Spermidine Regulates Vibrio Cholerae Biofilm Formation Via Transport And Signaling Pathways

By: Marcus W. McGinnis, Zachary M. Parker, Nicholas E. Walter, Alex C. Rutkovsky, Claudia Cartaya-Marin, and **Ece Karatan**

Abstract

Vibrio cholerae, the causative agent of the devastating diarrheal disease cholera, canform biofilms on diverse biotic and abiotic surfaces. Biofilm formation is important for the survival of this organism both in its natural environment and in the human host. Development of V. cholerae biofilms are regulated by complex regulatory networks that respond to environmental signals. One of these signals, norspermidine, is a polyamine that enhances biofilm formation via the NspS/MbaA signaling system. In this work, we have investigated the role of the polyamine spermidine in regulating biofilm formation in V. cholerae. We show that spermidine import requires PotD1, an ortholog of the periplasmic substratebinding protein of the spermidine transport system in Escherichia coli. We also show that deletion of the potD1 gene results in a significant increase in biofilm formation. We hypothesize that spermidine imported into the cell hinders biofilm formation. Exogenous spermidine further reduces biofilm formation in a PotD1-independent, but NspS/MbaA-dependent, manner. Our results suggest that polyamines affect biofilm formation in V. cholerae via multiple pathways involving both transport and signaling networks.

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INTRODUCTION

Biofilms are highly structured communities of microorganisms encased in a self-produced polymeric matrix containing polysaccharides, proteins, and sometimes DNA (Costerton et al., 1995; Davey & O'Toole, 2000). Most planktonic (free-swimming or free-floating) bacteria tend to form biofilms on suitable surfaces under favorable conditions. The biofilm state provides numerous advantages to a bacterium including protection from both chemical assaults such as antimicrobials and physical stresses such as UV light (Hoyle & Costerton, 1991; Kierek-Pearson & Karatan, 2005).

Vibrio cholerae, a Gram-negative aquatic bacterium responsible for the human intestinal disease cholera, can form biofilms on many aquatic surfaces such as crustaceans, zooplankton and aquatic plants (Huq et al., 1983; Islam et al., 1989; Tamplin et al., 1990). Biofilms have been proposed to aid in the persistence of V. cholerae in the environment by reducing osmotic stress (Faruque et al., 2006). In addition, biofilms have been correlated with enhanced infectivity of V. cholerae by providing protection from the body's natural defense mechanisms as well as providing a high dose of the pathogen (Faruque et al., 2006; Hung et al., 2006). Thus, biofilm formation is thought to be a survival strategy for V. cholerae both in its natural environment and in the human host.

The transition from the planktonic state to the biofilm state is a complex and highly regulated process. In V. cholerae, biofilm development is controlled by a variety of environmental signals such as osmolarity, nucleosides, sugars, quorum signals, and polyamines (Haugo &Watnick, 2002; Hammer & Bassler, 2003; Kierek & Watnick, 2003; Kapfhammer et al., 2005; Karatan et al., 2005). Polyamines, including cadaverine, norspermidine, putrescine and spermidine, are small organic hydrocarbons that are positively charged at physiological pH. They are pervasive at millimolar concentrations in both eukaryotes and prokaryotes and are required for normal cell growth (Tabor & Tabor, 1984). Polyamines are involved in numerous and diverse cellular processes including regulation of transcription and translation, stress tolerance, and virulence (Merrell & Camilli, 1999; Yoshida et al., 2004;Ware et al., 2006; Eraso & Kaplan, 2009). Polyamines also regulate biofilm formation.

For example, exogenous norspermidine enhances V. cholerae biofilm formation in the presence of NspS, a periplasmic protein that is thought to be the sensor for norspermidine (Karatan et al., 2005). NspS is hypothesized to form a complex with an integral membrane protein, MbaA, a putative c-di-GMP phosphodiesterase that contains tandem GGDEF and EAL domains. Thus, in this case, norspermidine acts as an extracellular signal that regulates biofilm formation via the putative NspS/MbaA complex. Maintenance of intracellular polyamine levels is also important for biofilm formation. Reduction in intracellular putrescine and norspermidine levels in Yersinia pestis and V. cholerae, respectively, inhibit biofilm formation (Patel et al., 2006; Lee et al., 2009). Thus, polyamines can act as both extracellular and intracellular regulators of biofilm formation.
In this work, we have studied the effect of the polyamine spermidine on V. cholerae O139 biofilm formation. Specifically, we have analyzed the role of a spermidine import system and a spermidine-responsive signaling system in the regulation of V. cholerae biofilm formation by spermidine.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and reagents

The bacterial strains and plasmids used in this study are listed in Table 1. Vibrio cholerae serotype O139 strain MO10 was used for all the experiments. Experiments were performed either in Luria–Bertani (LB) broth or minimal media as described previously, except the amino acid mix was replaced with 1% casamino acids (Kapfhammer et al., 2005). All primers used for this project are listed in Table 2. Norspermidine, spermidine, cadaverine, diaminopropane, benzoyl chloride, trichloroacetic acid, and HPLC-grade methanol were purchased from Sigma (St. Louis, MO). Putrescine was obtained from Research Organics (Cleveland, OH).

