Appalachian
STATE UNIVERSITY®
BOONE, NORTH CAROLINA

Elevated Levels Of The Norspermidine Synthesis Enzyme Nspc Enhance Vibrio Cholerae Biofilm Formation Without Affecting Intracellular Norspermidine Concentrations

By: Zachary M. Parker, Samuel S. Pendergraft, Jim Sobieraj, Marcus M. McGinnis & **Ece Karatan**

Abstract
Biofilm formation in Vibrio cholerae is in part regulated by norspermidine, a polyamine synthesized by the enzyme carboxynorspermidine decarboxylase (NspC). The absence of norspermidine in the cell leads to a marked reduction in V. cholerae biofilm formation by an unknown mechanism. In this work, we show that overexpression of nspC results in large increases in biofilm formation and vps gene expression as well as a significant decrease in motility. Interestingly, increased NspC levels do not lead to increased concentrations of norspermidine in the cell. Our results show that NspC levels inversely regulate biofilm and motility and implicate the presence of an effective feedback mechanism maintaining norspermidine homeostasis in V. cholerae. Moreover, we provide evidence that NspC and the norspermidine sensor protein, NspS, provide independent and distinct inputs into the biofilm regulatory network.

INTRODUCTION

Vibrio cholerae, the causative agent of the severe diarrheal
disease cholera, is a natural inhabitant of aquatic environments,
where it is believed to exist predominantly in biofilms
(Colwell & Huq, 1994; Colwell et al., 2003). Bacteria
in biofilms are more tolerant of environmental stresses
such as pH changes, UV irradiation, and antibiotics (Costerton
et al., 1995). Vibrio cholerae biofilm formation is
enhanced by bile acids, which are normally antibacterial
(Hung et al., 2006). In addition, growth in a biofilm has
recently been shown to induce a 'hyperinfectious phenotype'
in V. cholerae (Tamayo et al., 2010). Thus, formation
of a biofilm affords V. cholerae a survival advantage
both in its natural environment and in the host.

Biofilm formation is tightly regulated by numerous environmental
signals. One group of signals, polyamines, regulate
biofilm formation by a variety of bacteria including V.
cholerae, Yersinia pestis, and Bacillus subtilis (Karatan et al.,
2005; Patel et al., 2006; Lee et al., 2009; McGinnis et al.,
2009; Burrell et al., 2010). Polyamines are short hydrocarbon
chains containing two or more amine groups that are
positively charged at physiological pH. They are ubiquitous
molecules synthesized by virtually all organisms and are
essential for the normal growth of most prokaryotes and
eukaryotes (Tabor & Tabor, 1984).

For V. cholerae, the triamine norspermidine is a
positive signal for biofilm formation. Norspermidine is
synthesized by decarboxylation of carboxynorspermidine
by the enzyme carboxynorspermidine decarboxylase
encoded by the nspC gene (Lee et al., 2009). Maintaining
adequate levels of norspermidine in the cell is important
for V. cholerae biofilm formation as inhibition of norspermidine
biosynthesis severely hinders this process (Lee
et al., 2009). Exogenous norspermidine can also enhance
V. cholerae biofilm formation by a different mechanism
involving the periplasmic norspermidine sensor NspS.
NspS is hypothesized to interact with the GGDEF-EAL
family protein MbaA and regulate V. cholerae biofilm
formation in response to environmental norspermidine
(Karatan et al., 2005).

The purpose of the current study was to gain more
insight into how norspermidine and norspermidine synthesis

pathways regulate V. cholerae biofilm formation. We overexpressed the nspC gene and determined the effect of the increased levels of the NspC protein on biofilm formation, exopolysaccharide gene expression, motility, and cellular and extracellular polyamine levels in V. cholerae O139.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and reagents

The bacterial strains, plasmids, and primers used are listed in Table 1. Vibrio cholerae serotype O139 strain MO10 was used for all experiments. Experiments were conducted in Luria–Bertani (LB) media containing 100 μg mL^{-1} streptomycin and 2.5 μg mL-^{1} tetracycline. Primers were purchased from Eurogentec (San Diego, CA) or Eurofins MWG Operon (Huntsville, AL).

Cloning of the nspC gene

The nspC gene was amplified from chromosomal DNA using primers that annealed 40 bp upstream and 177 bp downstream of the coding sequence. Following amplification, the nspC gene was first cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and then subcloned into plasmid pACYC184 after verification of the sequence (Biotechnology Resource Center, Cornell University, Ithaca, NY).

Growth, biofilm, b-galactosidase, and motility assays

For assessing growth characteristics, overnight cultures were inoculated into fresh media at an OD$_{595nm}$ of 0.02. Cultures were divided into twelve 1-ml aliquots and incubated at 27 °C with shaking for 24 h. Samples were taken for 24 h at indicated intervals, and OD$_{595nm}$ of 100 uL of the cell suspension was measured in a microtiter plate. Growth rates were measured using the slopes of the trend lines fitted to data at exponential phase as described before using the formula: rate constant (k) = slope/0.301 (Slonczewski & Foster, 2011). Biofilm assays were performed

as described in the study of (Karatan et al., 2005). To determine vpsL promoter activity, 200 μL of stationary or one ml of exponential phase cultures grown at 27 °C were pelleted, washed once with Z buffer (Miller, 1992), and resuspended in 200 μL of Z-buffer. Protease inhibitors and Ortho-nitrophenyl-b-D-galactopyranoside were added to each lysate and incubated at 37 °C for 2 h. β-galactosidase activity was determined by measuring the A_{415nm}. To assess motility, three isolated colonies were stabbed on semi-soft LB-agar plates (0.3% agar). The swarm diameters were measured after incubation at 27 °C for 24 h. All assays were repeated multiple times to confirm reproducibility of the results.

