THE EFFECT OF POLYAMINES ON *VIBRIO CHOLERAE* VIRULENCE PROPERTIES

A Thesis

by

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ABSTRACT

THE EFFECT OF POLYAMINES ON *VIBRIO CHOLERAE* VIRULENCE PROPERTIES

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Vibrio cholerae is the causative agent of the severe enteric disease, cholera. To be a virulent strain, the bacterium must be able to synthesize both cholera toxin and toxin-coregulated pilus. Toxin-coregulated pilus has been shown to mediate microcolony formation, which is believed to aid in the colonization and concentration of *V. cholerae* within the small intestine. Only a few environmental signals have been shown to regulate *V. cholerae* virulence gene expression. Polyamines, which are ubiquitous in nature, and have been implicated in regulating virulence gene expression in other bacteria, have not been studied in *V. cholerae* for their effect on virulence properties. Performing agglutination assays I found that all polyamines tested had a negative effect on autoagglutination in a concentration dependent manner, yet cholera toxin synthesis did not appear to be effected. The polyamines tested did not appear to morphologically affect pili bundling as assessed by transmission electron microscopy. Putrescine and cadaverine appeared to significantly affect the synthesis of the major pilin subunit, TcpA; and interestingly, only

putrescine had an effect on the relative abundance of the toxin-coregulated pili found on the cell surface. CTXϕ transduction was also found to be inhibited by the addition of polyamines, indicating the dysfunction of the toxin-coregulated pili present on the cell surface. With data suggesting pili were present in equal numbers on the cell surface, yet transduction efficiency was inhibited by approximately 80% in the presence of polyamines, I hypothesized the inhibitory autoagglutination effect was caused by the positively charged amine groups on the polyamines electrostatically disrupting the pili-pili interactions which mediate microcolony formation. Additionally, only the polyamine spermidine was found to negatively regulate both autoagglutination and biofilm formation in the classical strain of *V. cholerae*, which to my knowledge has been the only molecule identified to do so.

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INTRODUCTION

Vibrio cholerae, a Gram negative, enteropathogenic organism, is the causative agent of the disease, cholera. Cholera is a severe and life threatening diarrheal disease, with a high mortality rate in regions with inadequate drinking water. The bacterium is known to inhabit brackish waters. Areas with poor sanitation and drinking water are at high risk for epidemic outbreaks due to the oral-fecal transmission route of the bacterium. *V. cholerae* is classified into distinct serogroups based upon the differences in the sugar composition of the "O" antigen present on the bacterial surface [1,2]. Currently, 206 distinct serogroups have been identified, with only two, O1 and O139, capable of causing pandemic cholera. The O1 serogroup can be divided into two biotypes, classical and El Tor. These biotypes can be further subdivided into distinct serotypes, Ogawa and Inaba, based upon differences in antigenic factors [2]. The mechanism of *V. cholerae* transmission and virulence has been studied extensively for over a century, with recent studies identifying two main virulence factors, cholera toxin (CTX) and toxin-coregulated pilus (TCP), as being essential for colonization and virulence within the host [3,4].

Cholera occurs when an infectious dose of *V. cholerae* is orally ingested, and subsequently colonizes the middle/distal portion of the small intestine [5-7]. To be considered a virulent strain of *V. cholerae*, the bacterium must be able to synthesize two main virulence factors: TCP, which is believed to aid in the colonization of the small intestine, and cholera toxin (CTX), an enterotoxin secreted by the bacterium that causes the

characteristic voluminous diarrhea [4,8]. In addition to TCP and CTX, *V. cholerae* synthesizes many other virulence factors which contribute to pathogenesis; however, when either TCP and/or CTX synthesis is reduced, or absent, colonization and virulence are markedly attenuated [4].

Toxin-coregulated pilus belongs to a class of type IVb pili, which have previously been shown to aid in the pathogenesis of other organisms [9,10]. The TCP is composed of a repeating homopolymer of the major pilin subunit, TcpA, which forms a helical arrangement originating in the inner membrane and protruding outward from the cell surface [9,11]. The genes which code for the proteins making up the TCP biogenesis apparatus and the major pilin subunit are located within the *tcp* operon found on a segment of the chromosome referred to as the *Vibrio* pathogenicity island [12]. Colonization and microcolony formation within the small intestine has been shown to be mediated by TCP [4,10,11,13-15]. Microcolony formation is composed of a number of sequential steps beginning with the assembly of functional pili on the cell surface. Once pili form on the bacterial surface they are believed to electrostatically interact with neighboring pili, forming pilus bundles [11,13,15]. These pilus bundles are then hypothesized to interact with pilus bundles from neighboring bacteria, in turn forming autoagglutinated microcolonies [15]. Autoagglutination can be assessed *in vitro* by culturing bacteria under optimal TCP expressing conditions [3,13]. Kirn *et. al*. showed that *in vitro* autoagglutination correlates with colonization in the infant mouse model, indicating the reliability of *in vitro* autoagglutination as a positive indicator of *V. cholerae* to effectively colonize the host [13].

TCP has also been shown to be the high affinity receptor for the filamentous, lysogenic bacteriophage CTXϕ [16]. CTXϕ carries the genes encoding the heterodimeric A-subunit and homopentameric B-subunit of cholera toxin, which is required to cause disease within a host. Once synthesized and secreted into the cultural supernatant, CTXϕ putatively interacts with charged residues on the TCP surface where it is taken into the cell by an unidentified mechanism [16,17]. Once in the cytosol, the single stranded DNA genome can either be incorporated into the host genome or become a replicative plasmid (pCTX) [16,17]. This has been proposed as the mechanism by which non-toxigenic strains of *V. cholerae* have horizontally acquired the *ctxAB* genes to become fully virulent strains.

The regulation of *V. cholerae* virulence genes is controlled through a complex pathway consisting of multiple proteins, each playing an integral role in the regulation of both *ctxAB* genes, and the *tcp* operon [18]. Virulence gene expression has been shown to respond to specific environmental stimuli *in vitro,* including temperature, pH, bile salts, osmolarity and amino acids [19-22]. Interestingly, the conditions required to promote maximal virulence gene expression *in vitro* are different than those encountered within the small intestine. Conditions for maximal virulence gene expression *in vitro* were found to be a temperature of 30°C, pH 6.5, and a salt concentration of approximately 66mM NaCl [23,24]. Conversely, environmental conditions within the small intestine are a temperature of 37°C, a more basic pH, and an osmolarity equivalent to approximately 300mM NaCl [23]. Within the intestinal environment only a few signals have been identified which regulate virulence gene expression. Animal studies have shown that wild-type *V. cholerae* preferentially colonize the middle/distal portion of the small intestine in a TCP dependent manner, indicating a potential gradient of either an attractant or repellent leading to the area

of colonization [25,26]. The purpose for this temporal/proximal colonization and virulence gene expression at the more distal portion of the small intestine has yet to be elucidated.

There are a plethora of signals within the small intestine which have yet to be studied for their effect on *V. cholerae* virulence properties. One class of host-derived molecules, polyamines have previously been implicated in regulating biofilm formation and virulence in a number of organisms including *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Francisella tularensis* and *Yersinia pestis* [27-32]. Also, both the polyamines spermidine and putrescine have previously been shown to attenuate MR/K hemagglutination, mediated by type 3 fimbriae, in species of Enterobacteria by a yet unidentified mechanism [33]. Polyamines are flexible hydrocarbon chains with interspersed, positively charged amine groups at physiological pH (Figure 1). These small molecules are ubiquitous in nature and are utilized by almost all living cells for normal cell proliferation [34-36]. Due to their cationic nature, polyamines have been shown to be important in modulating cellular processes by interacting with RNA, DNA, and proteins [34-39].

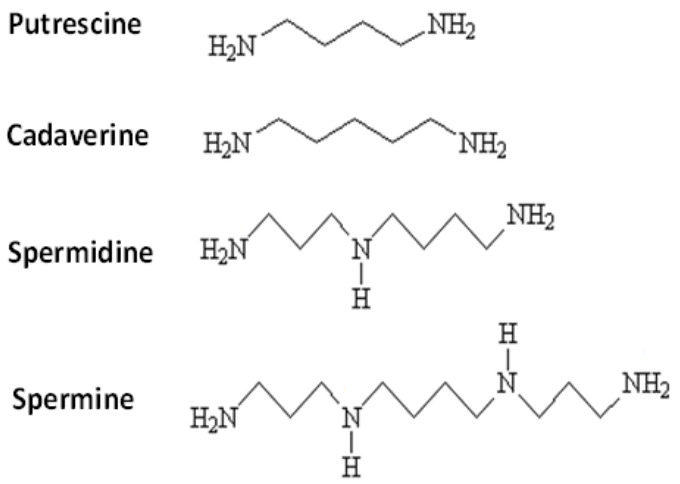

Figure 1. Structure of the major polyamines putrescine, cadaverine, spermidine and spermine.

Polyamines within the human gastrointestinal tract originate both endogenously and exogenously by means of three independent sources: polyamines which are synthesized by our own cells, those contributed by intestinal microflora, and those from dietary intake [34,40,41]. Due to the high proliferation rate of epithelial cells in the gastrointestinal tract, the gut inherently has a high demand for polyamines; and thus a relatively high concentration of polyamines compared to other tissues in the body [34,37,39]. The polyamines spermidine, spermine, putrescine and cadaverine are the most abundant in the human intestine [34]. Almost all cells are capable of *de novo* synthesis of the polyamines spermidine and putrescine, with limited exceptions; whereas, spermine synthesis is mainly confined to eukaryotic cells and cadaverine synthesis is mainly present in prokaryotic cells [34,42,43]. The largest portion of exogenous polyamines in the gastrointestinal tract comes from our diet [34]. The average human diet supplies the body with hundreds of micromoles of spermidine, spermine and putrescine per day [34,44]. Foods vary substantially in their polyamine content and distribution, with some foods having an extremely high concentration and diversity of polyamines, while others have very little [34]. Examining the contribution of polyamines by the intestinal microbiota is also essential when assessing polyamine concentrations in the gastrointestinal tract. Bacteria are believed to be the major contributors of polyamines, especially cadaverine, in the distal portion of the gastrointestinal tract [45,46].

Vibrio cholerae colonizing the distal portion of the small intestine are likely to encounter the four previously mentioned polyamines based on human and animal studies which found millimolar levels of polyamines at the distal portion of the small intestine where *V. cholerae* have been shown to preferentially colonize [45,47]. Since these host-derived molecules have been found at relatively high concentrations in the small intestine it is

possible that they play a role in cholera pathogenesis. Thus, the goal of this study was to explore whether or not these polyamines have an effect on *V. cholerae* virulence properties. We show that at high concentrations all of the polyamines tested negatively influenced autoagglutination of *V. cholerae in vitro*, suggesting that these host-derived molecules could modulate colonization of the intestines by this bacterium.