Table 1. Bacterial strains and plasmids

Strains/plasmids	Genotype	Reference/source
E. coli		
DH5α	F-ø80*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *phoA*, *endA*1, *hsdR*17(rk2, mk+), *supE*44, *thi*-1, *gyrA*96, *relA*1, λ-	Invitrogen
DH5αλpir	supE44 ΔlacU169 hsdR17, recA1 endA1 gyrA96 thi-1 relA1, λpir	Hanahan (1983)
SM10λpir	*thi thr leu tonA lacY supE recA*::*RP4-2-Tc*::*Mu*λ*pirR6K*; *Km*^{r}	Miller & Mekalanos (1988)
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)
PW444	MO10*lacZ*::*vpsLp* → *lacZ*, Δ*mbaA*, Sm^R	Karatan *et al.* (2005)
PW514	MO10*lacZ*::*vpsLp* → *lacZ*, Δ*nspS*, Sm^R	Karatan *et al.* (2005)
AK042	MO10Δ*potD2*, Sm^R	This study
AK061	MO10Δ*potD1*, Sm^R	This study
AK077	MO10Δ*potD2*Δ*potD1*, Sm^R	This study
Plasmids		
pWM91	*oriR6KmobRP4 lacI pTac tnp miniTn10Km*; Km^R, Ap^R	Metcalf *et al.* (1996)
pCR2.1-TOPO	Plasmid for TOPO cloning, Ap^R	Invitrogen
pMM9	pWM91 carrying an internal in-frame deletion of *potD1*	This study
pMM7	pWM91 carrying an internal in-frame deletion of *potD2*	This study
pMM12	pWM91 carrying an internal in-frame deletion of *potD1* and *potD2*	This study

Table 2. Primers

Primer	Description	Sequence
Construction of *V. cholerae potD1* deletion		
P332	Forward primer for the upstream fragment	5′-AGAAGGCGGTATCGGTTGGG-3′
P333	Reverse primer for the upstream fragment	5′-TTACGAGCGGCCGCATGCGCACAGAGCACTCGC-3′
P334	Forward primer for the downstream fragment	5′-TGCGGCCGCTCGTAAGGCGAGTGGCAAGATGAAG-3′
P335	Reverse primer for the downstream fragment	5′-AGCGCGATTCTCTGGTTCCC-3′
Construction of *V. cholerae potD2* deletion		
P336	Forward primer for the upstream fragment	5′-ATCCTGCCACTGGCGAAGCC-3′
P337	Reverse primer for the upstream fragment	5′-TTACGAGCGGCCGCACTCCAAACTAAACGTTGCCC-3′
P338	Forward primer for the downstream fragment	5′-TGCGGCCGCTCGTAATGGCAATCTGCCGTTGGCG-3′
P339	Reverse primer for the downstream fragment	5′-TCTCTTGCAGCATGCCTTC-3′
Analysis of *potD1* and *potD2* gene transcription		
PA62	Forward primer for *potD1*	5′-GCGCAGCAGCATTGTTCACC-3′
PA63	Reverse primer for *potD1*	5′-GATCATAACCAGCGCCTTGCG-3′
PA64	Forward primer for *potD2*	5′-GCAACGCCCAAAAGGGCAAC-3′
PA65	Reverse primer for *potD2*	5′-CCTTATCTTCCGCTGCCGC-3′

Construction of ΔpotD1, ΔpotD2 and ΔpotD1ΔpotD2 mutants

Fragments containing approximately 400 bp upstream and downstream of the potD genes were amplified by PCR from V. cholerae chromosomal DNA using Pfx polymerase. Antisense primers used to amplify the upstream fragment and the sense primers used to amplify the downstream fragment contained a complementary overhang that facilitated splicing of these fragments (Horton et al., 1990). Splicing of the

fragments generated in-frame deletions that removed 925 bp of the 1040-bp potD1 gene and 970 bp of the 1103-bp potD2 gene. To construct the ΔpotD1ΔpotD2 mutant, the downstream fragment was generated using primers P334 and 335 and the upstream fragment was generated using primers P336 and P337. The deletion constructs were cloned into pWM91; plasmids carrying the inserts were electroporated into SM10lpir and conjugated into V. cholerae. The deletion mutants were created by double homologous recombination and sucrose selection as described previously (Metcalf et al., 1996).

Biofilm assays and microscopy

Biofilm assays were performed as described in Karatan et al. (2005) using triplicates of each strain and repeated multiple times to confirm the results. For fluorescence microscopy, biofilms were formed on borosilicate coverslips inserted into 50-mL Falcon tubes containing 10mL of LB broth inoculated with V. cholerae to yield a starting $OD_{595\ nm}$ of 0.005. After an 18-h incubation, the media and the planktonic cells were discarded, and biofilms were incubated in fresh media containing 2.5 μM SYTO 9 (Invitrogen, Carlsbad, CA) for 30 min at room temperature, washed with fresh LB, and placed on a concave microscope slide filled with sterile medium. Biofilms were visualized using a Plan-Neofluar _40/1.3 oil objective lens. Biofilm images were acquired and analyzed using an LSM 510 confocal scanning system (Zeiss, Thornwood, NY). z-Sections were collected at 1-μm intervals.