Extraction, benzoylation, and detection of Polyamines

Extraction of polyamines from shaking cultures were performed as previously described (McGinnis et al., 2009). To extract cellular polyamines from biofilm cultures, biofilms were formed in glass bottles in 20 mL LB for 24 h, and planktonic cells were removed and pelleted. Biofilms were washed once with PBS, biofilm-associated cells were dispersed in 10 mL PBS by vortexing with 1-mm glass beads (Bartlesville, OK), and the cells were pelleted by centrifugation. Planktonic and biofilm-associated cells were resuspended in 10 μL of water per mg wet weight. Cells were lysed by sonication, cell debris was removed by centrifugation, and cellular protein was precipitated by the addition of trichloroacetic acid. The supernatant containing the polyamines were used for benzoylation. In addition, 500 μL of the conditioned media was set aside for benzoylation for all culture conditions. A standard mix containing 0.1 mM each of putrescine, diaminopropane, cadaverine, norspermidine, and spermidine was also prepared every time polyamines were quantified. Benzoylation procedure was performed as described previously (Morgan, 1998). Benzoylated polyamines were extracted with chloroform, evaporated to dryness, and dissolved in 100 μL of a 60% methanol and 40% water solution. HPLC was conducted using a Waters 1525 Binary Pump with a 2487 Dual Wavelength Absorbance Detector and a Waters Spherisorb ODS2 column (5 μm, 250 9 4.6 mm), fitted with a 50 9 4.6 mm guard cartridge (Waters Corporation, Milford, MA). The runs were performed using

a gradient of 45–60% methanol in water over a 30-min period followed by a 10-min isocratic re-equilibration step at 45% methanol in water.

Table 1. Bacterial strains, plasmids, and primers

Strains	Genotype	Reference/source
E. coli		
DH5α	F-ø80*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR, recA1, phoA, endA1, hsdR*17(rk2, mk+), *supE44, thi*-1, *gyrA*96, *relA*1, λ-	Invitrogen
V. cholerae		
PW249	MO10, clinical isolate of *V. cholerae* O139 from India, Sm^R	Waldor & Mekalanos (1994)
PW357	MO10*lacZ::vpsLp→lacZ*, Sm^R	Haugo & Watnick (2002)
AK083	PW249 with p*nspC*	This study
AK103	PW249 with pACYC184	This study
AK166	PW357 with p*nspC*	This study
AK169	PW357 with pACYC184	This study
AK089	PW357Δ*nspS* with p*nspC*	This study
AK007	PW357Δ*nspS* with pACYC184	This study
Plasmids	**Description**	**Reference/source**
pCR2.1-TOPO	Plasmid for TOPO cloning, Ap^R	Invitrogen
pACYC184	Tet^R, Cm^R	(Chang & Cohen, 1978)
p*nspC*	pACYC184::*nspC*	This study
Primer	**Description**	**Sequence**
Construction of *nspC* overexpression plasmid		
PA18	Forward primer for *nspC* amplification	5'-CCATTCTTGTTATCGAGATATTTG-3'
PA19	Reverse primer for *nspC* amplification	5'-CGACACTTCCTATGTAATCAC-3'
Analysis of *vpsL* gene transcription		
PA142	Forward primer for *vpsL*	5'-CGCACCATAGTGAATCGCTACAT-3'
PA143	Reverse primer for *vpsL*	5'-ATTGATCTGTGCCCATCCAGTA-3'
PA80	Forward primer for *rpoB*	5'-GCCGAGATCCTGGACATCTTCTT-3'
PA81	Reverse primer for *rpoB*	5'-CGCAGTTTCACCACGCAGAC-3'

Antibody production and Western blot

To produce antibodies against the NspC protein, the peptide GYDVEKLGAALKAFAERH corresponding to the amino acids 221–238 was synthesized and conjugated to KLH by Biosynthesis Inc. (Lewisville, TX). A male New Zealand White rabbit was immunized with an emulsion of 0.5 mL of a 2 mg mL^{-1} solution of the peptide in PBS and 0.5 mL of Freund's Complete Adjuvant (Sigma) and boosted on weeks 3, 5, 7, 9, and 11. The animal was sacrificed at week 11, and the serum was used directly for the Western blots. This procedure was approved by the Appalachian State University IACUC committee (protocol number 07-3). For Western blotting, the serum and the horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were used at a 1 : 1000 and 1 : 10000 dilutions, respectively. ECL Plus chemiluminescent (GE Healthcare, Piscataway, NJ) or Super Signal West Femto

(Thermo Fisher Scientific, Rockford, IL) HRP substrates were used for detection, and the X-ray films were developed manually.

RNA extraction, cDNA synthesis, and quantitative real-time PCR

Total RNA was extracted from 5 mL of cells (AK 083 and AK103) grown to mid-log phase using Ambion Ribo-Pure™-Bacteria kit (Applied Biosystems, Foster City, CA) and treated with DNase I for 2 h at 37 °C. One microgram of this RNA was reverse-transcribed using Protoscript® First Strand cDNA Synthesis kit (NEB, Ipswich, MA) with random primers. The cDNA from two biological replicates was then used in quantitative realtime PCR (qRT-PCR) using gene-specific primers and SYBR Green PCR Master Mix (Applied Biosystems). Reactions were performed in triplicate. All PCR efficiencies were tested using a log dilution curve and were 100% efficient ±10%. All qRT-PCR products were checked for accuracy using melt curve analysis. Data were analyzed using the relative expression software program, REST, which incorporates randomization and bootstrapping algorithms to analyze real-time quantitative PCR data (Pfaffl et al., 2002, available as freeware from www.qiagen.com). The rpoB gene encoding the RNA polymerase beta subunit was used as internal control (Quinones et al., 2005).

Statistical analysis

A minimum of three biological replicates were performed for all experiments (unless otherwise noted) to ensure reproducibility of the results. Data were analyzed using Student's t-test (two-tailed, unpaired, unless otherwise noted) and differences were deemed statistically significant for P-values of 0.05 and below.