MATERIALS AND METHODS

Bacterial Strains, Media and Reagents

Bacterial strains and plasmids used in this study are displayed in Table 1. Primers used for the deletion of *potD1* in O395 are found in Table 2. *E. coli* strains were grown in LB broth (1% Tryptone [Amresco®, Solon, O.H.], 0.5% Yeast Extract [Amresco®], and 0.5% NaCl [Amresco®]) at 37°C, unless otherwise noted. *Vibrio cholerae* strains were grown in 1% tryptone broth (1% [w/v] Tryptone, 66mM NaCl), pH 8.5, 200 rpm at 37°C. For the induction of the virulence genes, *V. cholerae* were grown in 1% tryptone broth, pH 6.5 with shaking at 150 rpm at 30°C [19]. Antibiotics used in this study were at the following working concentrations: 100 µg/ml Streptomycin (Sm) (FisherBiotech, Fair Lawn, N.J.), 100 µg/ml or 50 µg/ml Ampicillin (Ap) (FisherBiotech) and 50 µg/ml Kanamycin (Kn) (FisherBiotech). All strains were stored at -80° C in LB broth supplemented with 15% glycerol. Spermidine trihydrochloride, putrescine dihydrochloride, spermine tetrahydrochloride, and cadaverine dihydrochloride were from Sigma-Aldrich (St. Louis, M.O.). The monovalent and divalent salts used in this study were magnesium chloride hexahydrate (Sigma-Aldrich), calcium chloride dehydrate granular (Mallinckrodt, Paris, Kentucky), sodium chloride (Amresco®) and potassium chloride (Fisher Scientific, Fair Lawn, N.J.). Phusion and One*Taq* DNA polymerases, T4 Ligase, *Spe*I and *Xho*I were purchased from New England BioLabs (Ipswich, M.A.).

Agglutination Assay

V. cholerae strains O395, O395 (pSMC2), and O395::Δ*potD1* were streaked on fresh LB plates with 1.5% agar (Alfa Aesar, Ward Hill, M.A.) containing either streptomycin or 100 µg/ml ampicillin and incubated at 37°C overnight. Single colonies from overnight plates were inoculated into 1% tryptone broth containing either streptomycin or 100 µg/ml ampicillin and incubated overnight in a shaking incubator (220 rpm) at 37°C. Overnight cultures were diluted 1/100 into 2 ml 1% tryptone broth containing either streptomycin or 100 µg/ml ampicillin and incubated in a shaking incubator (250 rpm) at 37°C to mid-log phase (A_{595} 0.3 – 0.4) as determined by measuring 100 µl of the culture in microtiter plates using a Bio-Rad model 680 microplate reader (BioRad, Hercules, C.A.). *V. cholerae* day cultures were inoculated at a 1:10,000 dilution into 125 ml Erlenmeyer flasks containing 20 ml of 1% tryptone broth at either pH 6.5 or pH 8.5. To assess the effect polyamines and salts have on agglutination these cationic molecules were added to 20 ml 1% tryptone broth, pH 6.5, at increasing concentrations. Flasks were incubated in a shaking incubator (150 rpm) at 30°C for 18 hours. Following this incubation, 2 ml aliquots were removed from the cultures, in triplicate, briefly vortexed, and optical densities were measured at 595 nm. Cultures were allowed to sediment for 1 hour, and final optical density measurements were taken. Autoagglutination was quantified by subtracting the final A_{595} from the initial A_{595} and subtracting this number from 1.

$$\text{Sedimentation Index} = 1 - (\text{Final } A_{595} / \text{Initial } A_{595})$$

Construction of* Δ*potD1 Mutant

A 435bp fragment upstream of *potD1* and a 432bp fragment downstream of *potD1* were amplified by Phusion polymerase using primer pairs P332/P333 and P334/P335, respectively (Table 2). The antisense primer for amplification of the upstream fragment and the sense primer used for amplification of the downstream primer contained a complementary overhang which allowed for the splicing of the two fragments removing 925bp of the 1038bp *potD1* gene. The deletion construct was confirmed for correct size by PCR using primers P332 and P335. The deletion construct was gel purified by the Wizard® SV Gel and PCR Clean-Up System (Promega, Fitchburg, W.I.) using the manufacturer's protocol and then TOPO cloned into pCR2.1, using the manufacturer's protocol and finally transformed into One Shot® DH5α™ chemically competent cells (Invitrogen, Grand Island, N.Y.). The pCR2.1::Δ*potD1* plasmid was isolated from DH5α using the Wizard®Plus SV Minipreps Kit (Promega), and 1µg of this plasmid was sent to Cornell University Life Sciences Core Laboratories Center for sequencing (Ithaca, N.Y.). Once the deletion construct was confirmed, ~2µg of pCR2.1::Δ*potD1* plasmid was digested with *Spe*I and *Xho*I in a 40 µl reaction containing 0.4 µl 100X BSA, 1µl *Spe*I, 1µl *Xho*I, 1µ RNase and 4µl NEB 4 buffer for 2.5 hours at 37°C. The pCR2.1::Δ*potD1* digest was run on a 1% (w/v) agarose gel and the ~900bp Δ*potD1* insert was excised from the gel and purified using Wizard® SV Gel and PCR Clean-Up System (Promega). The digested Δ*potD1* insert was next ligated into the *Spe*I and *Xho*I digested and gel purified pWM91 plasmid. The ligation mixture contained 5µl T4 ligase buffer, 4.5µl pWM91 digested vector, 9.5µl Δ*potD1* insert and 1µl T4 ligase and was incubated at 16°C overnight. The ligation mixture was then transformed into chemically competent SM10λPir cells. Briefly, ligation mixture was added to the chemically

competent cells, and the mixture was heat shocked at 42°C for 70 seconds. Cells were immediately incubated on ice for 3 minutes, then allowed to recover for 1 hour with shaking (200 rpm) at 37°C in SOC media (2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ 7-hydrate, 20mM D-Glucose) and plated on LB agar plates containing 100 µg/ml ampicillin. Transformants were confirmed by colony PCR using primers P332 and P335.

Colony PCR was carried out as follows: a single colony was resuspended in 100µL sterile deionized water in a 0.2ml PCR tube. Bacterial cells were lysed by heating the PCR tube to 95°C for 5 minutes in an Eppendorf Mastercycler (Eppendorf, Hauppauge, N.Y.). Bacterial debris was pelleted by centrifugation for 5 minutes at 8,000 x g. Two µl of the supernatant, 16.4 µl deionized water, 0.5 µl 10mM dNTP, 5 µl One*Taq* buffer, 0.125 µl One*Taq*™ DNA polymerase, 0.5 µl 100mM P332 and 0.5 µl P335 were combined in a PCR tube and placed in an Eppendorf Mastercycler (Eppendorf). The cycling parameters were as follows: an initial 5 minute denaturing cycle at 95°C, 30 cycles with each cycle containing a 30 second denaturing step at 95°C, a primer annealing step at 60°C for 30 seconds, and an extension step for 1 minute at 72°C. Finally, an extension step was performed for 5 minutes at 72°C.

Once the transformation had been confirmed, pWM91::*ΔpotD1*, referred to hereafter as pBG2, was conjugated into *V. cholerae* by mating strains O395 and SM10λ Pir (pBG2) on nonselective LB agar at 37°C overnight. Bacteria from mate plates were streaked for isolation on selective LB agar plates containing streptomycin and 50 µg/ml ampicillin, at 37°C overnight. Individual colonies from selective agar plates were purified by plating on LB agar plates containing streptomycin and 50 µg/ml ampicillin and incubated at 37°C,

overnight. Single colonies were inoculated into 1ml LB and incubated in a shaking incubator (250 rpm) for 4 hours. Cultures were diluted 1/1000 into fresh LB media and plated on sucrose plates (1.5% Agar, 1% Tryptone, 0.5% Yeast Extract, 10% [w/v] sucrose) supplemented with 100mM spermidine and streptomycin. This protocol was attempted multiple times previously without supplementing the sucrose plates with spermidine and failed. Addition of spermidine resulted in successful double homologous recombination for unknown reasons. One possibility is that recovery of the wild-type gene, which occurs at approximately 50% of the time with this protocol, may have hindered the growth of the recombinant strains containing the wild-type gene in the presence of spermidine, thereby providing a positive selection effect. Sucrose plates were incubated overnight at room temperature, and then transferred to 30°C for approximately 12 hours. Translucent colonies were transferred to both streptomycin plates and 100µg/ml streptomycin / 50µg/ml ampicillin plates, and incubated at 37°C overnight. Streptomycin resistant ampicillin sensitive colonies, indicative of successful double homologous recombination, were used in a colony PCR using primers P332 and P335 to confirm the presence of the Δ*potD1* deletion on the chromosome.

O395 CTXϕKn Transduction Assay

Vibrio cholerae strain CL101 was streaked onto an LB agar plate containing 50 µg/ml kanamycin, and incubated at 37°C overnight. A single colony was inoculated into 2 ml LB broth containing streptomycin and incubated in a rotating incubator (220 rpm) for 16 hours at 37°C. Bacterial cells were pelleted in an Eppendorf 5412 D microcentrifuge (Eppendorf) at 16,100 x g. The supernatant was removed and filtered through a 0.2µm syringe filter

(Corning Inc., Corning, N.Y.) to remove any residual bacteria cells. Equal volumes of filtered supernatant containing CTXϕKn and cultures grown under virulence inducing conditions in the presence and absence of polyamines were mixed and incubated at room temperature for 30 minutes. Following the 30 minute incubation mixtures were serial diluted 1/10 eight times and plated on both streptomycin LB agar plates for total cell input, and kanamycin plates for total cells transduced. Plates were incubated 16 hours at 37°C. Transduction frequency was measured as the ratio of Kn^r colonies to the number of Sm^r colonies. Transduction efficiency was measured as the transduction frequency of the unknown sample compared to wild-type O395 transduction frequency. For statistical analysis, transduction assays were performed in triplicate.

