City, CA) with the intensity-dependent Lowess normalization. Genes were scored as differentially regulated if the ratio of the mutant to wild-type transcripts was >1.5 and <0.67 in three out of four technical replicates.

RESULTS

Deletion of the chromosomal locus VC0704 decreases *V. cholerae* biofilm formation. MbaA, a protein named for its role in the maintenance of biofilm architecture, was previously identified as a repressor of biofilm formation in a transposon mutagenesis screen designed to select biofilm-altered *V. cholerae* O1 El Tor mutants (3). *mbaA*, which is located on the large chromosome of *V. cholerae* at locus VC0703, is predicted to code for an integral membrane protein comprised of a periplasmic domain as well as cytoplasmic GGDEF and EAL domains. The ORF of *mbaA* is flanked by overlapping ORFs at loci VC0702 and VC0704. These genes are predicted to encode a cytoplasmic protein and a periplasmic protein, respectively (Fig. 1). The genomic arrangement of these three genes suggested to us that they might function together to regulate biofilm formation. To test this hypothesis, we constructed *V. cholerae* O139 mutants carrying in-frame deletions in each gene of this putative operon and quantified the growth rates of these mutants as well as their propensities to attach to surfaces and accumulate in biofilms. The growth rate of each mutant as well as its ability to attach to a surface and form a monolayer was similar to that of wild-type *V. cholerae* (data not shown); however, differences in biofilm formation were observed. (Fig. 2). As had previously been reported for the *V. cholerae* O1 El Tor strain N16961, the *V. cholerae* O139 Δ*mbaA* mutant demonstrated an increased propensity to accumulate in a biofilm. In contrast, surface accumulation by the ΔVC0704 mutant was greatly reduced compared to that of wild-type *V. cholerae* and was just slightly more than that of a Δ*vpsA-vpsK* deletion mutant. Surface accumulation by the ΔVC0702 deletion mutant was consistently slightly more than that of wild-type *V. cholerae*, but this difference was not always statistically significant ($P = 0.1$). We hypothesize that the environmental conditions utilized in these experiments may not highlight the role of VC0702 in the regulation of biofilm formation.

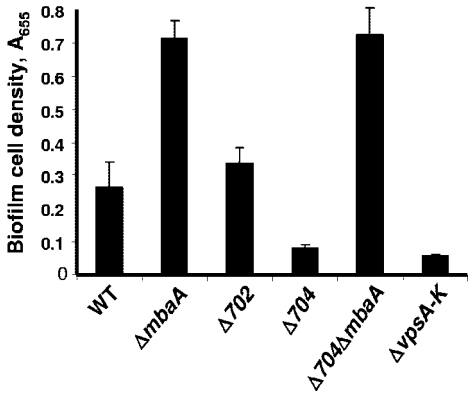

FIG. 2. Quantification of wild-type and mutant *V. cholerae* biofilms. Wild-type *V. cholerae* (WT) as well as Δ*mbaA*, ΔVC0702, ΔVC0704, ΔVC0704Δ*mbaA*, and Δ*vpsA-vpsK* (Δ*vpsA-K*) mutant biofilms were formed in borosilicate tubes overnight in LB broth and quantified as described in Materials and Methods. Error bars show standard deviations of three replicates.

Fluorescence microscopy was used to visualize the architectures of biofilms made by wild-type *V. cholerae* as well as by the three mutants described above (Fig. 3). As previously demonstrated in the El Tor background, the height of the Δ*mbaA* mutant biofilm was greater than that of the biofilm formed by wild-type *V. cholerae*. In contrast, the ΔVC0704 mutant biofilm was only a few cells in depth. The height of the ΔVC0702 mutant biofilm was similar to that of the wild-type *V. cholerae* biofilm. These results confirm that MbaA is a repressor of biofilm formation in *V. cholerae* O139 and suggest that the