Extraction, benzoylation, and detection of Polyamines

Extraction of polyamines from the cells was performed essentially as described previously (Morgan, 1998; Patel et al., 2006). Briefly, cells grown to mid-log phase at 27 1C were pelleted, washed with phosphate-buffered saline and resuspended in 10 μLwater mg^{-1} wet weight. Cells were lysed by sonication, cell debris was removed by centrifugation and cellular protein was precipitated by addition of trichloroacetic acid. The solution was centrifuged and the supernatant containing the polyamines was removed for benzoylation. To quantify the polyamines in LB, the broth was used directly for benzoylation. A standard mix containing

0.1mM of each polyamine was prepared every time cellular polyamines or polyamines in LB were quantified. The benzoylation procedure was performed as described previously (Morgan, 1998). Benzoylated polyamines were extracted twice with 1mL of chloroform, evaporated to dryness, and dissolved in 100 μL of the mobile phase used for HPLC (60% methanol and 40% water). 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 x 4.6 mm), fitted with a 50 x 4.6mm guard cartridge (Waters Corporation, Milford, MA). The run was performed isocratically using 60% methanol and 40% water as the mobile phase at a flow rate of 0.8mLmin^{-1} for 30 min. The detector was set to 254 nm. MS was used to verify the identity of each peak observed in HPLC fractions. HPLC–atmospheric pressure chemical ionization–MS analysis was performed on a Dionex Ultimate 3000 instrument coupled with a Dionex Ultimate 3000 photodiode array detector and a Dionex MSQ plus mass spectrometer (Bannockburn, IL).

RNA extraction, cDNA synthesis, and reverse transcriptase-PCR

Total RNA was extracted from 5mL of V. cholerae cells grown to mid-log phase and treated with DNAse I for 2 h at 37 1C to rid the sample of any genomic DNA. One microgram of this RNA was reverse transcribed using random primers. Negative controls were also performed without reverse transcriptase to ensure a lack of genomic DNA contamination. The cDNA was then used in a PCR reaction with gene-specific primers.

RESULTS AND DISCUSSION

Putative spermidine and norspermidine transport systems in V. cholera

We have previously shown that V. cholerae O139 biofilm formation is enhanced by the polyamine norspermidine (Karatan et al., 2005). Norspermidine was shown to exert its effect as an environmental signal that is thought to be detected and processed by the putative NspS/MbaA complex. This result suggested to us that transport of norspermidine

or other similar polyamines into the cell might also serve as a biofilm regulatory mechanism. Studies on norspermidine transport in V. cholerae or other microorganisms have not yet been reported. However, norspermidine is very similar to spermidine, another triamine that is one methylene group longer than norspermidine (Fig. 1a). In Escherichia coli, spermidine is imported into the cell by an ABC-type transporter composed of PotD (the periplasmic substrate-binding protein), PotB and PotC (the integral membrane permeases), and PotA (the ATPase). We hypothesized that norspermidine and/or spermidine might be transported into the cell via a transporter similar to the PotABCD transporter. Analysis of the V. cholerae genome (available at http://cmr.jcvi.org/tigrscripts/CMR/CmrHomePage.cgi) yielded a locus (VC1428-VC1424) annotated as potABCD2D1 (Heidelberg et al., 2000). Sequence comparisons between the proteins encoded by these genes and their orthologs in E. coli showed that they share 59–72% identity. Therefore, this putative operon is highly likely to encode a spermidine and/or norspermidine transport system. The presence of two genes annotated as potD was intriguing. To determine whether this arrangement was conserved in other closely related bacteria, we compared the genomic regions of a number of bacteria in Vibrionaceae whose genome sequences are available at the Comprehensive Microbial Resource website (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). We found putative potABCD2D1 loci in all of the members of this group [classical V. cholerae strain O395 (VCO395-A1039-1035), Vibrio fischeri ES114_5 (VF1315-1319), Vibrio vulnificus strains YJ01_5 and CMCP6_5 (VV1685-90 and VV_12604-2600); Vibrio parahaemolyticus 2210633_5 (VP1529-25), and Photobacterium profundum SS9_5 (PBPRA1852-1856)]. One potential explanation for having both PotD1 and PotD2 is that these two periplasmic binding proteins might recognize different substrates with similar structures and yet work through the same permeases and the ATPase.