SDS-PAGE and Immunoblotting

One ml aliquots were removed from bacterial cultures and pelleted in an Eppendorf centrifuge 5415D microcentrifuge (Eppendorf) at 16,100 x g for 30 seconds. The supernatant was transferred to a fresh microfuge tube for cholera toxin detection (described below), and bacterial cells were resuspended in 500 µl 1X PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, and 2mM KH_2PO_4, pH 7.4). Whole cell extracts were obtained by sonication of bacterial suspension for 10 seconds at 30% power, duty cycle 40, output 4 using a Heat Systems – Inc. Ultrasonic Processor (Farmingdale, N.Y.), followed by centrifugation at 16,100 x g for 20 minutes at 4°C. Total cell protein concentrations were quantified by Bradford assay. Ten µl of cell extracts and supernatants were aliquoted into a 96-well polystyrene microtiter plate along with 300 µl Coomassie Protein Assay Reagent

(Thermo Scientific). The solution was mixed in a Bio-Rad model 680 microplate reader for 30 seconds and then incubated at room temperature for 10 minutes. Protein concentrations for whole cell extracts and supernatants were quantified by comparing the A_{595} of unknown protein samples to the A_{595} of known protein concentrations.

Three µg of whole cell extract was mixed 1:1 with 2X Laemmli Sample Buffer (0.5M Tris-HCl pH 6.8, 20% [w/v] glycerol, 10% [w/v] Sodium Dodecyl Sulfate [SDS] 0.5% [w/v] bromophenol blue, 1.4% [v/v] β-Mercaptoethanol) and then heated in boiling water for 5 minutes. Samples were separated by SDS-PAGE with a 12% acrylamide resolving gel and 4% stacking gel at a constant 250 volts for 60 minutes using a Mini-Protean 3 cell system (BioRad) in 1X Glycine Tris running buffer (25mM Tris, 192mM glycine, 0.1% [w/v] SDS). A 2 x 3.5 inch piece of PVDF membrane was soaked in methanol for 30 seconds and equilibrated in transfer buffer (25mM Tris, 192mM Glycine, 0.1% [w/v] SDS) along with the gel for 15 minutes. Proteins were transferred to the PVDF membrane using a semi-dry transfer cell (BioRad) at 15 volts for 15 minutes. The PVDF membrane was blocked overnight in 100 ml 5% (w/v) non-fat dry milk in 1X PBST (0.1% [v/v] Tween 20 solution in 1X PBS) with constant shaking at 4°C. The PVDF membrane was then incubated with a rabbit monoclonal anti-TcpA antibody, at a 1:50,000 dilution in 10 ml 1X PBST for 1 hour with constant rotation, followed immediately by three consecutive 1X PBST washes for 5 minutes each. Next, the membrane was incubated with an HRP-conjugated goat anti-rabbit antibody at a 1:10,000 dilution in 10 ml 1X PBST for 1 hour with constant rotation followed by three subsequent 1X PBST washes for 5 minutes each. The membrane was then incubated with SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) for approximately 5 minutes. Following incubation, membranes were exposed to X-ray film

(BioExpress, Kaysville, U.T.) for 10, 30, and 45 second intervals and developed in a Konica Minolta SRX-101A developer (Konica Minolta Medical & Graphic, Inc., China).

Whole cell ELISA

Experimental procedure was followed as previously described, with minor modifications [48]. Five ml of cultures grown under virulence inducing conditions in the presence and absence of polyamines were pelleted at 3,320 x g in a clinical centrifuge (International Equipment Co., Needham, M.A.) in 15-ml conical tubes. Cells were resuspended in 500 µl 1X PBS in a microcentrifuge tube and vortexed vigorously for 1 minute using a Vortex Genie 2 (Scientific Industries, Bohemia, N.Y.). Large microcolonies which were not dispersed by vortexing were pelleted by centrifugation at 300 x g for 2 minutes in an Eppendorf Centrifuge 5415D (Eppendorf). Four hundred µl of the top layer was removed and placed in a fresh microcentrifuge tube. Optical density of 100 µl of the samples was measured using Bio-Rad model 680 microplate reader at A_{595}. Cells were resuspended in 250 µl 1X PBS at an optical density of 0.1 and aliquoted into 0.4 ml Nunc 96-well Flat Bottom Immuno Plates (Thermo Scientific). Aliquots were serial diluted 1/5 into 200 µl 1X PBS for the remaining wells and incubated at 37°C for 1 hour. Wells were washed three times with 1X PBST for 5 minutes each. The wells were blocked with 200 µl 5% (w/v) Bovine Serum Albumin (BSA) in 1X PBS and incubated at 37°C for 1 hour. Wells were washed as described previously followed by the addition of 1 µl anti-TcpA antibody diluted 1:5,000 in 1X PBS and subsequent incubation at 37°C for 1 hour. Following incubation with the primary antibody, wells were washed as previously described, followed

by the addition of 1 ul of HRP-conjugated goat anti-rabbit antibody diluted 1:5,000 in 1X PBS. Plates were then incubated at 37°C for 1 hour. Wells were again washed as previously described, followed by a single 1X PBS wash for 5 minutes. Following the removal of the final 1X PBS wash, enzymatic detection was performed by the addition of 100 µl TMB Super Sensitive One Component HRP Microwell Substrate (*SurModics*®, Eden Prairie, M.N.) and allowed to incubate at room temperature approximately 5 minutes. Enzymatic reactions were stopped by the addition of 100 µl 1N HCl. Optical densities were measured at A_{415} for wells using a Bio-Rad model 680 microplate reader (BioRad). Assays were performed in triplicate for statistical analysis.

Cholera Toxin ELISA

Agglutination assays were performed as described above. A 1 ml aliquot was removed from cultures grown under virulence inducing conditions in the presence and absence of polyamines and pelleted in an Eppendorf centrifuge 5415D microcentrifuge (Eppendorf) at 16,100 x g for 30 seconds. The supernatant was transferred to a fresh microfuge tube and stored at -20°C until used for cholera toxin detection.

Nunc 96-well Flat Bottom Immuno Plates were coated with 100µl of 2 µg/ml Monosialoganglioside GM_1 (Sigma-Aldrich) and incubated at room temperature for 4 hours. Following incubation, plates were blotted on an absorbent cloth to remove liquid. Wells were washed three times with 1X PBST for 5 minutes each. The wells were blocked with 200µl binding buffer (1X PBS, 0.05% [v/v] Tween 20, 0.5% [w/v] BSA) and incubated at room temperature for 1 hour. Liquid was removed and wells were washed as previously

described. Supernatants to be analyzed were diluted 1:10 in 1X PBS, and 100µl was added to the wells, in quadruplicate. Plates were then incubated at room temperature for 1 hour. Following the removal and washing of wells, 100µl of anti-CtxB antibody (Abcam, Cambridge, M.A.), diluted 1:5,000 in binding buffer, was added to each well. Plates were then incubated at room temperature for 1 hour. Following the removal and washing of wells, 100µl of an HRP conjugated goat anti-rabbit antibody, diluted 1:20,000 in binding buffer, was added to each well. Plates were then incubated at room temperature for 1 hour. Following the removal and washing of wells, enzymatic detection was performed by the addition of 100µl TMB Super Sensitive One Component HRP Microwell Substrate (*SurModics*®, Eden Prairie, M.N.) and incubated at room temperature for approximately 5 minutes to allow enzymatic reaction to occur. Enzymatic reactions were stopped by the addition of 100 µl 1N HCl. Absorbances were measured at A_{415} for wells using a Bio-Rad model 680 microplate reader (BioRad).

Transmission Electron Microscopy

Five µl aliquots were removed from bacterial cultures and placed on a section of 5 cm x 5 cm parafilm. Three hundred-mesh formvar coated copper grids (Ted Pella Inc., Redding, C.A.) were inverted on top of the 5 µl aliquots for 5 minutes to absorb bacteria to the grids. Remaining bacteria was wicked from grids using VWR Light-Duty Tissue Wipers (VWR, West Chester, P.A.) and grids were washed by inverting onto 5 µl sterile deionized water for 10 seconds. Grids were negatively stained for 5 minutes by inverting onto 5 µl filter sterilized 1% (w/v) Phosphotungstic acid (EMD, Gibbstown, N.J.) pH 7. Remaining stain

was wicked from the grids as previously described, and grids were allowed to air dry at room temperature for 2 days. Grids were imaged in a JEM-1400 S/TEM (JEOL Inc., U.S.A.) at 120 keV.

Confocal Microscopy

V. cholerae strain O395 (pSMC2) was grown under agglutinating and non-agglutinating conditions. Ten µl from each sample was analyzed by fluorescent microscopy on 25 x 75 x 1 mm micro slides (VWR International, U.S.A) with 22 x 22 mm micro cover glass (VWR International). Microcolonies were visualized with a Plan-Apochromat 10X/0.45 objective lens. Images were obtained from a LSM 510 confocal scanning system (Zeiss, Thornwood, N.Y.).

Static Biofilm Assays

Vibrio cholerae strain O395 was streaked for isolation onto a fresh LB agar plate containing streptomycin, and incubated overnight at 37°C. LB media containing streptomycin was inoculated with a single colony from the freshly streaked plate, and incubated in a rotating incubator (220 rpm) overnight at 37°C. The overnight culture was diluted 1/1000 into LB media with streptomycin, and grown in a rotating incubator (220 rpm) at 37°C until mid-log phase. Optical densities for day cultures were measured in a Bio-Rad model 680 microplate reader (BioRad) at A_{595}, and diluted into fresh LB media containing streptomycin, with a starting optical density of 0.04. Polyamines were then added to the

media at a concentration of 10mM. Three hundred µl aliquots from master mixes were inoculated into sterile 10x75mm borosilicate glass tubes (Fisher Scientific), in triplicate. Tubes were incubated at 30°C under static conditions for 24 hours. One hundred and fifty µl of planktonic cells were removed and quantified by measuring A_{595} from biofilm tubes. The remaining planktonic cells were removed, and biofilms were washed with 300 µl 1X PBS. Biofilms were stained for 25 minutes with 350 µl of a 1% (v/v) crystal violet solution (EMD, Gibbstown, N.J.). The crystal violet solution was removed and biofilms were washed three times with 450 µl deionized water. Crystal violet was solubilized in 400 µl dimethyl sulfoxide (Thermo Scientific) and 150 µl of the solubilized crystal violet solution was quantified by measuring A_{595}.