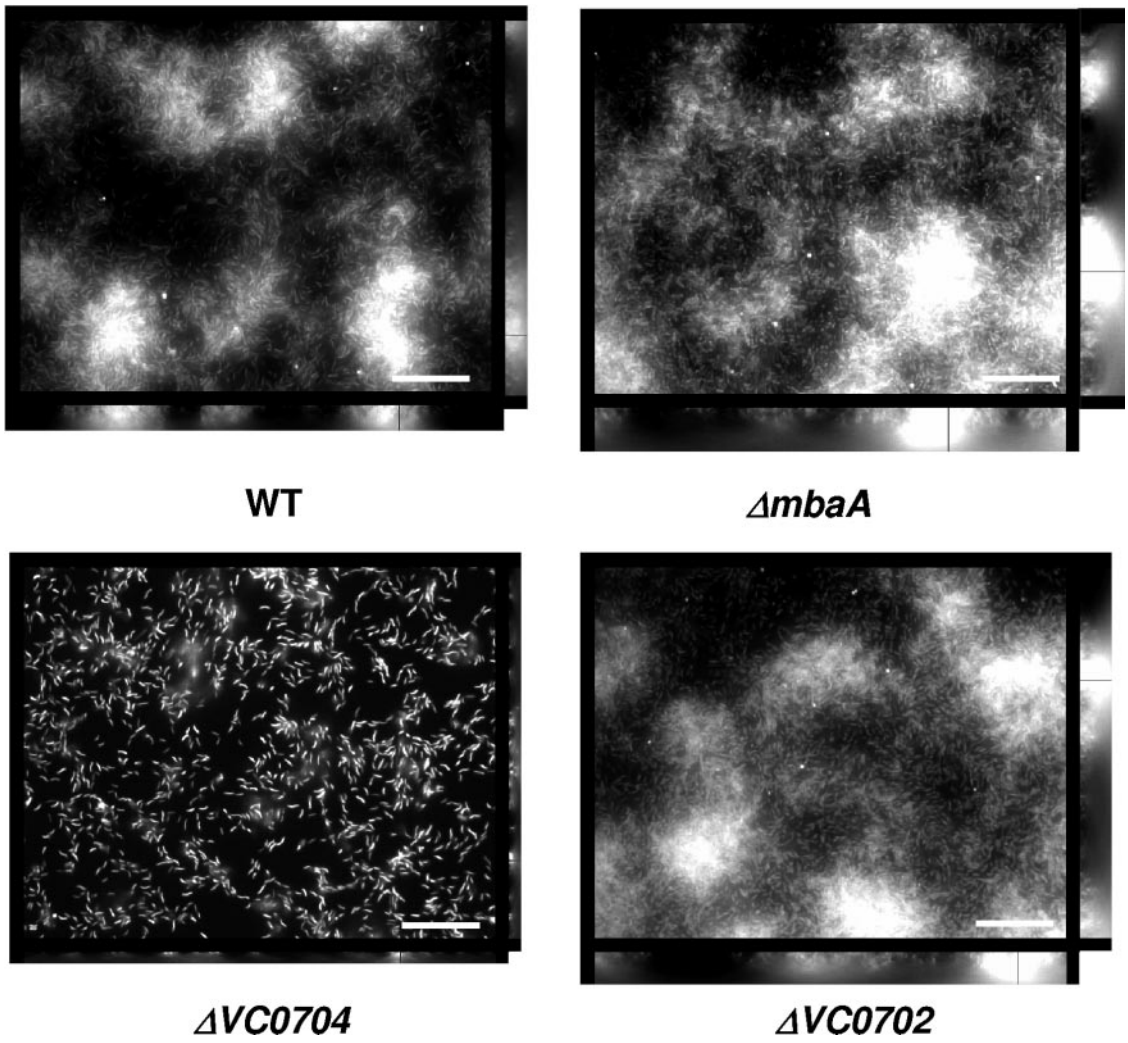

FIG. 3. Architecture of wild-type *V. cholerae* and mutant biofilms. Transverse and vertical cross sections through DAPI-stained wild-type *V. cholerae* (WT) as well as Δ*mbaA*, ΔVC0702, and ΔVC0704 mutant biofilms formed after overnight incubation in LB broth. The transverse sections were obtained at the level of the substratum. (Bar ≈ 10 μm.)

protein encoded at locus VC0704 is an activator of biofilm formation.