RESULTS

High levels of NspC enhances growth, biofilm formation, and vps gene transcription and decreases motility

Deletion of the nspC gene has been shown to be deleterious to biofilm formation in V. cholerae O1 El Tor (Lee et al., 2009). The inhibition of biofilm formation was attributed to the lack of norspermidine in the cell; however, the mechanism of this effect was not elucidated. Our repeated attempts to delete the nspC gene in V. cholerae O139 proved unsuccessful; therefore, we overexpressed the nspC gene from a multicopy plasmid (pACYC184::nspC, hereafter referred to as pnspC) to gain more insight into regulation of biofilm formation by polyamine synthesis pathways. We first confirmed the increased amounts of the NspC protein in this strain (Fig. 1a); however, under these conditions, we were not able to detect NspC in cells that did not overexpress this protein. To detect wild-type levels of NspC, we had to use a more sensitive detection system, which allowed us to visualize the NspC protein in cells that did not contain the pnspC plasmid. This result ensured that NspC was being expressed from its chromosomal location under our experimental conditions (Supporting Information, Fig. S1). We then assayed the effect of elevated NspC levels on various aspects of V. cholerae physiology. The presence of pnspC altered growth characteristics of the cells such that the lag time was much shorter, the growth rate 1.5-fold higher, and the cell density at stationary phase also higher (Fig. S2). Thus, the presence of pnspC appears to impart a growth advantage to V. cholerae under the conditions of our experiment.

Biofilm assays showed that increased production of NspC resulted in an approximately fivefold increase in biofilm cell density (Fig. 1b). This result is in contrast to a previous study which reported an inhibitory effect of ectopic expression of nspC on biofilms formed by V. cholerae O1 El Tor (Lee et al., 2009). The reasons for this disagreement are not known but can potentially be a result of different genetic backgrounds or plasmid systems used in these experiments. Planktonic cell density showed a very small but statistically significant reduction in the strain containing the pnspC plasmid. In most cases, strains that have a high propensity to form biofilms show reduced densities of planktonic cells. The fact that we did not see a large reduction in planktonic cells overexpressing nspC may be accounted for by the fact that this strain can grow slightly faster and to higher cell densities.
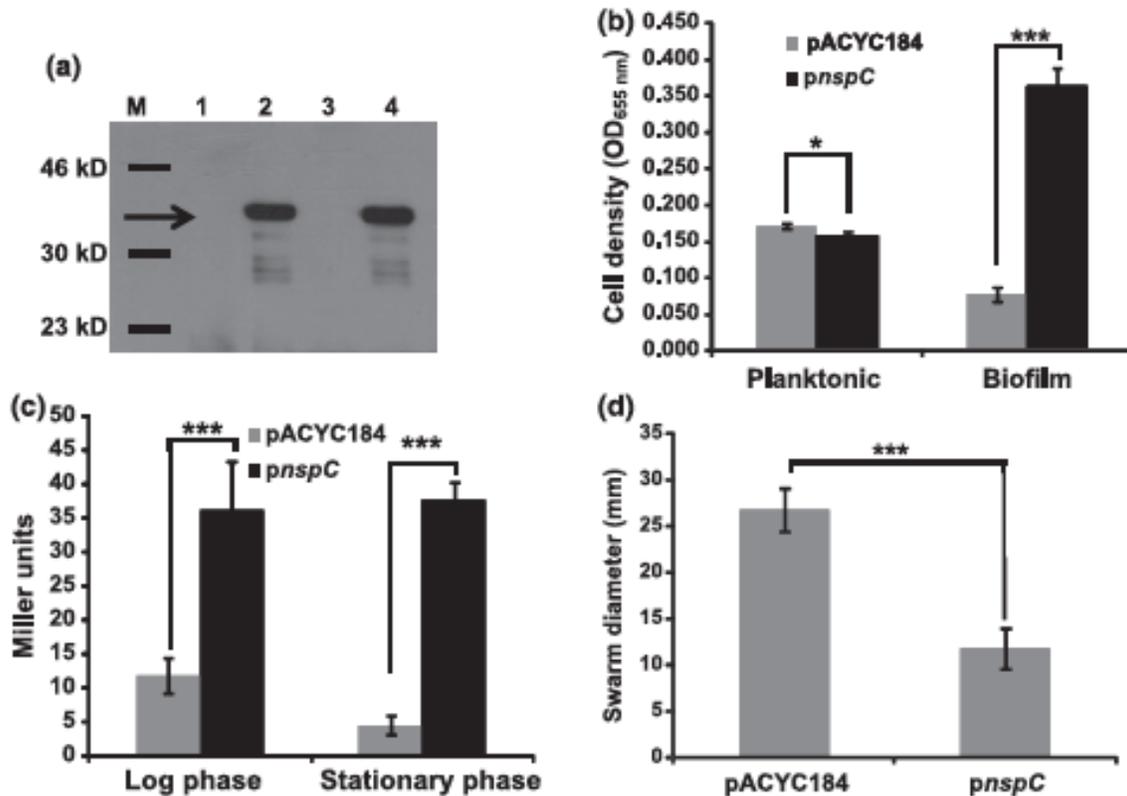
Fig. 1. Effect of nspC overexpression on NspC levels, biofilms, vps gene transcription, and motility. (a) Western blot. Whole-cell extracts were separated by SDS-PAGE, blotted on PVDF membrane, and detected with anti-NspC serum. Under the conditions of this experiment, the anti-NspC antibody is not able to detect endogenous levels of NspC, which is predicted to be 42 kD and is indicated by an arrow. M: marker, lane 1: wild-type with pACYC184; lane 2: wild-type with pnspC; lane 3: ΔnspS with pACYC184; ΔnspS with pnspC. (b) Biofilm assays. Biofilms were formed in borosilicate tubes in LB broth for 18 h and quantified as described in Materials and methods. Error bars show standard deviations of three replicates. (c) vps gene transcription. vpsL promoter activity was measured making use of a chromosomal vpsL-lacZ fusion in log and stationary-phase cells. Error bars show standard deviations of three biological replicates. (d) Motility. Motility assays were performed as described in Materials and methods. Error bars show standard deviations of three biological replicates. *P < 0.05; ***P < 0.0005.