Table 1. Strains and Plasmids

Strain/Plasmid	Genotype	Reference
***E. coli* Strains**		
DH5α™-T1^R	F$^-$ ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17 (r_k^-, m_k^+) *pho*A*sup*E44 *thi*-1 *gyr*A96 *rel*A1 *ton*A	Invitrogen
SM10λ Pir	*thi rec*A thr leu *ton*A *lac*Y *sup*E RP4-2-Tc::Mu λ::pir	Lab Strain
AK284	DH5α (pBG1), Ap^r	This Study
AK286	SM10λ Pir (pBG2), Ap^r	This Study
***V. cholerae* Strains**		
AK171	Classical O395, Sm^r	[3]
AK307	O395::Δ*potD*1, Sm^r	This Study
AK305	O395 (pSMC2), Sm^r Ap^r	This Study
CL101	O395 (pCTX-Knϕ), Sm^r Kn^r	[13]
Plasmids		
pCR2.1- TOPO	Plasmid for TOPO cloning, Ap^r	Invitrogen
pWM91	oriR6K mobRP4 lacI Ptac tnp mini-Tn10Km; Kn^r, Ap^r	[49]
pBG1	pCR2.1::Δ*potD*1	This Study
pBG2	pWM91::Δ*potD*1	This Study
pSMC2	pKEN carrying a bright mutant of gfp and 1.8 kb stabilizing fragment from pUC181.8; Ap^r	[50]

Table 2. Primers

Primer	Sequence
Construction of potD1 deletion	
P332	5’ AGAAGGCGGTATCGGTTGGG 3’
P333	5’ TTACGAGCGGCCGCATGCGCACAGAGCACTCGC 3’
P334	5’ TGCGGCCGCTCGTAAGGCGAGTGGCAAGATGAA 3’
P335	5’ AGCGCGATTCTCTGGTTCCC 3’

RESULTS

Polyamines inhibit autoagglutination of V. cholerae

In order to assess one property associated with *V. cholerae* virulence, autoagglutination, a simple agglutination assay was performed on cultures grown in the presence and absence of polyamines. When *V. cholerae* is cultured under optimal virulence factor inducing conditions, cells begin synthesizing TCP which inherently leads the cells to autoagglutinate and form microcolonies. Since microcolonies are heavier, they sediment to the bottom of the media leaving any cells which did not form microcolonies suspended in the media (Figure 2). A relative quantification of sedimentation is achieved by measuring the absorbance of the media before and after sedimentation of the microcolonies in the media. A sedimentation index for each sample can then be calculated from the sedimentation measurements, which can be used to compare samples cultured in the presence and absence of polyamines. When *V. cholerae* was cultured in the presence of the polyamines spermidine, spermine, putrescine and cadaverine we found these polyamines to have a concentration dependent inhibitory effect on autoagglutination (Figure 3). The concentration of each polyamine added to the media was normalized to one another to take into account the different number of charged amine groups found on each polyamine. In the presence of spermidine, autoagglutination was inhibited by nearly 50% at 10mM and 20mM, and 80% at 30mM. Spermine inhibited autoagglutination by approximately 50, 70, and 85% at concentrations of 7.5, 15, and 22.5mM, respectively.

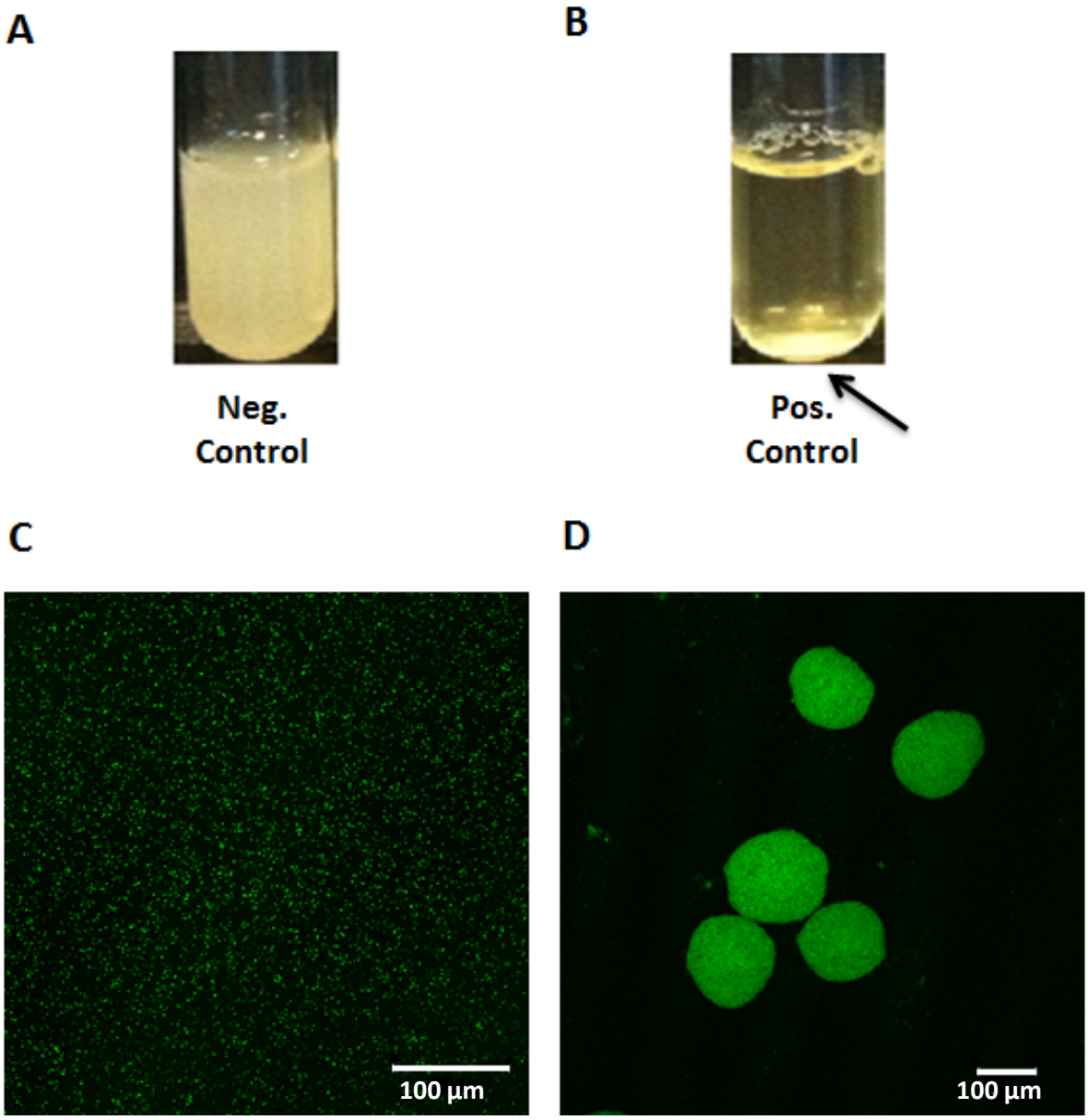

Figure 2. Appearance of agglutinated and unagglutinated *V. cholerae* as assessed by sedimented microcolonies and confocal microscopy. Appearance of *V. cholerae* cells after culturing bacteria under non-virulence (**A**) and virulence expressing (**B**) conditions. Black arrow points to sedimented microcolonies. Confocal microscopy images of *V. cholerae* cells after culturing bacteria under non-virulence (**C**) and virulence gene expressing (**D**) conditions. *V. cholerae* used for confocal microscopy contained a GFP-expressing plasmid.

This same effect was witnessed with cadaverine, although the inhibitory effect this polyamine had on autoagglutination was not as significant as the others tested since autoagglutination was only inhibited 10, 20, and 70% from concentrations of 15, 30, and 45mM cadaverine, respectively. Putrescine had the most drastic effect by inhibiting autoagglutination at all concentrations tested by 50-95% compared to cultures grown in the absence of polyamines. The data show that spermidine, spermine, putrescine, and cadaverine all have an inhibitory effect on autoagglutination in a concentration dependent manner.

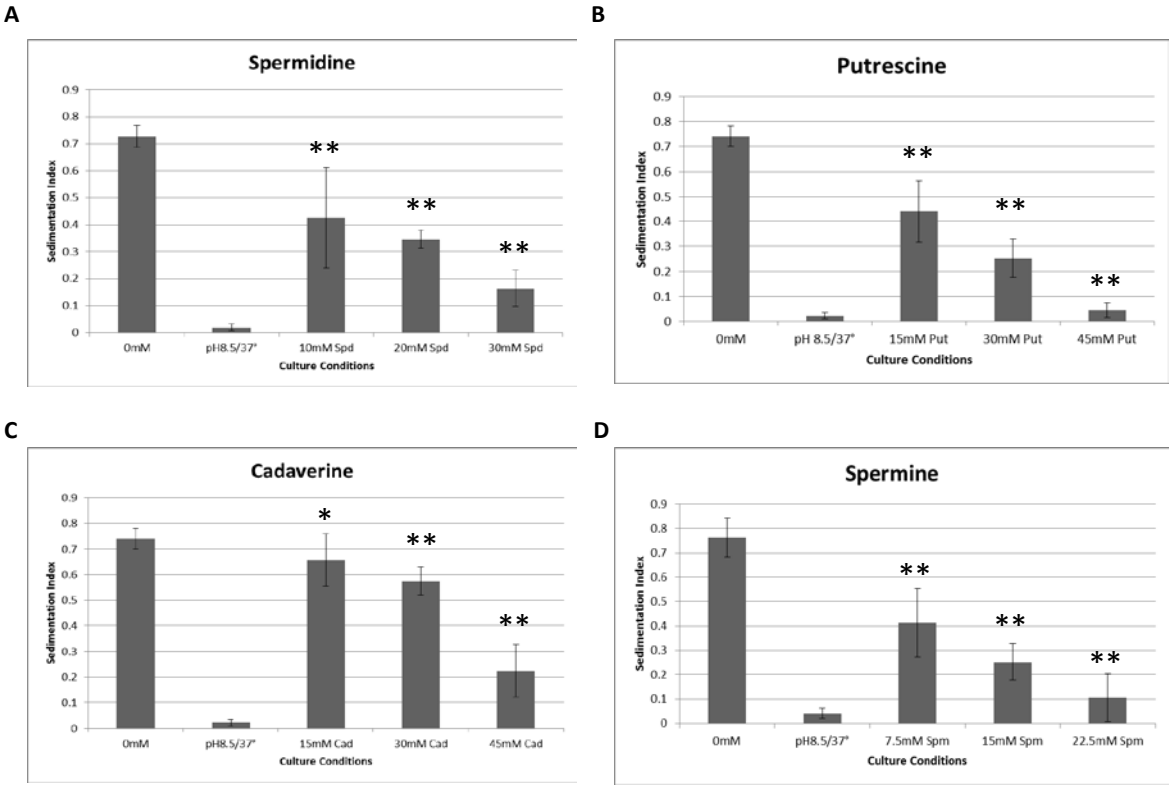

Figure 3. The effect of polyamines on *V. cholerae* autoagglutination. (**A**) *V. cholerae* cultured in the presence of increasing concentrations of Spermidine (**B**) Putrescine (**C**) Cadaverine (**D**) Spermine. Sedimentation index is calculated as 1 – (Final A_{595} / Initial A_{595}). Negative controls are represented as samples grown in media of pH 8.5 at 37°C. Each sample is the average of three biological replicates with error bars representing the standard deviation. To obtain *P* values Student *t* tests were performed comparing samples to cultures grown in the absence of polyamines (* represents a *P* value <0.05, ** represents a *P* value <0.001).