We hypothesized that the protein encoded at locus VC0704, a putative periplasmic protein, might interact with the periplasmic domain of MbaA to block the repression of biofilm formation by MbaA. We predicted that if this were the case, then the deletion of VC0704 would have no effect on biofilm accumulation in the absence of MbaA. To test this prediction, we constructed a Δ*mbaA*ΔVC0704 double mutant and quantified its ability to accumulate in a biofilm. As shown in Fig. 2, the Δ*mbaA*ΔVC0704 double mutant displayed a phenotype similar to that of the Δ*mbaA* mutant. This is consistent with the hypothesis that protein encoded at locus VC0704 modulates the regulation of biofilm development by MbaA.

Norspermidine enhances biofilm formation in a VC0704-dependent manner. Numerous studies have shown that *V. cholerae* biofilm formation is an environmentally regulated process. We hypothesized that the VC0704-encoded protein might serve as the ligand-binding sensory component of the MbaA regulatory system. In order to identify potential ligands for the VC0704-encoded protein through homologies with other bacterial proteins, a BLAST search was performed. This demonstrated 22% sequence identity and 44% similarity at the amino acid level between the VC0704-encoded protein and PotD, the periplasmic component of the *Escherichia coli* ABC-type spermidine transport system (Fig. 4). Furthermore, three amino acids that have been shown to be essential for spermidine binding by PotD (E171, W255, and D257) are conserved in the VC0704-encoded protein as E173, W261, and D263 (38).

We first tested the effect of spermidine, the ligand of PotD, on *V. cholerae* biofilm formation. In wild-type *V. cholerae* and the Δ*mbaA* mutant, the addition of exogenous spermidine at concentrations greater than or equal to 1 mM resulted in decreases in biofilm formation (data not shown). No effect was seen at lower concentrations of spermidine. We were unable to document an effect of spermidine on the ΔVC0704 mutant biofilm, most likely because the biofilm formed by this mutant is already quite minimal. The high exogenous concentration of

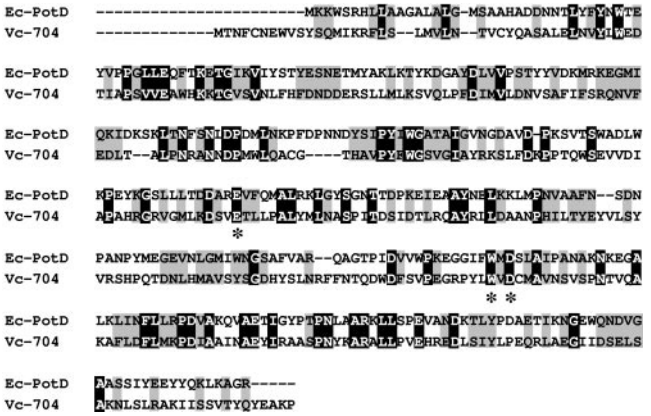

FIG. 4. Multiple sequence alignment of *E. coli* PotD and the protein encoded by *V. cholerae* VC0704. Identical residues are boxed in black, conserved residues are boxed in gray, and nonconserved residues are shown on a white background. The positions of three conserved amino acids (E173, W261, D263) are depicted with asterisks below the sequences. Sequence analysis and alignment was done using the online tools at Biology Workbench (http://workbench.sdsc.edu). Ec, *E. coli*; Vc, *V. cholerae*.

spermidine required to cause an effect on biofilm formation led us to hypothesize that a related polyamine might alter *V. cholerae* biofilm formation at lower concentrations. Because norspermidine is very similar to spermidine, differing only by one carbon residue, we tested the effect of norspermidine on *V. cholerae* biofilm formation. As shown in Fig. 5A, wild-type *V. cholerae* biofilm formation was enhanced at concentrations of norspermidine as low as 10 μM, and, at 100-μM concentrations, a threefold increase in biofilm formation was observed. In contrast, the addition of norspermidine to the growth medium had no effect on the biofilms formed by the ΔVC0704 and Δ*mbaA* mutants. We questioned whether norspermidine might affect biofilm formation by a Δ*mbaA* mutant at earlier times in biofilm development. To address this possibility, we quantified biofilm development by wild-type *V. cholerae* and a Δ*mbaA* mutant in the presence and absence of 10 μM norspermidine after 6, 8, 10, and 12 h of incubation (Fig. 5B). In these experiments, the activation of wild-type *V. cholerae* biofilm formation by norspermidine was observed after only 8 h. In contrast, even at early times, norspermidine had no effect on biofilm formation by the Δ*mbaA* mutant. Planktonic cell densities were unaffected by the addition of norspermidine in all the experiments performed (data not shown). These results suggest that the protein encoded at locus VC0704 specifically binds norspermidine and that this interaction results in the activation of biofilm formation. For this reason, we have named the gene at locus VC0704 *nspS* (for *n*ors*p*ermidine *s*ensor). Furthermore, because norspermidine had no effect on the biofilm formed by Δ*mbaA*, we hypothesize that MbaA also forms part of this regulatory pathway.