Formation of biofilms usually requires the presence of an exopolysaccharide in the biofilm matrix whose synthesis and export is achieved by proteins encoded by the vps genes (Watnick & Kolter, 1999; Yildiz & Schoolnik, 1999). Under most conditions, increases in biofilm formation are accompanied by increases in vps gene transcription. These genes are found on the V. cholerae large chromosome in two operons: vpsA-K and vpsL-Q (Watnick & Kolter, 1999; Yildiz & Schoolnik, 1999). To test whether increased nspC gene expression also leads to an

increase in vps gene transcription, we assayed the activity of the vpsL promoter, making use of a chromosomal vpsLp-lacZ fusion in our strains (Haugo & Watnick, 2002). This insertion does not change the physiological characteristics of the wild-type bacteria such as growth, motility, and biofilm formation under the conditions of our experiments. Increased levels of the NspC protein resulted in a threefold and an eightfold increase in b-galactosidase activity in exponential and stationaryphase cells, respectively (Fig. 1c). We also confirmed the increase in the activity of the vpsL promoter using an independent assay; we quantified the levels of the vpsL gene transcript using quantitative real-time PCR. We found that vpsL mRNA levels were approximately 15-fold higher in the strain with increased NspC levels (Table S1). These results indicate that increased NspC levels affect biofilms through a vps-dependent mechanism. Moreover, because the measurements in these assays are normalized to cell density and an internal standard, respectively, they support the conclusion that the effect of NspC on biofilms is in addition to and independent of its effect on growth.

In most cases, biofilm formation and motility are inversely regulated such that signals or mutations that lead to increases in biofilm formation lead to decreases in motility (Watnick et al., 2001; Moorthy & Watnick, 2005). To determine the effect of increased NspC levels on motility, we performed motility assays using semisolid agar plates. Increased NspC levels led to a twofold decrease in the swarm diameter (Fig. 1d), indicating that increased NspC levels affect biofilms and motility in an inverse manner.

Increased NspC levels do not lead to increases in intracellular norspermidine production

Because decreases in intracellular norspermidine levels lead to a decrease in biofilm formation in V. cholerae O1 El Tor, we hypothesized that the increase in biofilm formation may be a consequence of increased levels of norspermidine in these cells. To test this hypothesis, we extracted and quantified the cellular polyamines from shaking cultures grown to log phase (Fig. 2a and b). Norspermidine levels did not increase in the strain overexpressing

nspC. Under the conditions of our experiment,
V. cholerae also contains significant amounts of putrescine,
diaminopropane, spermidine, and a small amount
of cadaverine. The levels of these polyamines were also
not different between the two strains. Next, we quantified
polyamines in the planktonic cells and biofilm-associated
cells of static biofilm cultures. Again, we observed no
differences in the levels of the various polyamines in the
two strains (Fig. 2c and d). However, cadaverine levels
were increased in both the planktonic and biofilmassociated
cells as compared to shaking cultures. These
results indicate that the increased biofilm levels seen in
this strain did not result from increased levels of norspermidine
or changes to levels of other polyamines in the
cells.

Norspermidine does not accumulate in the spent medium

We next hypothesized that cells could indeed produce
increased amounts of norspermidine as a result of
increased NspC levels; however, the excess norspermidine
might be exported to maintain norspermidine homeostasis
in the cell. To test this hypothesis, we quantified the
polyamines in the spent media of these strains as well as
sterile LB medium. We did not detect any norspermidine
in LB or the spent media. In addition, we found that the
spent medium of these strains contained putrescine, diaminopropane,
cadaverine, and spermidine; however, only
putrescine levels were higher in the spent media of either
of the strains as compared to LB, in shaking cultures
(Fig. 3). We next quantified polyamines in the spent
media of biofilm cultures (Fig. 3). Again, we did not
detect any norspermidine in the spent medium. Putrescine,
diamiopropane, and spermidine levels in the biofilm
spent media were similar to those of shaking cultures.
However, the spent media of the biofilm cultures contained
approximately 2 mM cadaverine, as compared to
about 3 μM cadaverine in the spent media of shaking
cultures. In the static biofilm cultures, both biofilm-associated
and planktonic cells can potentially contribute to
extracellular cadaverine levels; therefore, the increase in
cadaverine levels seen under these conditions can simply
be a result of contribution from higher numbers of cells.
Alternatively, the increase in cadaverine could reflect a