(a)

Diaminopropane: $H_2N(CH_2)_3NH_2$

Putrescine: $H_2N(CH_2)_4NH_2$

Cadaverine: $H_2N(CH_2)_5NH_2$

Norspermidine: $H_2N(CH_2)_3NH(CH_2)_3NH_2$

Spermidine: $H_2N(CH_2)_4NH(CH_2)_3NH_2$

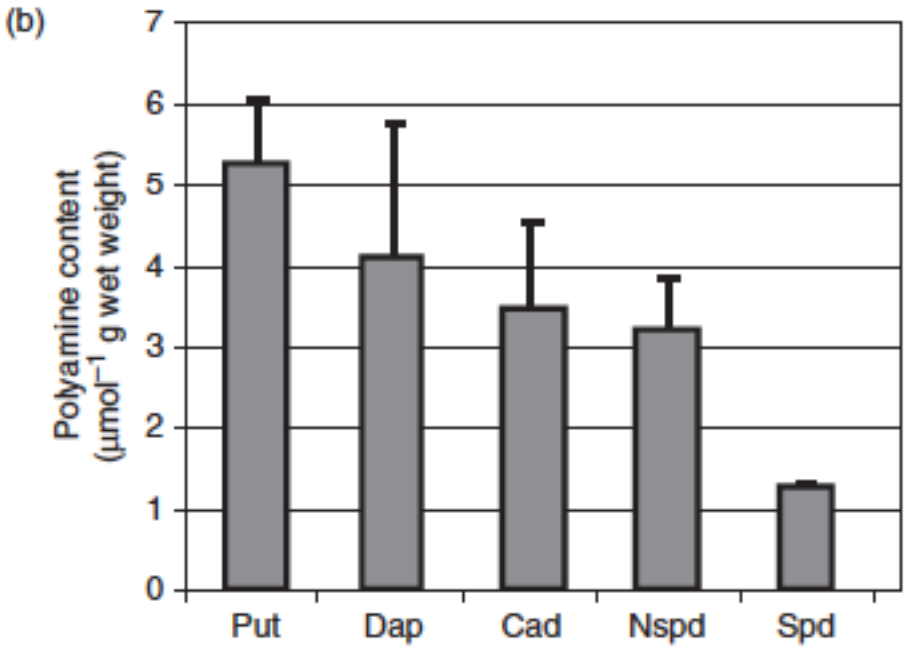


Fig. 1. (a) Major polyamines found in Vibrio cholerae. (b) Polyamine composition of V. cholerae grown in LB broth. Polyamines extracted from the cells were derivatized, separated by HPLC, and quantified as described in Materials and methods. LB broth contains approximately 10 mM putrescine and 40 mM spermidine. Diaminopropane, cadaverine, and norspermidine are either not present in LB or below the detection limit under our experimental conditions. Error bars show SDs of three replicates. Put, putrescine; Dap, diaminopropane; Cad, cadaverine; Nspd, norspermidine; and Spd, spermidine.

Role of PotD1 and PotD2 in polyamine import

In order to determine whether either of the PotD proteins is responsible for importing spermidine or norspermidine into the cell, we first identified the polyamines in the wild-type V. cholerae O139 cells that were grown in LB broth. Extracts of V. cholerae cells consistently showed five types of polyamines (Fig. 1a). Identities of these polyamines were determined using MS in tandem with high-performance liquid chromatography (LC-MS) (data not shown). Molecular weights of the major species yielded by LC–MS analysis were 297, 283, 211, 444, and 458, which correspond to benzoylated molecular weights of putrescine, diaminopropane, cadaverine, norspermidine, and spermidine. Quantification of these polyamines in the cell and a representative HPLC chromatogram are shown in Figs 1b and 2a, respectively. This composition is similar to the polyamine composition of V. cholerae O1 El Tor grown in LB reported recently (Lee et al., 2009). The presence of the biosynthetic pathways for cadaverine, norspermidine, and diaminopropane has been demonstrated in this organism (Merrell & Camilli, 1999; Lee et al., 2009). Presence of a biosynthetic pathway for putrescine can be inferred as V. cholerae genome encodes homologs of putrescine biosynthetic enzymes. Putrescine is synthesized either by decarboxylation of ornithine by ornithine decarboxylase, a homolog of which is encoded by the VCA1063 locus, or through the formation of agmatine by arginine decarboxylase followed by the conversion of agmatine to putrescine by agmatinase, homologs of which are encoded at loci VCA0815 and VCA0814, respectively (Shah & Swiatlo, 2008). However, the V. cholerae genome does not encode S-adenosylmethionine decarboxylase or spermidine synthase enzymes, which are responsible for spermidine synthesis (Tabor et al., 1986; Anton & Kutny, 1987). Despite the lack of these enzymes, V. cholerae does possess the ability to synthesize spermidine via the metabolic pathway used for norspermidine synthesis. Norspermidine is synthesized by decarboxylation of carboxynorspermidine by carboxynorspermidine decarboxylase encoded by the nspC gene (VC1623) (Lee et al., 2009). Synthesis of carboxynorspermidine is carried out by carboxynorspermidine synthase, which catalyzes the reductive condensation of L-aspartate b-semialdehyde and diaminopropane. It has been demonstrated that carboxynorspermidine synthase can use putrescine in place of diaminopropane to produce spermidine instead of norspermidine (Lee et al., 2009). However, this

reaction is relatively inefficient and is unlikely to account for the substantial amount of spermidine in V. cholerae grown in LB. In fact, polyamines extracted from V. cholerae grown in minimal medium contain putrescine, diaminopropane, cadaverine, and norspermidine, but not spermidine (data not shown) (Lee et al., 2009). Therefore, the most likely source of the spermidine found in the cell is the LB medium, which contains approximately 40 mM spermidine (Supporting Information, Fig. S1).