Even in LB broth alone, the NspS mutant biofilm was decreased in comparison to the wild-type biofilm. One possibility is that LB broth itself may contain enough norspermidine to activate this signal transduction pathway. Alternatively, while norspermidine may enhance the function of NspS in activating biofilm formation, it is possible that NspS has a basal level of function even in the absence of norspermidine.

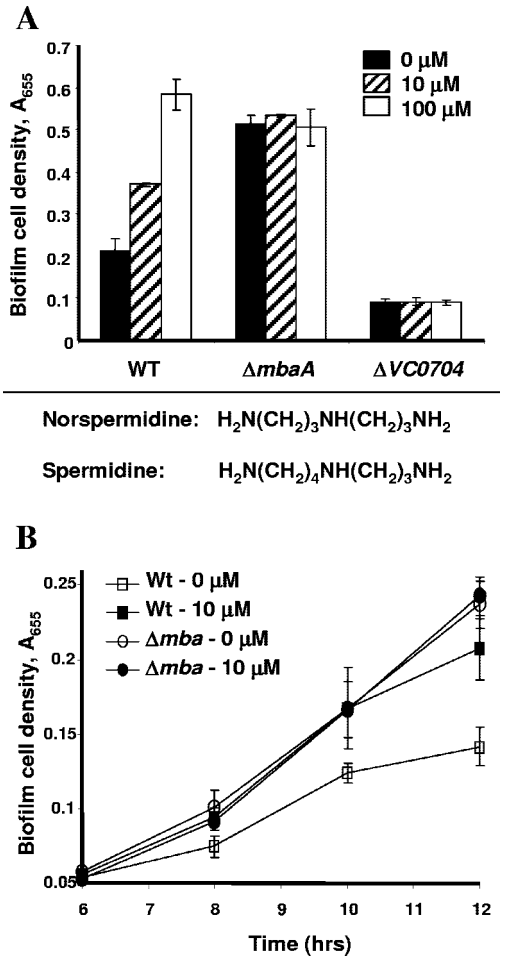

FIG. 5. Effect of norspermidine on biofilm cell densities. A. Effect of norspermidine on surface accumulation by wild-type *V. cholerae* (WT) as well as on that by Δ*mbaA* and ΔVC0704 mutants. Biofilms were formed in borosilicate tubes in LB broth for 20 h with indicated quantities of norspermidine and quantified as described in Materials and Methods. Error bars show standard deviations of three replicates. The chemical formulas of norspermidine and spermidine are shown below the graph. B. Time course of wild-type *V. cholerae* and Δ*mbaA* mutant biofilm formation in the presence and absence of norspermidine. Wild-type or Δ*mbaA* mutant biofilms were formed in borosilicate tubes in LB broth alone or supplemented with 10 μM norspermidine and quantified after 6, 8, 10, or 12 h. Error bars show standard deviations of three replicates.