change in cellular physiology brought about by growth conditions used for the biofilm cultures. To differentiate between these two possibilities, we calculated the ratio of the cells in the biofilm cultures to that of shaking cultures. We found that the biofilm cultures contained only 1.5- to 2.5-fold more cells than shaking cultures (Table S2). We conclude that the approximately 600-fold increase in extracellular cadaverine levels observed in the biofilm cultures is predominantly a result of changes to cellular physiology.
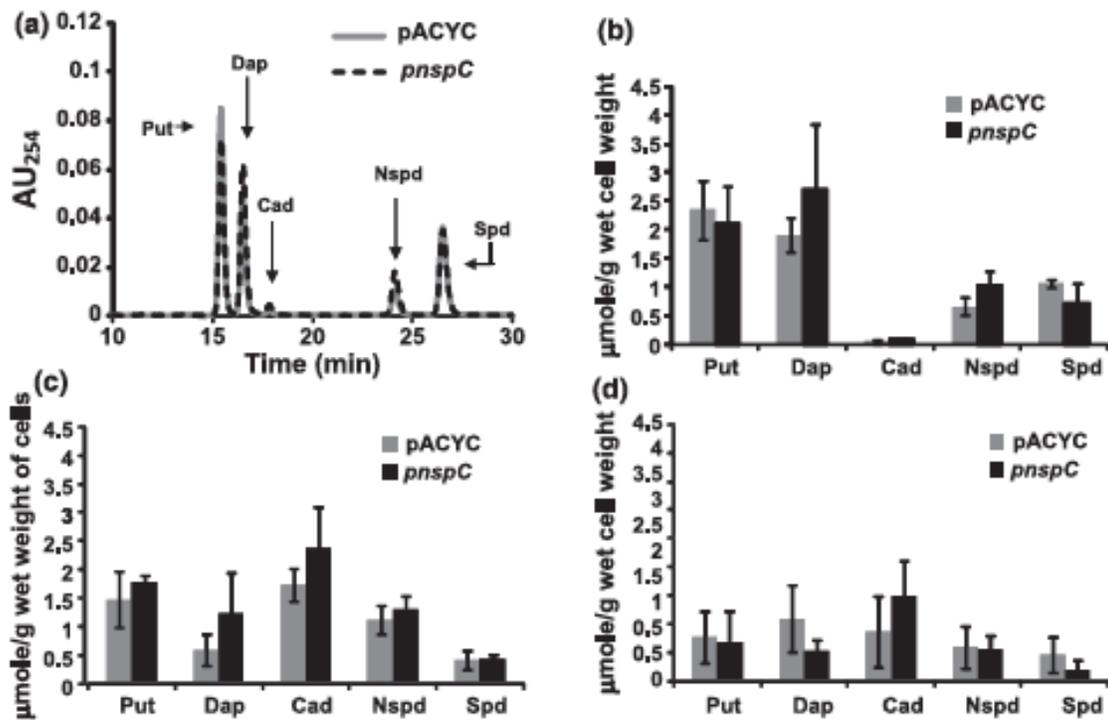
Fig. 2. Effect of nspC overexpression on cellular polyamine levels. Polyamines were extracted from cells, derivatized by benzoylation, and analyzed by HPLC as described in Materials and methods. (a) Polyamine composition of wild-type cells with pACYC184 or pnspC. Labeled peaks on the chromatogram correspond to putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd). AU254, absorbance units at 254 nm. Only 10–30 min of a 40-min run are plotted for clarity. (b, c) Quantification of cellular polyamines extracted from shaking cultures (b), planktonic cells (c), and biofilm-associated cells (d). Error bars indicate the standard deviation of three biological replicates.
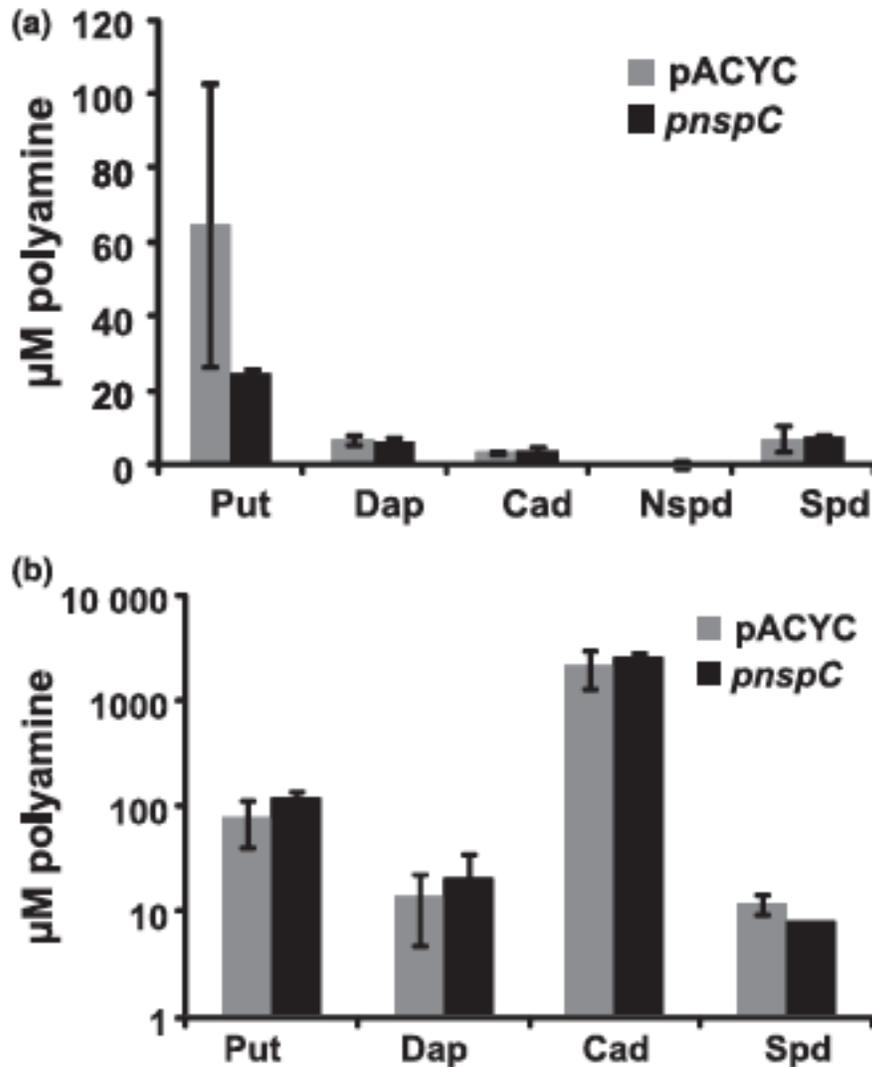
Fig. 3. Effect of nspC overexpression on extracellular polyamine levels. Polyamines were extracted from spent media of either shaking (a) or biofilm cultures (b), derivatized by benzoylation, and analyzed by HPLC as described in Materials and methods. Error bars indicate the standard deviation of three biological replicates. Putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd).