To determine whether PotD1, PotD2, or both are responsible for transporting spermidine or norspermidine into the cell, we constructed mutant strains of V. cholerae lacking potD1, potD2 or both and analyzed their polyamine content. The ΔpotD2 mutant contained all five of the polyamines found in the wild-type strain, whereas the DpotD1 and the ΔpotD1ΔpotD2 mutants contained all except for spermidine (Fig. 2). These results suggest that PotD1 is responsible for spermidine transport into V. cholerae, while PotD2 does not appear to play a role in the import of this polyamine.

Next, to determine whether either of the PotD proteins is responsible for the import of norspermidine, we added 1mM norspermidine into the growth medium. However, we were not able to observe an increase in the intracellular norspermidine levels in the wild-type cells (Fig. S2). Intracellular levels of polyamines have been shown to be regulated tightly by a variety of mechanisms (Shah & Swiatlo, 2008). Therefore, it is possible that even if the cell can import norspermidine, the total levels of this polyamine may be adjusted by decreasing the de novo synthesis of this molecule. However, even though the addition of norspermidine into the culture medium did not result in an increase in intracellular norspermidine levels, it did inhibit the uptake of spermidine by the cells (Fig. S2). A similar observation has been recently reported for V. cholerae O1 El Tor, corroborating our findings (Lee et al., 2009). It is possible that PotD1 has a higher affinity for norspermidine and that the presence of large amounts of norspermidine competitively inhibits spermidine binding to PotD1. While this result strongly argues for a role for PotD1 in norspermidine transport, definitive proof must be obtained using mutant strains that are unable to synthesize norspermidine.