Modulation of *vps* gene transcription contributes to the biofilm phenotypes of the Δ*mbaA* and Δ*nspS* mutants. Wild-type *V. cholerae* biofilm formation in freshwater, monosaccharide-supplemented minimal medium, and LB is dependent on *vps* gene transcription (40, 52). Furthermore, most transcriptional regulators that alter *V. cholerae* biofilm formation effect this change through the regulation of *vps* gene transcription (4, 24, 25). Thus, we hypothesized that Δ*mbaA* and Δ*nspS* mutants would also show altered levels of *vps* gene transcription. To test this hypothesis, Δ*mbaA* and Δ*nspS* mutations were inserted into the chromosome of a previously constructed *V. cholerae* strain carrying a chromosomal promoter fusion of the *vpsL* operon to the *E. coli lacZ* gene, and transcription at the *vpsL* promoter was measured in the resulting strains (25). As shown

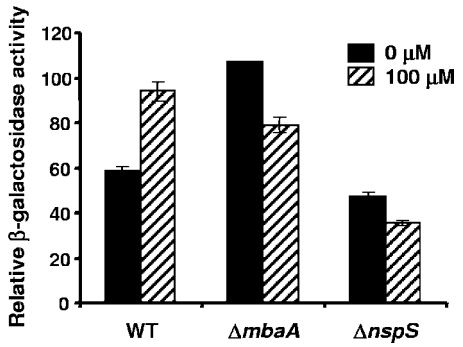

FIG. 6. *vpsL* transcription in wild-type and mutant *V. cholerae* strains. Wild-type *V. cholerae* (WT) as well as Δ*mbaA* and Δ*nspS* mutants containing a chromosomal fusion of the *E. coli lacZ* gene to the *vpsL* promoter were grown to logarithmic phase with shaking in the absence (black bars) or presence (striped bars) of 100 μM norspermidine. β-Galactosidase measurements were done on the planktonic cultures as described in Materials and Methods. Error bars show standard deviations.

in Fig. 6, the level of *vpsL* transcription in the Δ*mbaA* mutant was twice that observed in wild-type *V. cholerae*. The level of *vpsL* transcription in the Δ*nspS* mutant showed a small but statistically significant decrease ($P = 0.01$). This result is consistent with our hypothesis that Δ*nspS* and Δ*mbaA* mutants have altered *vps* gene transcription levels. The addition of norspermidine increased *vpsL* transcription in wild-type *V. cholerae* almost to the level observed in the Δ*mbaA* mutant. This result suggests that norspermidine interacts with NspS to relieve the repression of *vpsL* transcription by MbaA. Interestingly, norspermidine repressed *vpsL* transcription in the Δ*mbaA* and Δ*nspS* mutants.

The NspS-MbaA regulatory pathway specifically regulates *V. cholerae* biofilm formation. Proteins in the GGDEF and EAL protein families, such as MbaA, have been shown to affect intracellular levels of c-di-GMP, which is thought to be a secondary messenger (35). Secondary messengers often have global effects on gene transcription. Thus, we questioned whether other *V. cholerae* genes might be regulated by MbaA. To identify additional genes regulated by MbaA, we compared the whole-genome transcriptional profiles of wild-type *V. cholerae* and of a Δ*mbaA* mutant grown to logarithmic phase in shaking cultures. Data were collected from four separate microarray experiments on RNA extracted on different days (four technical replicates). In an analysis of the microarray data, the gene transcription in the Δ*mbaA* mutant was used as the test and the gene transcription in wild-type *V. cholerae* was used as the reference. We identified 23 genes with 1.5-fold-or-greater increases in transcription in the Δ*mbaA* mutant and 6 genes with 1.5-fold-or-greater decreases in transcription (Table 3). As predicted from our *vpsL* reporter fusion experiments, 14 of the genes demonstrating transcriptional activation in the Δ*mbaA* mutant were located in or between the two *vps* operons. Genes at loci VC1585 (*katB*), VC1888, VCA0849, and VCA0952 (*vpsT*), whose transcriptions were also found to be increased in previous microarray studies of a *V. cholerae* rugose-phase variant (76), demonstrated increased transcription in the Δ*mbaA* mutant. VC0930 and VC1888, both of

TABLE 3. Genes differentially regulated in Δ*mbaA* relative to wild-type *V. cholerae*