Biofilms have been shown to share some characteristics with stationary-phase cultures (Beloin & Ghigo, 2005; An & Parsek, 2007). To determine whether the increased extracellular cadaverine levels was a result of stationaryphase characteristics, we quantified polyamines in the spent medium of stationary-phase shaking cultures. We

found that the polyamine profiles of these media were very similar to that of log-phase cultures and contained very low levels of cadaverine, indicating that the increased cadaverine in the spent media of biofilm cultures is a specific response to growth in the biofilm (data not shown). Overall, these results show that the increase in biofilm cell density resulting from increased nspC levels is not a consequence of changes to the levels of these polyamines in the external environment.

The norspermidine sensor protein, NspS, is not required for the NspC-induced increase in biofilm cell density

We have previously demonstrated that exogenous norspermidine increases biofilm formation and that this increase is dependent on the presence of the protein NspS (Karatan et al., 2005). NspS and MbaA are thought to constitute a signaling system that regulates biofilm formation through their effect on local or global c-di-GMP pools in response to the polyamine norspermidine. NspS is a positive regulator of biofilms as ΔnspS mutants are significantly inhibited in biofilm formation. We wanted to determine whether the NspS-dependent norspermidine sensory pathway interacts with the norspermidine synthesis pathway to regulate biofilms. To do this, we transformed pnspC into a ΔnspS mutant and first confirmed the increased NspC levels in this strain (Fig. 1a, lanes 3 and 4, Fig. S1, lanes 2 and 4). Biofilm assays showed that overexpression of nspC in the DnspS strain did lead to a statistically significant increase in biofilm cell density (P < 0.001); however, this increase was only able to restore the biofilm defect of the ΔnspS strain to levels of the wild-type cells that did not overexpress nspC (Fig. 4a). Planktonic cell density was not affected. To determine whether vps gene transcription was also affected by increased NspC levels, we measured the activity of the vpsL promoter making use of a vpsL-lacZ chromosomal fusion in this strain. Increased NspC levels led to 4.7- and 2.5-fold higher β-galactosidase activity in log- and stationary-phase cells, respectively (Fig. 4b). To determine whether the increases in biofilm cell density and vps gene transcription could be explained by an effect on the intraor extracellular polyamine pools, we quantified the polyamines in these strains and the spent medium and found that increased levels of NspC did not lead to any alterations

in polyamine levels (Fig. 4c and d). These results indicate that NspS is not required for the stimulatory effect of increased NspC levels on biofilms and vps gene expression.
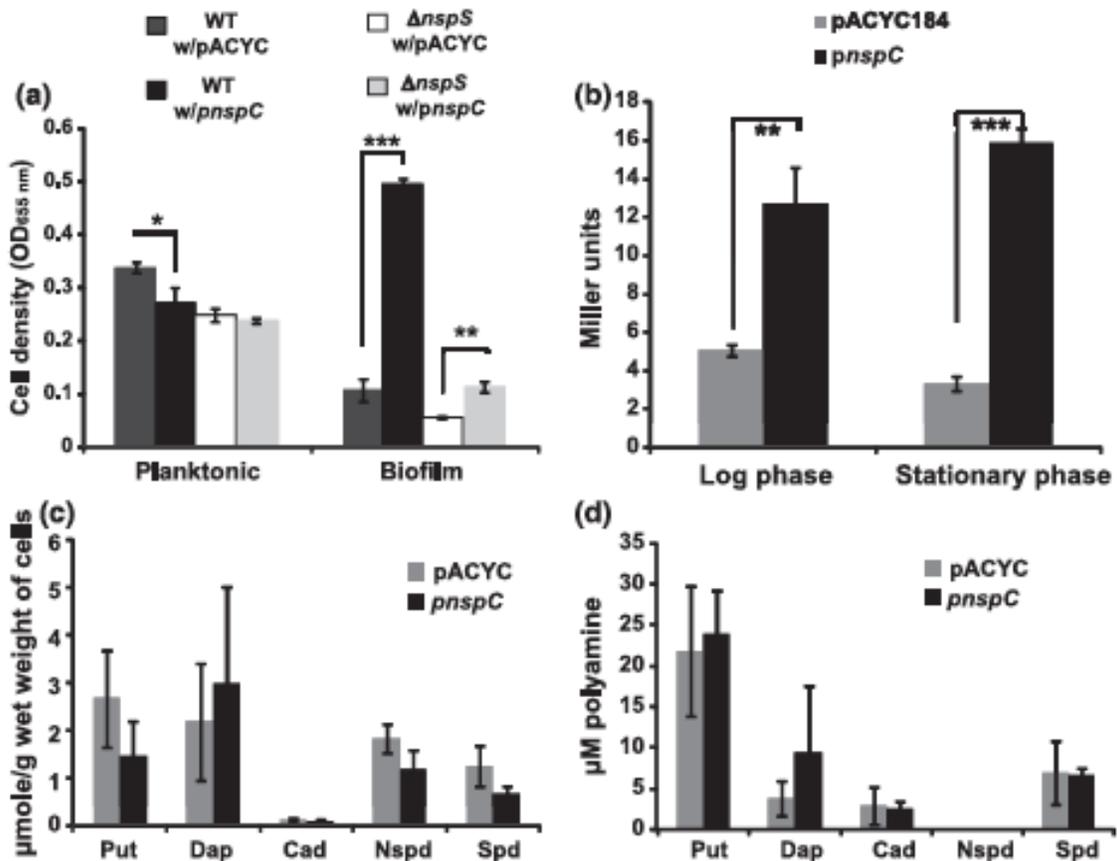

Fig. 4. Effect of nspC overexpression on biofilms, vps gene expression, and polyamine composition of a DnspS mutant. (a) Biofilm. Biofilms were formed in borosilicate tubes in LB broth for 18 h and quantified as described in Materials and methods. Error bars show standard deviations of six replicates. *P < 0.05; **P < 0.005; ***P < 0.0005. (b) vps gene transcription. vpsL promoter activity was measured making use of a chromosomal vpsL-lacZ fusion in log- and stationary-phase cells. Error bars show standard deviations of three biological replicates. **P < 0.005; ***P < 0.0005. (c, d) Quantification of cellular (c) and media polyamines (d). Polyamines were extracted from cells or the conditioned media, derivatized by benzoylation, and analyzed by HPLC as described in Materials and methods. Error bars show standard deviations of three biological replicates. Putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd).