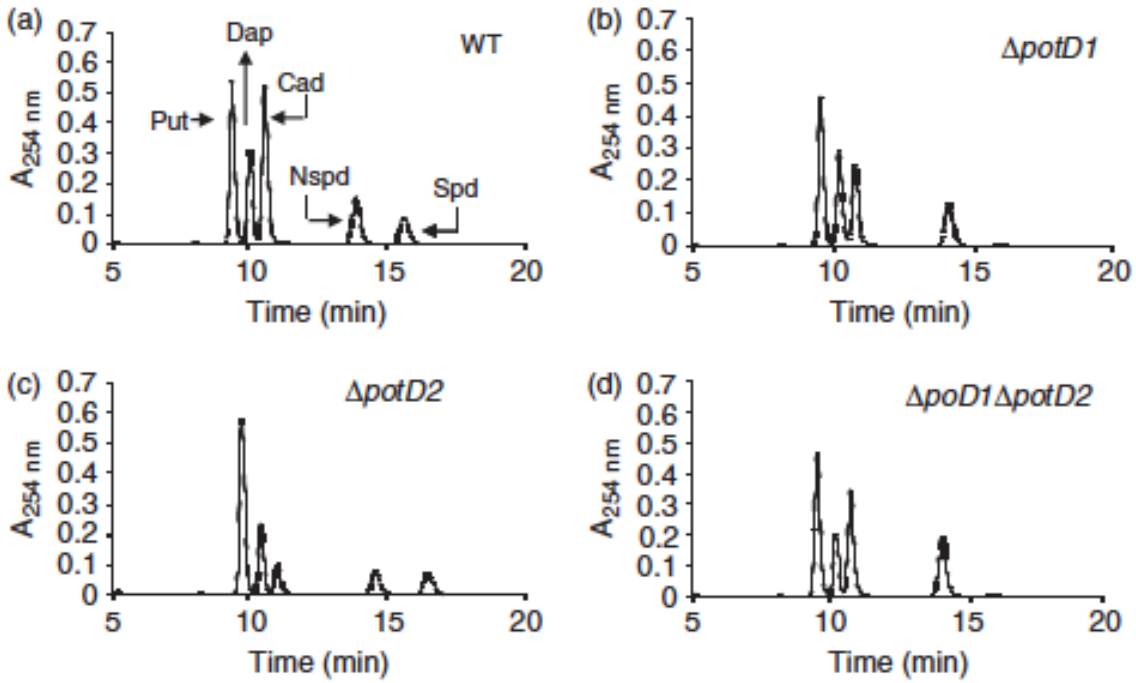


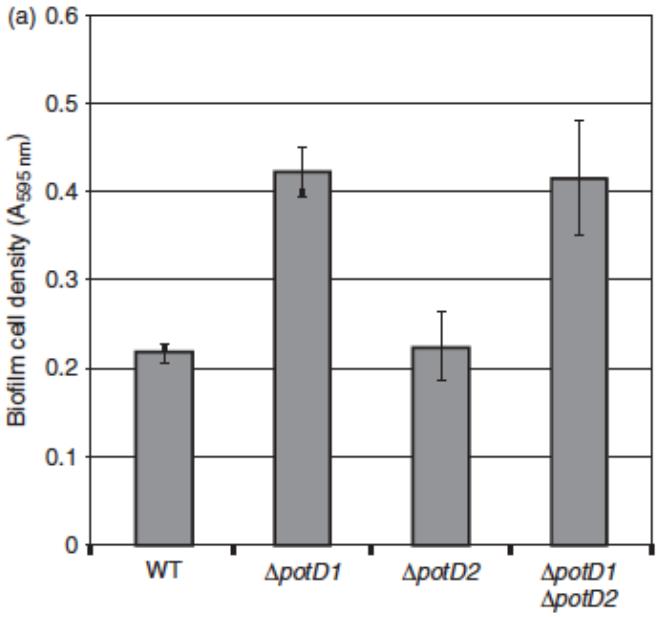
Fig. 2. Polyamine composition of wild-type Vibrio cholerae (a) and the potD mutants (b-d). Polyamines were extracted, derivatized by benzoylation, and analyzed by HPLC as described in Materials and methods. For clarity, only data obtained between 5 and 20 min of a 30-min run are plotted. Peaks labeled in the wild-type (WT) chromatogram correspond to putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd).

Role of PotD1 and PotD2 in biofilm formation

To determine whether either of these PotD orthologs plays a role in V. cholerae biofilm formation, we performed biofilm assays. Both the DpotD1 and the DpotD1DpotD2 mutants displayed significantly increased biofilm formation in comparison with the wild type, while the ΔpotD2 mutant did not demonstrate a difference from the wild type (Fig. 3a). We then used confocal scanning laser microscopy (CSLM) to visualize the differences between the wild-type and the mutant biofilms. The biofilms made by both the DpotD1 and the ΔpotD1ΔpotD2 mutants had greater depth in comparison with the wild type and the ΔpotD2 mutant biofilm was similar to that of the wild type, confirming the

results of the biofilm assays (Fig. 3b). In addition, growth curves generated for all of the strains showed that the observed effects were not due to an overall increase in bacterial numbers (data not shown). These results suggest that PotD1 represses V. cholerae biofilm formation, whereas PotD2 is not involved in regulating biofilm formation under the conditions tested.

The lack of involvement of PotD2 in spermidine import and biofilm formation prompted us to ask whether the potD2 gene is expressed. To determine whether potD1 and potD2 genes are both being transcribed, total RNA was extracted from V. cholerae cells and reverse transcribed. PCR amplification of this cDNA using gene-specific primers resulted in distinct products, indicating that both of these genes are transcribed under our experimental conditions (Fig. S3). In addition, we were able to detect the presence of potD2 in the ΔpotD1 mutant and, conversely, the presence of potD1 in the ΔpotD2 mutant, indicating that deletion of one of the potD genes did not affect the expression of the other (Fig. S3).


(a)
0.6
0.5
0.4
0.3
0.2
0.1
0
Biofilm cell density ($A_{595\ nm}$)
WT
ΔpotD1
ΔpotD2
ΔpotD1
ΔpotD2

(b)