Deletion of the V. cholerae potD1 gene effects autoagglutination in comparison to wild-type

Polyamines are transported across the bacterial inner membrane via ATP binding cassette transport systems [51]. The *potD1* gene, encoding the ligand binding protein of the spermidine transporter [29], was deleted to determine whether the inhibitory effect witnessed in the presence of spermidine was due to an internal or external effect. In the absence of PotD1, cells would be unable to import spermidine into the cell, thus any internal effect spermidine may have on autoagglutination would be abolished. Agglutination assays were performed on the mutant strain, O395::Δ*potD1*, and the wild-type strain in the presence and absence of increasing concentrations of spermidine. The Δ*potD1* mutant strain autoagglutinated significantly better than wild-type strains at concentrations of 5mM and 10mM of exogenous spermidine. This difference was not observed in the presence of 30 mM spermidine in the media (Figure 4). These results suggest spermidine is affecting autoagglutination by both an internal and external mechanism.

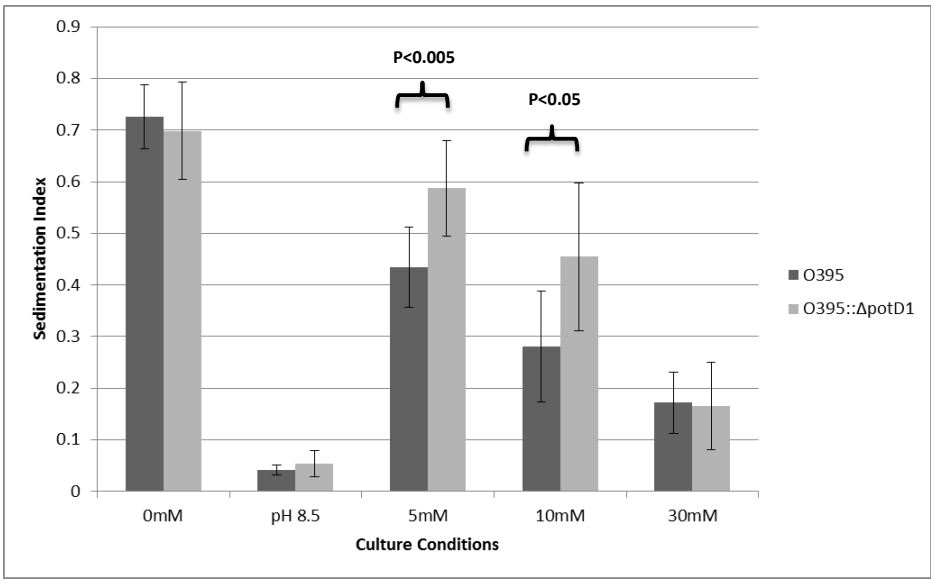

Figure 4. Effect of spermidine on autoagglutination of *V. cholerae* strain O395::Δ*potD1* and wild-type O395. Each sample is the average of three biological replicates with error bars representing the standard deviation.

Putrescine and cadaverine affect TcpA synthesis, but spermidine and spermine do not have a significant effect

Since *V. cholerae* autoagglutination was shown to be inhibited by polyamines in a concentration dependent manner, I next wanted to determine whether these small molecules were inhibiting the synthesis of the major pilin subunit, TcpA. Like many type IV pili, TCP is composed of a major pilin subunit which is synthesized in the cytosol and transported to the periplasm. They are then incorporated into a growing pilus filament which is held together by hydrophobic and electrostatic interactions. Since polyamines have been shown to bind to DNA, RNA, and ribosomes in the cell where they are found at millimolar levels [34], it is plausible these molecules could be inhibiting TcpA synthesis by disrupting either the transcription of *tcpA* or the translation of *tcpA* mRNA.

Western blot analysis of whole cell extracts from samples grown in the presence and absence of polyamines showed that putrescine and cadaverine were the only polyamines to have a significant effect on TcpA levels compared to cultures grown in the absence of polyamines (Figure 5). Densitometry analysis from three biological replicates showed putrescine and cadaverine significantly reduced TcpA levels at all concentrations tested (Table 3). Interestingly, putrescine had a more pronounced inhibitory effect on TcpA synthesis than cadaverine with an approximate 50% reduction in TcpA levels compared to only a 30% reduction seen with cultures supplemented with cadaverine. These results suggest that spermidine and spermine affect agglutination by a mechanism that does not involve reduction of TcpA production, whereas the effect of putrescine and cadaverine on autoagglutination can be partially explained by their effect on TcpA levels.

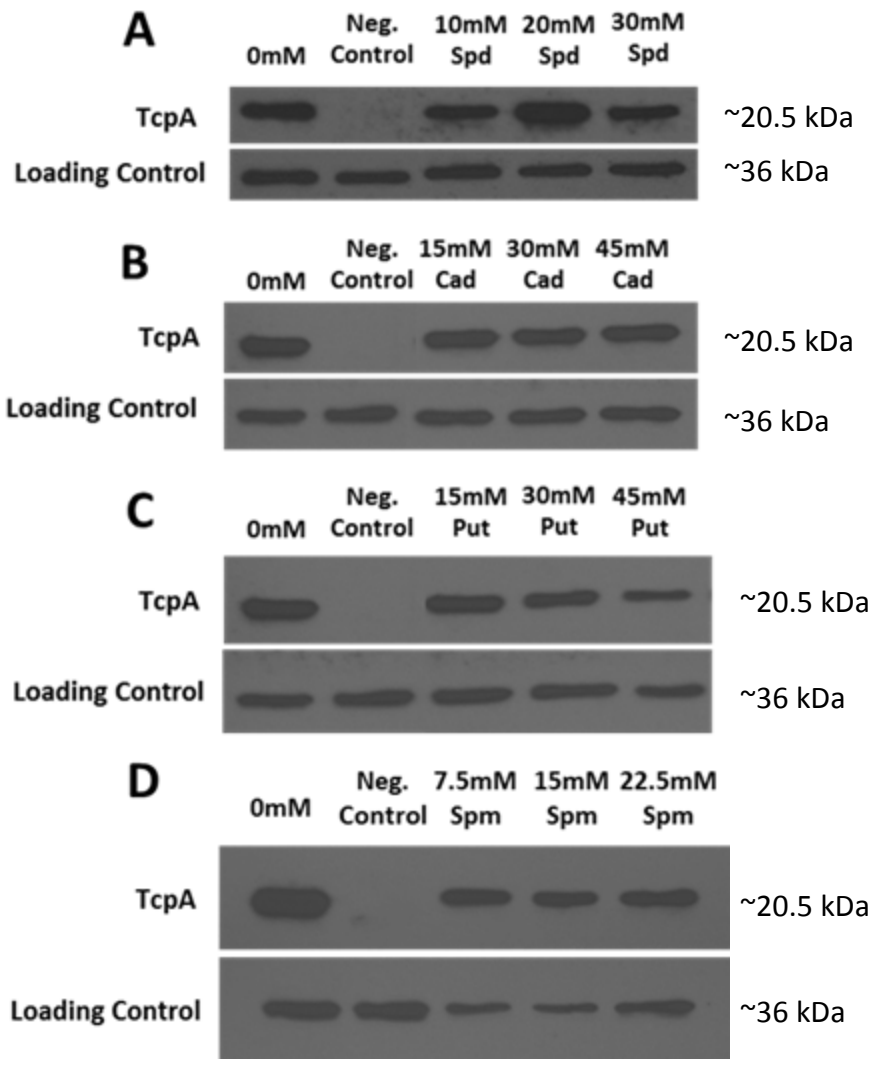

Figure 5. Effect of polyamines on TcpA levels. Western blot analysis of whole cell extracts from samples grown in the absence or presence of Spermidine (**A**) Cadaverine (**B**) Putrescine (**C**) Spermine (**D**). Negative controls were grown under non-virulence inducing conditions. The loading control was probed using an antibody against the α-subunit of the *E. coli* RNA polymerase.

Only putrescine has a significant effect on TCP biogenesis

The proteins which make up the TCP biogenesis apparatus are coded for by genes which are present downstream of the *tcpA* gene and are co-transcribed with *tcpA*. If the polyamines are disrupting the biogenesis of TCP by inhibiting a portion of the biogenesis

apparatus this may explain why spermidine and spermine inhibit agglutination and not decrease TcpA levels. To assess if polyamines affected synthesis of TCP, a whole cell ELISA assay was performed to quantify cell-associated TCP (Table 3). Putrescine, at a concentration of 45mM, led to an approximately 25% reduction in surface associated TCP compared to cultures grown in the absence of polyamines. Interestingly, cadaverine, another diamine, had no significant effect on TCP biogenesis even though TcpA levels were found to be inhibited by approximately 25% in a concentration of 45mM cadaverine compared to cultures grown in the absence of polyamines. Spermidine and spermine also did not influence the number of cell associated TCP present on the cell surface. This result confirms that cells are able to synthesize TCP filaments in the presence of spermidine, spermine, and cadaverine at levels comparable to cultures grown in the absence of polyamines, and thus they do not affect the TCP biogenesis apparatus. Conversely, putrescine does appear to inhibit the relative number of cell-associated TCP on the cell surface indicating this polyamine affects the TCP biogenesis apparatus.

Polyamines do not affect pilus morphology or bundling but do affect pilus functionality

TCP from the classical strains of *V. cholerae* which have undergone autoagglutination have been shown to associate laterally into rope-like bundles [3,11]. These pilus bundles are thought to be held together by electrostatic interactions between neighboring TcpA. Mutations in charged residues on TcpA have been shown to alter pilus bundling morphology by inhibiting the formation of rope-like bundles [11]. It is this association of pilus bundles from neighboring cells which are believed to mediate microcolony formation. Kirn *et. al.* showed that mutating charged residues within TcpA inhibits autoagglutination, suggesting that these charged residues interact to form the characteristic pilus bundles. Since

spermidine, spermine, and cadaverine do not appear to be affecting TCP biogenesis I hypothesized these cationic molecules may be disrupting a step associated with pilus bundling. Using transmission electron microscopy (TEM) I examined whole cell cultures grown in the presence and absence of polyamines to identify whether these molecules were affecting pilus bundling. To maximize the effect on autoagglutination, the polyamines were used at the concentration which inhibited ≥70% of agglutination. TEM analysis revealed that laterally associated rope-like bundles were found in all samples, indicating that polyamines have no effect on the pilus bundling morphology (Figure 6). No discernible morphological differences were observed when *V. cholerae* was cultured in the presence of polyamines compared to cultures devoid of polyamines.