Locus (gene name[s])[a]	Predicted function	Fold change
VC0916	Phosphotyrosine protein phosphatase	1.7–5
VC0918 (*vpsB*, *epsD*)	UDP-*N*-acetyl-D-mannosaminuronic acid dehydrogenase	2.7–5.4
VC0919 (*vpsC*)	Serine acetyltransferase-related protein	1.6–6.3
VC0921 (*vpsE*)	Polysaccharide export protein, putative	1.7–4.8
VC0922* (*vpsF*)	Hypothetical protein	1.5–4
VC0926 (*vpsJ*)	Hypothetical protein	1.5–5.6
VC0927 (*vpsK*, *cpsF*)	UDP-*N*-acetyl-D-mannosamine transferase	1.5–6.1
VC0928*	Hypothetical protein	1.9–6.2
VC0930*	Hemolysin-related protein	2.1–2.6
VC0932*	Hypothetical protein	1.5–15.6
VC0933*	Hypothetical protein	1.8–16.2
VC0934 (*vpsL*)	Capsular polysaccharide biosynthesis glycosyltransferase, putative	1.5–7
VC0935* (*vpsM*)	Hypothetical protein	2.1–18.6
VC0936 (*vpsN*)	Polysaccharide export-related protein	2.2–6.1
VC1216	GGDEF family protein, heme-erythrin domain	1.6–2.2
VC1585 (*katB*)	Catalase	2–2.5
VC1888*	Hemolysin-related protein	1.8–6.4
VC2134 (*fliE*)	Flagellar hook-basal body complex protein FliE	1.7–2.3
VC2455	Hypothetical protein	1.5–1.7
VC2456	Hypothetical protein	1.8–2.2
VCA0470	Acetyltransferase, putative	1.6–2.4
VCA0480*	Hypothetical protein	1.5–2.1
VCA0849a	Hypothetical protein, bp 6531–9792	2.1–2.7
VCA0952* (*vpsT*)	Transcriptional regulator, LuxR family	1.5–5.5
VC2046	Hypothetical protein	0.4–0.49
VC2735	Conserved hypothetical protein	0.53–0.64
VCA0188	Hypothetical protein	0.55–0.65
VCA0689	Conserved hypothetical protein	0.51–0.57
VCA0734	Hypothetical protein	0.39–0.6

[a] *, differentially regulated in all four experiments.

which demonstrated transcriptional activation in our microarray study, are annotated as encoding putative hemolysins. Because these genes encode secreted proteins, we hypothesize that they may contribute to the formation of the biofilm matrix. VC1585, which is 59% identical and 72% similar to the *P. aeruginosa katB* gene, was also activated in our microarray studies. The *P. aeruginosa katB* gene has been shown to be induced in biofilms (17). In this work, the catalase KatA was shown to be mainly responsible for the resistance of *P. aeruginosa* biofilms to H_2O_2. Because *V. cholerae* does not appear to encode KatA, we propose that KatB may account for the resistance of the rugose *V. cholerae* variant to H_2O_2 (69, 77). Two response regulators, VpsR and VpsT, regulate biofilm formation (4, 75). Transcription of *vpsT* but not of *vpsR* was found to be increased in the Δ*mbaA* mutant. VC1216, a gene predicted to encode a GGDEF protein with an N-terminal hemerythrin histidine-histidine-glutamate cation-binding domain, was also upregulated in the Δ*mbaA* mutant. These domains, which usually bind oxygen, have been shown to be important when low oxygen concentrations are encountered (66). The induction of VC1216 suggests that low oxygen tensions in the environment may also direct biofilm formation. The majority of genes identified in our microarray study had increased transcription levels. Furthermore, approximately 84% of these genes are likely to be directly involved in biofilm formation. Thus, we conclude that the NspS-MbaA regulatory pathway does not have global effects on gene transcription but more likely is specifically regulating *V. cholerae* biofilm formation.

DISCUSSION

In bacteria, changes in gene transcription are usually the result of the sensing of a signal in the environment. The genome of *V. cholerae* codes for 31 proteins with GGDEF domains, 12 proteins with EAL domains, and 10 that contain both GGDEF and EAL domains. More than half of these are linked to sensory or signaling domains. Despite the growing number of functionally characterized proteins in the GGDEF and EAL families, the signals that they process remain largely unknown.