DISCUSSION

In this work, we have demonstrated that increased levels of the enzyme NspC lead to a significant increase in biofilm formation in a vps-dependent manner in V. cholerae O139. In addition, increased NspC levels result in a decrease in motility, indicating that NspC levels have opposing effects on biofilms and motility. Norspermidine concentrations in the cells do not change in response to increased NspC levels. This finding corroborates previous studies on polyamine metabolism in other organisms; for example, overexpression of S-adenosylmethionine decarboxylase, which is involved in spermidine biosynthesis in plants, does not lead to changes in polyamine levels in the cell (Hanfrey et al., 2002). In both prokaryotes and eukaryotes, polyamine homeostasis is maintained by a variety of regulatory mechanisms including import, export, degradation, and interconversion of polyamines, feedback inhibition of polyamine synthesis enzymes by end products, and transcriptional regulation of genes encoding proteins involved in polyamine metabolism and transport (Persson, 2009; Igarashi & Kashiwagi, 2010). In Vibrio alginolyticus, norspermidine was shown to inhibit all three enzymes involved in the synthesis of norspermidine (Nakao et al., 1991). The V. cholerae and V. alginolyticus enzymes share approximately 82% amino acid sequence identity; therefore, it is likely that the V. cholerae enzymes are also regulated by feedback inhibition by norspermidine. Therefore, product feedback inhibition could contribute to maintaining norspermidine levels and partially account for the lack of an increase in cellular norspermidine levels in the nspC overexpression strain. It is also highly likely that limitations in the levels of the NspC substrate carboxynorspermidine could also prevent increased production of norspermidine. In addition, increases in the amount and activity of potential norspermidine-modifying/norspermidine-degrading enzymes such as acetyltransferases may be involved. Thus, multiple mechanisms are likely to contribute to maintaining intracellular norspermidine concentrations in response to increases in NspC levels.

We also quantified the polyamines in the spent medium of the various cultures to test the possibility that excess norspermidine might be transported out of the cell. We did not detect any norspermidine in any of the

samples, indicating that norspermidine is either not secreted out of the cell or secreted in a modified form, which might be undetectable by our methods. While the levels of intra- and extracellular polyamines did not change in response to increases in NspC, we did find a large increase in cellular cadaverine levels in biofilm cultures and a drastic increase in extracellular cadaverine levels in the spent media of biofilm cultures. While this finding does not explain why increased NspC levels lead to increases in biofilms, it indicates that cadaverine metabolism and export are likely to be regulated differently in biofilms. Increased cadaverine synthesis has been demonstrated in uropathogenic Esherichia coli in response to nitrosative stress; it is possible that increased cadaverine production seen in biofilms is a similar response to stress such as anaerobiosis (Bower & Mulvey, 2006).

The increase in the NspC levels appears to be responsible for signaling a positive environment for vps gene transcription and biofilm formation for V. cholerae O139. While the mechanism of this effect is unknown, one possible explanation may be that increased amounts of NspC sequester a biofilm inhibitory molecule, thereby relieving the repression on biofilm formation. A potential candidate for this molecule is spermidine. We have previously reported that reduction in intracellular spermidine levels leads to a large increase in biofilm formation (McGinnis et al., 2009). NspC can also use carboxyspermidine as a substrate and produce spermidine albeit at a much reduced rate (Nakao et al., 1991; Lee et al., 2009). In addition, spermidine has been shown to inhibit the specific activity of NspC, which shares 82% sequence identity with V. cholerae NspC, in V. alginolyticus (Nakao et al., 1991). It is possible that increased numbers of NspC protein can sequester free spermidine in the cell, leading to an increase in biofilm formation. Polyamines are known to modulate translation of proteins (Igarashi & Kashiwagi, 2010). In Y. pestis, putrescine enhances translation of the HmsHFRS proteins responsible for the synthesis of the polysaccharide component of the biofilm matrix (Wortham et al., 2010). In a similar way, spermidine can potentially affect the translation of VPS proteins either directly by associating with the mRNA or the translational machinery or indirectly by modulating translation of upstream effectors biofilm formation.

Finally, our experiments demonstrated that NspS is not required for the stimulatory effect of NspC on vps gene transcription and biofilms. However, in the absence of NspS, this effect is less pronounced. Conversely, the large reduction seen in biofilm formation in the nspS mutant with high NspC levels suggests that NspC is not required for the effect of NspS on biofilm formation. The fact that biofilm formation is maximal when both pathways are intact may imply a direct or an indirect interaction between these two pathways that enhance the effect of the other. One possible interaction could involve an autocrine-type signaling mechanism where a modified form of norspermidine is secreted by V. cholerae; this molecule is detected by NspS and activates the NspS signaling pathway. Polyamines can be modified by acetylation and exported to maintain polyamine homeostasis in cells (Igarashi & Kashiwagi, 2010). This process has not been studied in V. cholerae; however, an ortholog of the speG gene encoding spermidine acetyltransferase is found in the V. cholerae genome. It is possible that this protein is capable of acetylating norspermidine; acetylated norspermidine could then potentially interact with NspS. Alternatively, norspermidine signaling and norspermidine biosynthesis pathways can act independently of each other and provide additive inputs into regulation of V. cholerae O139 biofilm formation. The distinction between these two possibilities will require more in-depth studies of these pathways.