Since TCP has been implicated as being the high affinity receptor for the lysogenic phage CTXϕ, an assay was performed which identifies the functionality of TCP by measuring the ability of CTXϕ to transduce the cell. If TCP are not present or dysfunctional, then transduction of CTXϕ will be inhibited. Taking advantage of this fact, a CTXϕ was designed in which the *ctxAB* genes normally located within the phage genome were replaced with a kanamycin cassette [16]. This replacement created a selectable marker allowing for the identification of cells which had been transduced by CTXϕKn. All polyamines when used at concentrations which inhibited autoagglutination by ≥70%, led to an approximately 70-80% reduction in CTXϕKn transduction as compared to cultures grown in the absence of polyamines (Table 3). Data from TEM images and transduction assays suggests that the TCP may not be fully functional in the presence of polyamines.

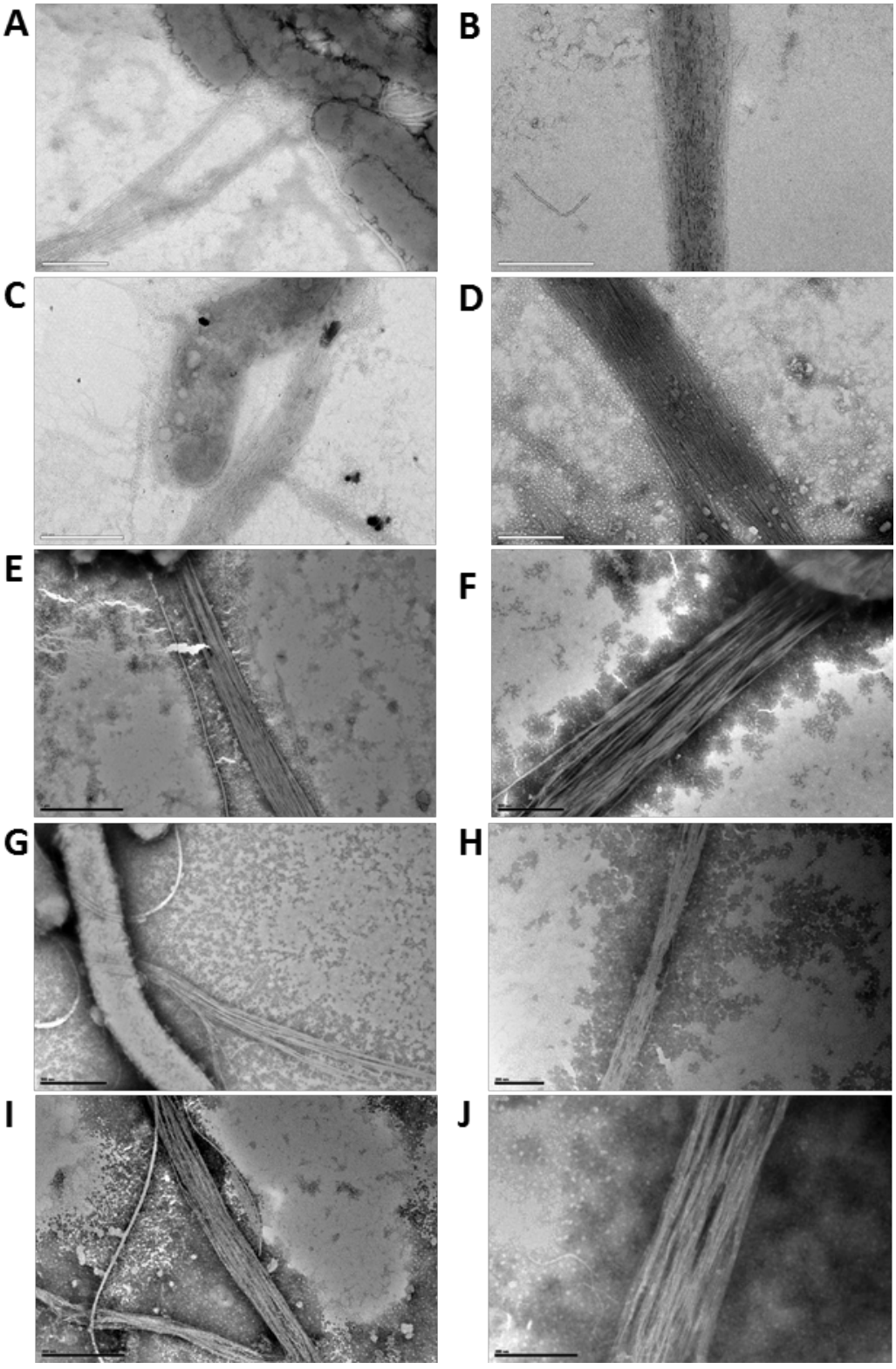

Figure 6. TEM images from cultures following agglutination assays. TEM images showing rope-like bundles of TCP from cultures grown in the presence and absence (**A,B**) of the polyamines spermidine (**C,D**) putrescine (**E,F**) cadaverine (**G,H**) and spermine (**I,J**).

Divalent cations inhibit autoagglutination more drastically than monovalent cations

Since polyamines are charged molecules at physiological pH, the effects on agglutination may be non-specific due to their charge. To determine whether the inhibitory effect on autoagglutination was polyamine specific, or simply due to an increase in charged molecules within the media, I performed agglutination assays with a number of monovalent and divalent cations. The salts for this study were used at the chosen concentrations to mimic the charge ratio of polyamine supplemented media. Autoagglutination was inhibited by both monovalent (sodium and potassium) and divalent cations (calcium and magnesium) although divalent cations had a more drastic effect compared to monovalent cations (Figure 7). Cultures grown in the presence of the salts sodium chloride and potassium chloride at concentrations of 30, 60, and 90mM inhibited autoagglutination by an average of approximately 5, 17, and 30% relative to cultures not supplemented with additional salts. Conversely, cultures grown in the presence of the salts calcium chloride and magnesium chloride at concentrations of 30, 60, and 90mM inhibited autoagglutination by an average of approximately 15, 43, and 80% relative to cultures not supplemented with additional salts. These results indicate the divalent cations had a similar effect to polyamines in modulating autoagglutination, whereas the monovalent cations did not decrease autoagglutination to the same extent as the polyamines.

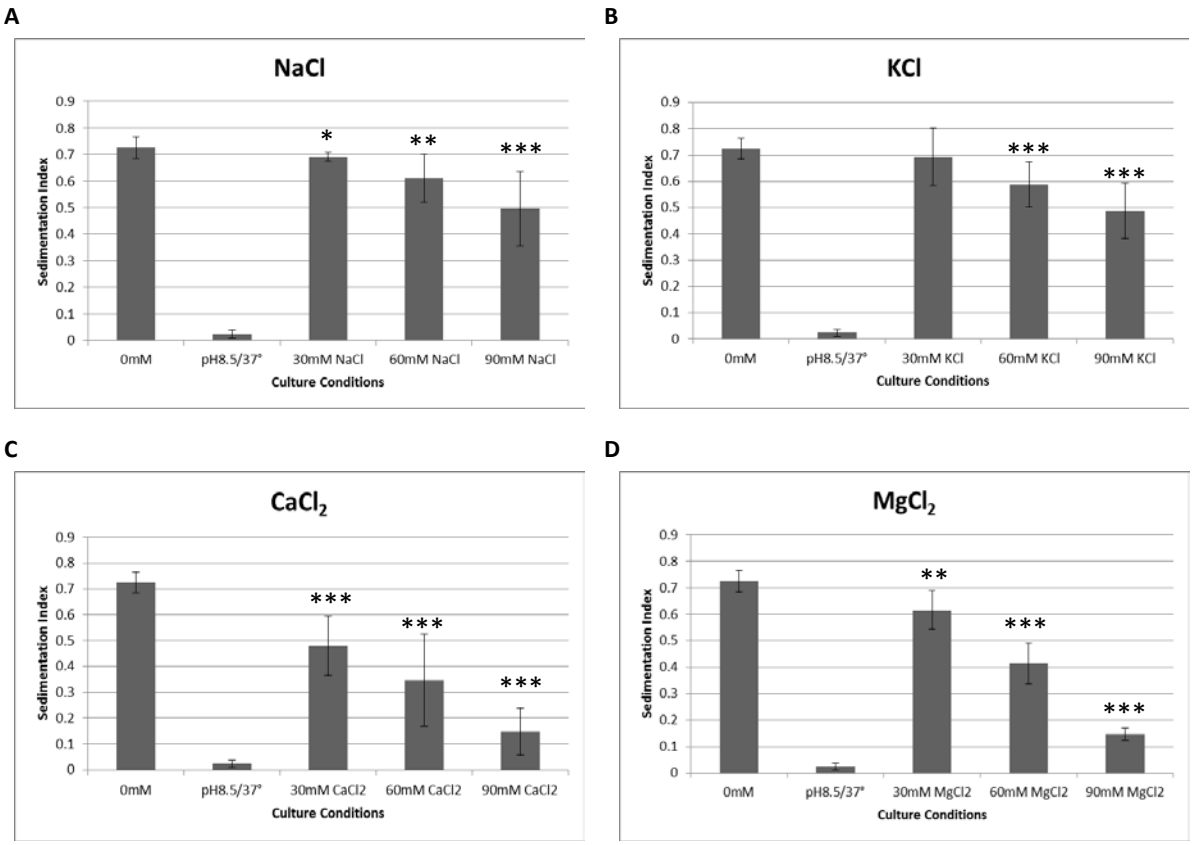

Figure 7. Effect of monovalent and divalent cations on *V. cholerae* autoagglutination. Effect of increasing concentrations of sodium chloride (**A**) potassium chloride (**B**) calcium chloride (**C**) and magnesium chloride (**D**) on *V. cholerae* autoagglutination. Sedimentation index is a representation of the sample's ability to autoagglutinate. Each sample is the average of three biological replicates with standard deviation. Negative controls were grown under non-virulence inducing conditions. To obtain *P* values Student *t* tests were performed comparing samples to cultures grown in the absence of salts (* represents a *P* value <0.05, ** represents a *P* value <0.005, and *** represents a *P* value <0.0005).