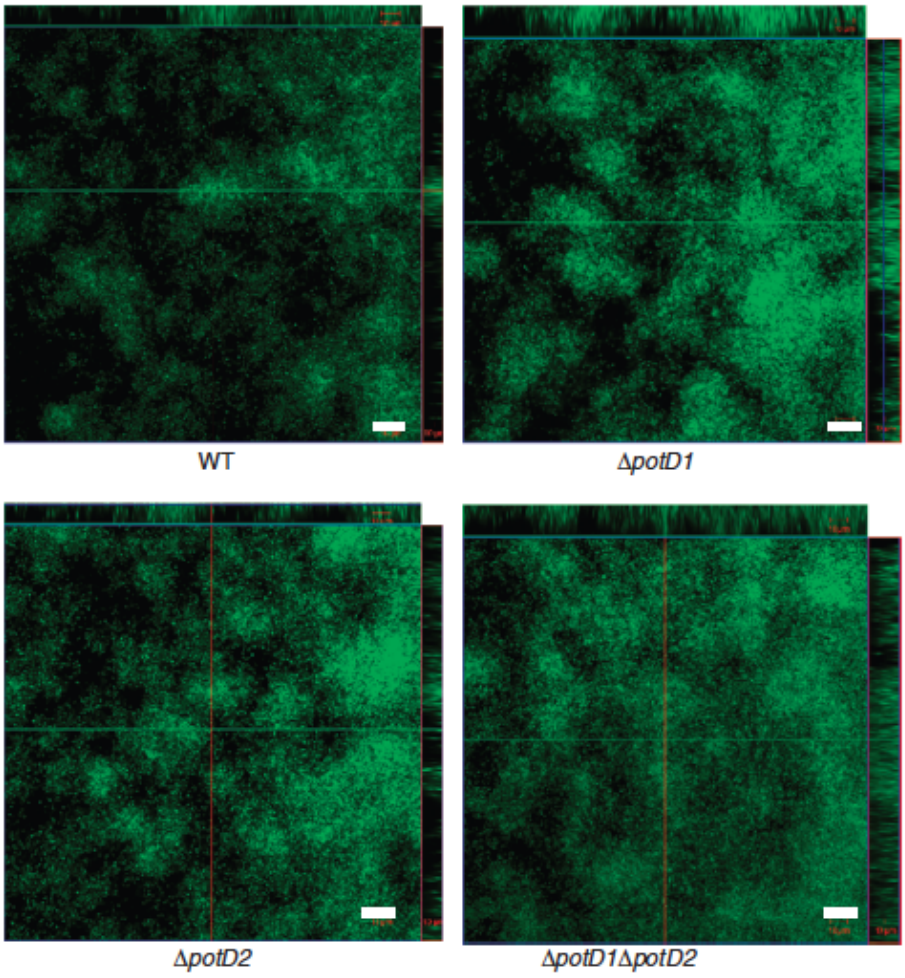

WT
ΔpotD1
ΔpotD2
ΔpotD1ΔpotD2

Fig. 3. Biofilm formation of WT and potD mutant biofilms. (a) Quantification of wild-type and mutant Vibrio cholerae biofilms. Wild-type V. cholerae (WT) and ΔpotD1, ΔpotD2, and ΔpotD1ΔpotD2 mutant biofilms were formed in borosilicate tubes overnight in LB broth and quantified as described in Materials and methods. Error bars show SDs of three replicates. (b) Architecture of biofilms made by wild-type V. cholerae and the potD mutants. Transverse and vertical cross sections through SYTO 9-stained wild-type V. cholerae (WT) as well as ΔpotD1, ΔpotD2, and ΔpotD1ΔpotD2 mutant biofilms formed after overnight incubation in LB broth. The transverse sections were obtained at the level of the substratum (scale bar = 10 μm).

Effect of exogenous spermidine on biofilm formation

We have previously shown that addition of spermidine into the culture medium decreases V. cholerae biofilm formation (Karatan et al., 2005). To determine whether this effect was a result of the import of spermidine into the cell by PotD1, we performed biofilm assays in the presence and absence of exogenous spermidine. Surprisingly, 1mM exogenous spermidine decreased biofilm formation in all of the strains tested regardless of the presence of potD1, arguing for an alternative mechanism for the spermidine-associated decrease (Fig. 4a). One possibility for this alternative mechanism is the NspS/MbaA signaling system. NspS is a paralog of PotD1 and PotD2 and shares 25% and 22% sequence identity with these proteins, respectively. NspS is thought to be a sensor for norspermidine as exogenous norspermidine enhances biofilm formation only in the presence of this protein (Karatan et al., 2005). NspS is believed to be in association with MbaA, a putative integral membrane protein, which is a repressor of biofilm formation (Karatan et al., 2005). Because spermidine is very similar in structure to norspermidine, it might bind NspS and regulate biofilm formation via the NspS/MbaA system. To test this hypothesis, we conducted biofilm assays with strains lacking either NspS or MbaA, in the presence and absence of spermidine. The deletion of either of these proteins abolished the negative effect of spermidine on biofilm formation (Fig. 4b).