AUTHORS' CONTRIBUTION

Z.M.P. and S.S.P. have contributed equally to this work.

REFERENCES

An D & Parsek MR (2007) The promise and peril of transcriptional profiling in biofilm communities. Curr Opin Microbiol 10: 292–296.

Beloin C & Ghigo JM (2005) Finding gene-expression patterns in bacterial biofilms. Trends Microbiol 13: 16–19.

Bower JM & Mulvey MA (2006) Polyamine-mediated resistance of uropathogenic Escherichia coli to nitrosative stress. J Bacteriol 188: 928–933.

Burrell M, Hanfrey CC, Murray EJ, Stanley-Wall NR & Michael AJ (2010) Evolution and multiplicity of arginine decarboxylases in polyamine biosynthesis and essential role in Bacillus subtilis biofilm formation. J Biol Chem 285: 39224–39238.

Chang AC & Cohen SN (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol 134: 1141–1156.

Colwell RR & Huq A (1994) Environmental reservoir of Vibrio cholerae. The causative agent of cholera. Ann NY Acad Sci 740: 44–54.

Colwell RR, Huq A, Islam MS et al. (2003) Reduction of cholera in Bangladeshi villages by simple filtration. P Natl Acad Sci USA 100: 1051–1055.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR & Lappin-Scott HM (1995) Microbial biofilms. Annu Rev Microbiol 49: 711–745.

Hanfrey C, Franceschetti M, Mayer MJ, Illingworth C & Michael AJ (2002) Abrogation of upstream open reading frame-mediated translational control of a plant S-adenosylmethionine decarboxylase results in polyamine disruption and growth perturbations. J Biol Chem 277: 44131–44139.

Haugo AJ & Watnick PI (2002) Vibrio cholerae CytR is a repressor of biofilm development. Mol Microbiol 45: 471–483.

Hung DT, Zhu J, Sturtevant D & Mekalanos JJ (2006) Bile acids stimulate biofilm formation in Vibrio cholerae. Mol Microbiol 59: 193–201.

Igarashi K & Kashiwagi K (2010) Modulation of cellular function by polyamines. Int J Biochem Cell Biol 42: 39–51. Karatan E, Duncan TR & Watnick PI (2005) NspS, a predicted polyamine sensor, mediates activation of Vibrio cholerae biofilm formation by norspermidine. J Bacteriol 187: 7434–7443.

Lee J, Sperandio V, Frantz DE, Longgood J, Camilli A, Phillips MA & Michael AJ (2009) An alternative polyamine biosynthetic pathway is widespread in bacteria and essential for biofilm formation in Vibrio cholerae. J Biol Chem 284: 9899–9907.

McGinnis MW, Parker ZM, Walter NE, Rutkovsky AC, Cartaya-Marin C & Karatan E (2009) Spermidine regulates Vibrio cholerae biofilm formation via transport and signaling pathways. FEMS Microbiol Lett 299: 166–174.

Miller JH (1992) A Short Course in Bacterial Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Moorthy S & Watnick PI (2005) Identification of novel stage-specific genetic requirements through whole genome transcription profiling of Vibrio cholerae biofilm development. Mol Microbiol 57: 1623–1635.

Morgan DM (1998) Polyamine Protocols. Humana Press, Totowa, NJ.

Nakao H, Shinoda S & Yamamoto S (1991) Purification and some properties of carboxynorspermidine synthase participating in a novel biosynthetic pathway for norspermidine in Vibrio alginolyticus. J Gen Microbiol 137: 1737–1742.

Patel CN, Wortham BW, Lines JL, Fetherston JD, Perry RD & Oliveira MA (2006) Polyamines are essential for the formation of plague biofilm. J Bacteriol 188: 2355–2363. Persson L (2009) Polyamine homoeostasis. Essays Biochem 46: 11–24.

Pfaffl MW, Horgan GW & Dempfle L (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 30: e36.

Quinones M, Kimsey HH & Waldor MK (2005) LexA cleavage is required for CTX prophage induction. Mol Cell 17: 291–300. Slonczewski JL & Foster JW (2011) Microbiology: An Evolving Science. W. W. Norton & Company, New York.

Tabor CW & Tabor H (1984) Polyamines. Annu Rev Biochem 53: 749–790.

Tamayo R, Patimalla B & Camilli A (2010) Growth in a biofilm induces a hyperinfectious phenotype in Vibrio cholerae. Infect Immun 78: 3560–3569.

Waldor MK & Mekalanos JJ (1994) Emergence of a new cholera pandemic: molecular analysis of virulence determinants in Vibrio cholerae O139 and development of a live vaccine prototype. J Infect Dis 170: 278–283.

Watnick PI & Kolter R (1999) Steps in the development of a Vibrio cholerae El Tor biofilm. Mol Microbiol 34: 586–595.

Watnick PI, Lauriano CM, Klose KE, Croal L & Kolter R (2001) The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in Vibrio cholerae O139. Mol Microbiol 39: 223–235.

Wortham BW, Oliveira MA, Fetherston JD & Perry RD (2010) Polyamines are required for the expression of key Hms proteins important for Yersinia pestis biofilm formation. Environ Microbiol 12: 2034–2047.

Yildiz FH & Schoolnik GK (1999) Vibrio cholerae O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. P Natl Acad Sci USA 96: 4028–4033.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Effect of nspC overexpression on NspC levels.
Fig. S2. Effect of elevated levels of NspC on growth.
Table S1. Real-time PCR results.
Table S2. Average wet weight of cells (mg).