In previous work, Bomchil and colleagues demonstrated that the protein MbaA is a repressor of *V. cholerae* biofilm formation (3). However, the environmental signal and the sensory component of the MbaA signal transduction system were not identified. In this work, we have identified norspermidine as the environmental signal sensed by the MbaA regulatory cascade. Furthermore, we present evidence that the protein NspS is the sensory component of this signal transduction system. We propose that NspS interacts with the periplasmic domain of MbaA to regulate its enzymatic activity and that this interaction is modulated by binding of norspermidine to NspS. MbaA contains both GGDEF and EAL domains. Both of these domains have been shown to dimerize, and dimerization is thought to be required for the activity of the GGDEF domain (2, 5). Interestingly, PotD, a protein that is homologous to NspS, forms a dimer that is disrupted in the presence of its ligand, spermidine (62). Thus, we hypothesize that one possible mechanism by which the norspermidine-NspS complex decreases the repression of biofilm formation by MbaA may be through the destabilization of the MbaA dimer. Alternatively, the binding of norspermidine to NspS may induce a conformational change in MbaA that reduces its function. Experiments to test these various possibilities directly are under way.

We have also demonstrated that exogenous norspermidine results in an increase of *vpsL* gene expression in wild-type *V. cholerae* but a decrease in *vpsL* gene expression in the absence of NspS or MbaA. We hypothesize that norspermidine affects biofilm formation through multiple pathways. While the NspS-MbaA pathway is dominant in wild-type *V. cholerae*, alternative pathways may predominate in the absence of a functional NspS-MbaA pathway.

Polyamines such as spermidine, putrescine, and spermine are abundant in nature, and their roles in a wide variety of cellular processes have been studied extensively. Polyamines have been previously shown to act as intercellular signaling molecules both in prokaryotes and eukaryotes. For example, putrescine, a diamine, can act as an extracellular signal that is required for the swarming cell differentiation and migration ability of *Proteus mirabilis* (61). In eukaryotes, extracellular polyamines can increase fluid secretion in rat distal colonic crypts via their interactions with the calcium-sensing receptors, which in turn affects levels of cyclic secondary messengers in the cell (8). Furthermore, the intestinal polyamine pool, which may be modulated by diet and the commensal bacterial population, affects epithelial cell development and apoptosis (44, 48). Norspermidine has also been shown to have inhibitory effects on immunoglobulin M production by lipopolysaccharide-stimulated murine splenocytes by interfering with the polyamine metabolism pathway (42). Thus, commensal or pathogenic gut bacteria may influence gut epithelial development and immunity through the synthesis and export of polyamines.

V. cholerae contains very small amounts of spermidine, a polyamine found in species as diverse as humans, yeast (*Saccharomyces cerevisiae*), and *E. coli*. Instead, the major polyamine in *Vibrio* species is norspermidine (74). The genes for norspermidine synthesis are present in the genomes of *V. cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* as well as in those of many other members of the proteobacteria and of a few members of the deinococci, bacteroidetes, cyanobacteria, and gram-positive bacteria (7, 27, 47). Furthermore, norspermidine has been identified as a major polyamine in a number of species of archaea and thermophilic bacteria (19, 20, 30). Norspermidine has also been identified as a major polyamine in a number of aquatic plants and in other aquatic organisms such as algae, bivalve mollusks, sea urchins, and sea cucumbers as well as in terrestrial plants and insects (18, 21–23). These studies suggest that norspermidine is present in a wide range of prokaryotes and eukaryotes.

Relatively little is known about the role of norspermidine in *Vibrio* species. Vibriobactin and vulnibactin, the iron-scavenging molecules of *V. cholerae* and *V. vulnificus*, respectively, contain norspermidine, suggesting an essential role for this molecule in iron-limiting environments (39, 55). It is also likely that norspermidine is essential for normal growth in these organisms, as is the case for spermidine or spermine in *Saccharomyces cerevisiae* (1).

Our results suggest that norspermidine promotes *V. cholerae* surface accumulation. In seawater, the concentration of norspermidine is in the nanomolar range (54). Therefore, global

pools of norspermidine in the marine environment would not be sufficient to promote the association of *V. cholerae* with biotic or abiotic surfaces. However, it is likely that *V. cholerae* associates with species of bacteria, archaea, plants, and animals that maintain norspermidine gradients at their surfaces. Thus, we suggest that polyamines serve as a form of communication that enhances close associations between *V. cholerae* and its prokaryotic and eukaryotic neighbors.

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