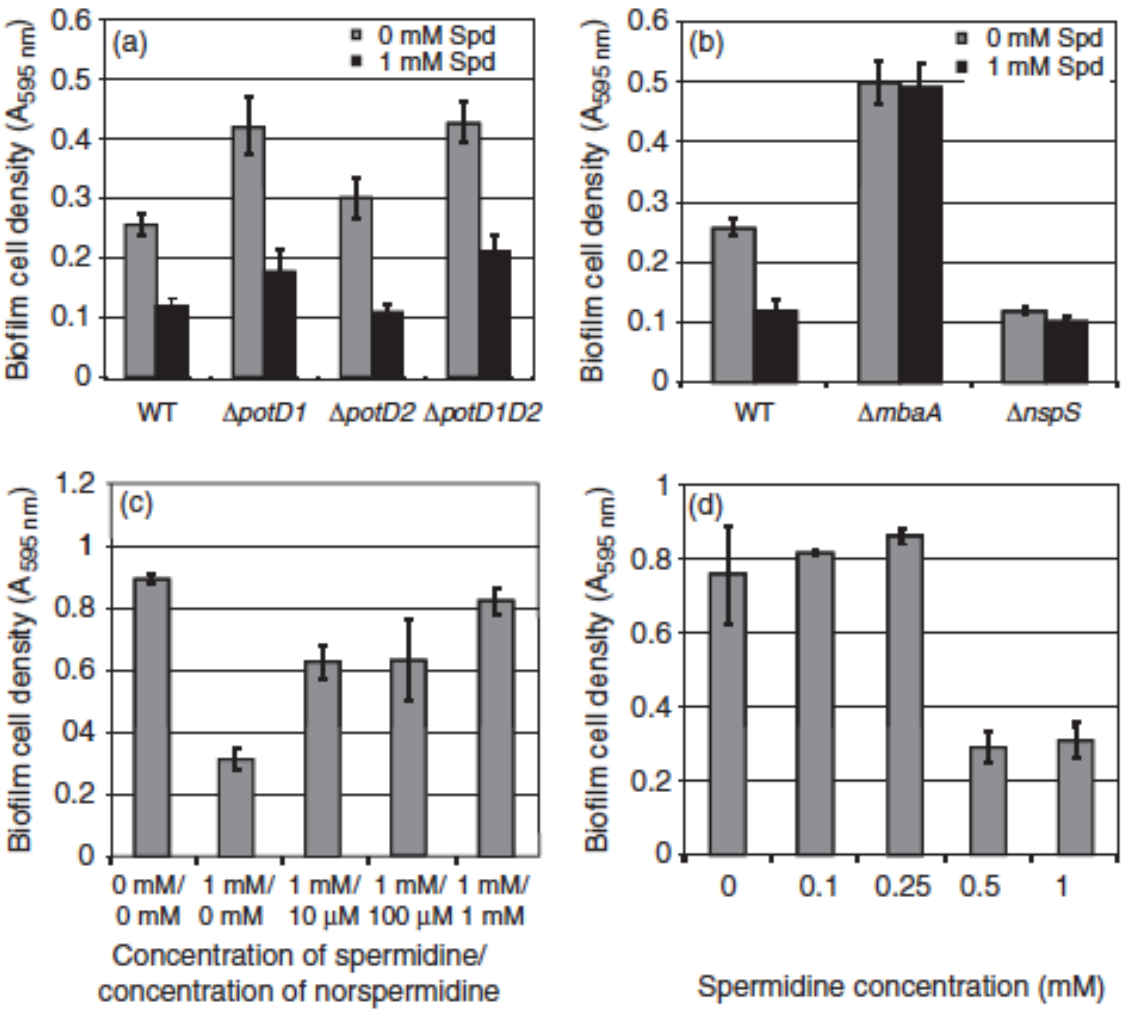


Fig. 4. Effects of exogenous polyamines on biofilm formation. (a) Effect of spermidine on wild-type (WT) and potD mutant biofilms. (b) Effect of spermidine on the wild-type (WT), DnspS, and DmbaA mutant biofilms. (c) Biofilm formation of ΔpotD1 mutant in the presence of both spermidine and norspermidine. (d) Biofilm formation of ΔpotD1 mutant in the presence spermidine. Biofilms were formed in borosilicate tubes in LB broth for 18 h with indicated quantities of polyamines and quantified as described in Materials and methods. Error bars show SDs of three replicates. In order to eliminate any contribution from spermidine imported into the cells, experiments in (c) and (d) were performed in the ΔpotD1 mutant; however, the same trends were observed in the wild type as well.

Next, we hypothesized that if spermidine and norspermidine both bind NspS, then the presence of norspermidine in the media could reverse the spermidine-associated decrease in biofilm formation. To test this hypothesis, we performed biofilm assays in LB medium containing 1mM spermidine and increasing amounts of norspermidine. Norspermidine concentrations as low as 10 mM could reverse the inhibitory effect of exogenous spermidine on biofilms (Fig. 4c). These results are consistent with the hypothesis that norspermidine and spermidine compete for the same sensor protein that is in direct communication with the external environment of the cell. While we cannot completely rule out the presence of other yet unidentified proteins mediating this effect, NspS is the most likely candidate for this sensor.
Finally, to determine whether spermidine can signal through the NspS/MbaA system when it is present at lower concentrations in the environment, we performed biofilm assays with different spermidine concentrations. The inhibitory effect of spermidine on biofilms was observed only at concentrations Z500 mM (Fig. 4d). These findings also indicate that in LB broth, where concentration of spermidine is approximately 40 mM, spermidine is likely to affect biofilm formation only through an intracellular mechanism and not through the NspS/MbaA system. Our data also suggest that PotD1 has a higher affinity for spermidine than NspS has for this molecule, as PotD1 can bind spermidine and import it into the cell under these conditions.
In conclusion, our results suggest that spermidine regulates biofilm formation in V. cholerae through two different pathways. Spermidine imported by PotD1 may decrease biofilms through an intracellular mechanism, while the decrease in biofilms associated with exogenous spermidine may be due to the ability of this polyamine to bind to NspS and interfere with its activation of biofilm formation. Therefore, spermidine is likely to inhibit biofilm formation in V. cholerae as both an intracellular and an extracellular effector. Further studies will help to elucidate the molecular details of these effects.

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Authors'contribution

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

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SupportingInformation

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

Fig. S1. Polyamine profile of LB.
Fig. S2. Effect of exogenous norspermidine on intracellular polyamine composition.
Fig. S3. Transcription of potD1 and potD2.