Polyamines do not affect cholera toxin synthesis

The genes responsible for the synthesis of cholera toxin (CTX) and TCP have been shown to be co-regulated by the major transcriptional regulator ToxT [18]. Since all polyamines tested significantly affected autoagglutination I wanted to determine whether these molecules were also affecting cholera toxin synthesis. Levels of cholera toxin in the

culture medium were assessed using an ELISA assay. The results showed that polyamines did not have a significant effect on cholera toxin synthesis (Table 3).

Table 3. Effect of polyamines on autoagglutination, TCP synthesis, and functionality relative to cultures with no polyamines.

Culture Conditions	SI	TcpA	TCP	Trans. Eff.	CTX
No polyamines	1	1	1	1	1
10mM Spd	0.49±0.15	1.12±0.02	--	--	0.99±0.02
20mM Spd	0.48±0.04	1.42±0.50	--	--	0.97±0.02
30mM Spd	0.23±0.11	1.17±0.06	1.2±0.13	0.20±0.11	0.99±0.02
15mM Put	0.53±0.19	0.85±0.15	--	--	1.00±0.00
30mM Put	0.34±0.12	0.57±0.11	--	--	0.99±0.01
45mM Put	0.06±0.05	0.55±0.09	0.72±0.08	0.21±0.26	0.97±0.01
15mM Cad	0.89±0.19	0.78±0.14	--	--	0.97±0.01
30mM Cad	0.78±0.11	0.73±0.05	--	--	0.97±0.02
45mM Cad	0.31±0.17	0.70±0.09	1.0±0.04	0.23±0.11	0.98±0.01
7.5mM Spm	0.53±0.17	0.89±0.16	--	--	0.91±0.02
15mM Spm	0.33±0.10	0.95±0.12	--	--	0.91±0.01
22.5mM Spm	0.14±0.13	0.80±0.25	0.95±0.07	0.31±0.15	0.88±0.02

- Numbers are the average of 3 biological replicates +/- standard deviation. Values were normalized to those obtained in the absence of polyamines, which are reported as "1". SI: sedimentation index, Trans. Eff.: Transduction efficiency, CTX: cholera toxin.

Spermidine is the only polyamine which significantly affects biofilm formation

Previous data has shown that polyamines affect biofilm formation in the O139 serogroup of *V. cholerae* [27,29]. Since polyamines have previously been implicated in the regulation of biofilm formation in another strain of *V. cholerae* we wanted to determine if this same affect would occur in the classical strain of *V. cholerae.* Following crystal violet

staining of biofilms attached to the edge of borosilicate tubes, differences in biofilm formation were observed both qualitatively and quantitatively as the level of crystal violet stained biofilm. Biofilm assays performed in the presence and absence of 10mM polyamines showed only spermidine had a significant effect on biofilm formation (Figure 8).

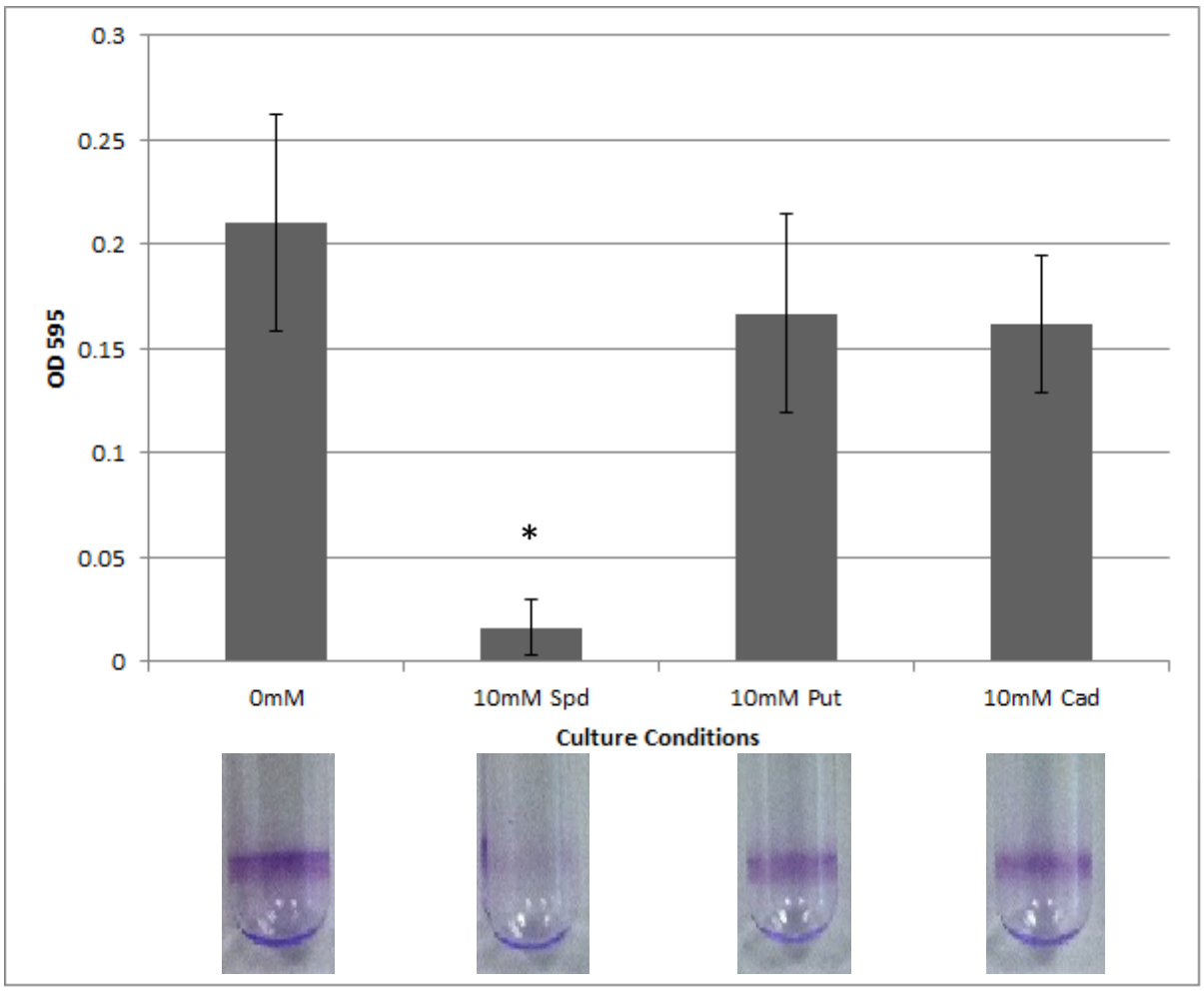

Figure 8. Biofilm assay of the classical strain of *V. cholerae* in the presence and absence of 10mM polyamines. Crystal violet stained biofilms are shown below the graph. Data is the average of three biological replicates with error bars representing standard deviation. To obtain *P* values Student *t* tests were performed comparing samples to 0mM (* represents a *P* value <0.000001).

DISCUSSION

The purpose of this study was to determine if polyamines, molecules that are abundant in the host intestinal environment, would modulate virulence properties of *V. cholerae*. I have shown that *V. cholerae* autoagglutination is inhibited by the four host-derived molecules spermidine, spermine, putrescine, and cadaverine. At the highest concentration tested, all polyamines inhibited agglutination by ≥70% compared to cultures grown in the absence of polyamines. To determine the mechanism by which polyamines inhibit agglutination, I then investigated several avenues by which polyamines could be modulating autoagglutination.

Autoagglutination is dependent upon the presence of TCP which putatively interact to mediate microcolony formation. The first step in the TCP biogenesis apparatus is the synthesis of functional TcpA, the major pilus subunit, which is then incorporated into the growing pilus filament. Therefore, reduction in TcpA levels could lead to lower amounts of TCP on the cell which in turn can result in a decrease in autoagglutination. Western blot analysis on whole cell extracts showed that putrescine and cadaverine had a significant effect on TcpA synthesis. Spermidine and spermine did not affect TcpA synthesis, indicating an alternative mechanism independent of TcpA synthesis.

The next step was to determine if these polyamines were affecting autoagglutination by disrupting the biogenesis of functional TCP on the cell surface. Quantification of cell surface associated TCP in the absence and presence of the polyamines showed that only

putrescine had a significant effect on the amount of TCP on the cell surface. This reduction of TCP by putrescine could be explained by the fact that TcpA levels are also reduced approximately 50% in the presence of putrescine compared to those grown in the absence of polyamines. This decrease in the availability of the major pilus subunit would inevitably lead to a reduction in the number of surface associated TCP present on the cell surface. In contrast, cadaverine did not affect cell-associated TCP amounts, which is in contradiction with its effect on TcpA levels in the cell. Contradicting data in which cadaverine appears to modulate TcpA levels in the cell yet has no effect on cell associated TCP levels may be explained by a potential threshold effect. A 30% reduction in TcpA levels seen in the presence of cadaverine may not be enough of a reduction to inhibit TCP levels whereas a 50% reduction may reduce TcpA levels enough to effect TCP levels.

I next hypothesized that the polyamines, especially spermidine and spermine, may affect the structural integrity of the pili rather than the amount on the cell surface. Recent data showed that mutating specific charged TcpA residues produced pili that did not form the characteristic rope-like bundles as seen with wild-type TCP but rather were wavier and not as tightly bundled [11,13]. TEM was utilized to determine whether polyamines affected formation of the TCP bundles. No morphological difference in TCP between cultures grown in the presence and absence of polyamines were observed. This observation confirms that polyamines were not affecting pilus bundling at a level that I could ascertain by TEM. A recent study has shown the appearance of rope-like bundles does not necessarily correlate with autoagglutination as mutations in several charged residues of TcpA still formed laterally associated rope-like bundles, yet autoagglutination was significantly impaired [11]. Also, recent work suggests there is a hierarchy to pilus bundling which involves pilus filaments

from individual cells laterally associating to form higher ordered rope-like bundles [15]. Once bundles are formed on individual cells, these smaller bundles go on to form larger bundles caused by a putative supertwist between neighboring pilus bundles. Since I was unable to discern these higher ordered supertwisted structures from single cell pilus bundles by TEM analysis, it is possible that polyamines may be inhibiting autoagglutination by disrupting these higher ordered, supertwisted structures. A piece of data supporting this hypothesis is that the transduction of the cells by CTXϕKn was found to be attenuated by nearly 80% in the presence of polyamines indicating that the TCP is not fully functional. It has been shown that transduction of CTXϕ is mediated by electrostatic interactions between the phage and TCP [17]. Point mutations of charged residues on TcpA were shown to either disrupt or enhance the transduction of CTXϕ. Thus, I hypothesize that these polyamines are inhibiting autoagglutination by electrostatically disrupting the pili-pili interactions which mediate autoagglutination.

The results presented so far suggest polyamines affect autoagglutination by an external mechanism. However, polyamines can also be imported into the cells and can potentially influence virulence properties by affecting intracellular processes. To determine if uptake of one of the polyamines, spermidine, influenced its effect on autoagglutination, agglutination assays were performed with a strain which cannot transport spermidine. This strain, Δ*potD1*, autoagglutinated significantly better at 5 and 10mM spermidine compared to wild-type. Interestingly, when the media was supplemented with 30mM spermidine, both wild-type and Δ*potD1* were equally inhibited in their ability to autoagglutinate. These results suggest spermidine has a dual internal and external effect on autoagglutination, although

there appears to be a concentration threshold in which the external mechanism outweighs the internal mechanism since at 30mM autoagglutination was inhibited equally in both strains.

In *V. cholerae*, production of the TCP and CTX are thought to be co-regulated as the *tcp* operon and *ctxAB* genes are under the control of the major transcriptional regulator, ToxT. Because putrescine and cadaverine were shown to decrease cellular TcpA levels, I hypothesized that they may also decrease CTX production. Interestingly, polyamines did not affect cholera toxin levels. Another intestinal compound, bile acids, were recently identified as modulators of cholera toxin synthesis in a ToxT-independent manner, indicating the paradigm where the ToxT protein co-regulates both *ctxAB* and the *tcp* operon does not necessarily apply under all circumstances [21].

One possible explanation of the effect polyamines are having on autoagglutination is that these molecules, which are positively charged under these experimental conditions, are electrostatically inhibiting pilus bundling. Therefore, I wanted to test whether other cationic molecules could potentially have a similar effect on autoagglutination. I found that the divalent cations, calcium and magnesium, had a similar inhibitory effect as polyamines on autoagglutination, whereas the monovalent cations, sodium and potassium, did not have a drastic effect. Effects of high concentrations of salts on virulence gene expression in *V. cholerae* have previously been reported. It has been shown that virulence gene expression by *V. cholerae* is very sensitive to the osmolarity of the media [24]. Osmolarity is the measure of solute concentration of a solution, and can be determined by multiplying the osmotic coefficient of a particular solute by the number of particles the solute dissociates into by the molar concentration of the solute. Each solute has its own osmotic coefficient which is simply the degree of dissociation of that particular solute, and is a number between 0 and 1,

with 1 being 100% dissociation of the solute in solution. Studies have shown that a NaCl concentration between 66-86mM was optimal for virulence gene expression *in vitro*, which is equivalent to an osmolarity of 123-160 mOsm [19,24]. Raising the NaCl concentration from 66mM to 132mM, which is equivalent to an osmolarity of approximately 246 mOsm, significantly reduced both cholera toxin and TcpA synthesis [19]. With this knowledge, I determined that supplementing a media containing 66mM NaCl with 90mM of either NaCl or KCl would raise the osmolarity of the media from 123 mOsm to approximately 266 mOsm. Similarly, when a media containing 66mM NaCl was supplemented with 90mM of either calcium or magnesium chloride the osmolarity was calculated to be approximately 330 mOsm. Thus osmolarity of media supplemented with both monovalent and divalent cations were at levels which have been previously shown to inhibit virulence gene expression. Therefore, it is not possible to discern whether the inhibitory effect on autoagglutination was due to the osmolarity of the media, or whether these cations were disrupting higher ordered pilus twists by electrostatic interactions. I propose that both of these mechanisms are likely to be at play.

Osmolarity of the media must also be taken into account with the addition of polyamines. Since the polyamines added were in the form of salts, these compounds would dissociate into their individual solute particles in an aqueous environment, thus increasing the osmolarity of the media. Since the osmotic coefficient was unknown for the polyamines, the osmolarity of these cultures were calculated using an osmotic coefficient of 1, which would signify a 100% dissociation of the compound, and thus the highest osmolarity possible for that solution. With putrescine and cadaverine containing dihydrochloride, this compound would dissociate into 3 solute particles. At maximum dissociation of the polyamine salts the

osmolarity of a 66mM NaCl solution supplemented with 15, 30, and 45mM would be approximately 165, 207, and 247 mOsm, respectively. Since the spermidine salt contained three hydrochlorides this compound would then dissociate into 4 solute particles meaning a 66mM NaCl solution supplemented with 10, 20, and 30mM of spermidine would be approximately 161, 199, and 236 mOsm, respectively. Since spermine would dissociate into 5 solute particles the osmolarity of a 66mM NaCl solution supplemented with 7.5, 15, and 22.5mM spermine would be approximately 158, 192, and 225 mOsm, respectively. Thus, the osmolarity of the media, supplemented with the highest concentration of polyamines were within the inhibitory range of virulence gene expression. Therefore, an increase in osmolarity alone does not explain why all the polyamines tested were having a more significant effect on autoagglutination than the monovalent cations. Osmolarity concentrations were calculated to be lower in the presence of spermidine and spermine rather than cadaverine and putrescine. This may explain why cadaverine and putrescine have an inhibitory effect on TcpA synthesis whereas spermidine and spermine do not. Research showing the osmolarity of the small intestine to be equivalent to approximately 300mM NaCl illustrates how environmental signals which modulate virulence gene expression *in vitro* do not necessarily correlate with signals which enhance virulence gene expression *in vivo* [23]. This indicates the effect or concentration of polyamines needed to attenuate virulence properties *in vivo* may be completely different than the concentrations used in this study.

Studies which have examined the effect of polyamines on *V. cholerae* biofilm formation found these molecules to modulate biofilm formation by altering *vps* genes transcription which are responsible for the architecture of the biofilm [52]. These studies were performed in the MO10 strain of *V. cholerae,* which is part of the O139 serogroup. I

wanted to determine whether polyamines would have an effect in the classical strain of *V. cholerae*. Biofilm assays showed that spermidine was the only polyamine to significantly affect biofilm formation. Interestingly, to date spermidine has been the only molecule implicated as attenuating both biofilm formation and TCP-dependent microcolony formation.

In conclusion, it was found that the polyamines spermidine, spermine, putrescine, and cadaverine inhibited *V. cholerae* autoagglutination in a concentration dependent manner. For the polyamines spermine, spermidine, and cadaverine this inhibitory effect could not be explained by reduction in TCP levels, disrupting lower ordered pilus bundling or osmolarity of the media. These results suggest TCP are not fully functional in the presence of polyamines. Based on this hypothesis, I propose the major mechanism for this inhibition to be external in which polyamines disrupt higher ordered pilus twists believed to mediate autoagglutination. Conversely, there is evidence presented in this study supporting the hypothesis that both spermidine and putrescine are able to inhibit autoagglutination by both an internal and external mechanism.

IMPLICATIONS

Only a handful of environmental signals have been identified which modulate *V. cholerae* virulence properties. Polyamines have been previously shown to affect biofilm formation and virulence in many other species of bacteria. In this study I show that one group of host-derived molecules, polyamines, are able to attenuate *V. cholerae* autoagglutination in a concentration dependent manner. Since polyamines are found at relatively high concentrations in the small intestine these molecules have the potential to modulate *V. cholerae* pathogenesis. There has been no research which has found polyamine concentrations in the gut to be close to the inhibitory concentrations used in this study. This implies that under most conditions, the polyamine concentrations encountered in the gut by *V. cholerae* would not be inhibitory to colonization. This result is consistent with the observation that *V. cholerae* can easily colonize most individuals if ingested in sufficient amounts. However, there are very few human studies concerning the polyamine concentrations in the gut, especially polyamine concentrations in the distal portion of the small intestines, where *V. cholerae* preferentially colonize. Animal studies have found millimolar concentrations throughout the intestine lending to the notion that these levels may be attainable in humans [47]. Studies assessing *V. cholerae* pathogenesis within the small intestine found that microenvironments encountered by the bacteria during infection had a significant effect on the virulence gene expression which lead to bacteria having altered expression profiles within different microenvironments of the intestine [7]. This study presents an infection model in which microenvironments encountered by the bacteria within

the small intestines are able to modulate virulence gene expression. It is plausible that temporal increases in polyamine levels in these microenvironments, such as increases in epithelial cell turnover and the resulting release of polyamines into the environment, could potentially be one of the factors affecting *V. cholerae* colonization.

Putrescine and cadaverine were the only polyamines identified which inhibited TcpA synthesis *in vitro*. A hypothesis can be drawn for their role during pathogenesis. A study with Male Sprague-Dawley rats found that putrescine and cadaverine concentrations were found at millimolar levels in the luminal portion of the proximal small intestine, compared with micromolar levels found in the distal portion of the lumen [47]. This same study found putrescine and cadaverine to be at micromolar concentrations in the mucosal chyme of the distal small intestine where wild-type *V. cholerae* have been shown to colonize and express their virulence genes [7,25]. With these two polyamines being found at relatively high concentrations in the proximal lumen, yet their levels diminish in the lumen and mucosal layer of the distal portion of the small intestine, putrescine and cadaverine may be a potential signal mediating the colonization and virulence gene expression within the human host.

To disrupt the colonization of *V. cholerae* in the small intestine one would need to increase the polyamine levels at the site of colonization. Physiological studies of polyamines in the intestine show that these molecules are rapidly absorbed in the proximal portion of the small intestine and dispersed throughout the body [37,53]. Knowing this, modulating one's diet to increase polyamine intake would most likely not alter *V. cholerae* colonization dynamics since these polyamines would not even make it to the distal portion of the small intestine at the concentrations ingested. The identification of an enterohepatic portal which circulates polyamines from the distal portion of the small intestine to the proximal portion

could lend to the idea of combating cholera by altering the gut microbiota, which is a large contributor to polyamine levels in the distal portion of the small intestine [47]. A recent paper suggests that altering the intestinal microflora with probiotics would increase the polyamine concentrations in the distal portion of the gastrointestinal tract by increasing the number of polyamine synthesizing/excreting bacteria compared to bacteria which absorb polyamines for their own use [54]. By increasing the concentration of the polyamine secreting bacteria, the infection dynamics of *V. cholerae* may be altered by inhibiting intestinal microcolony formation, thus allowing the body to quickly passage the bacteria through the intestines before the onset of serious infection.

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