Construction of a Δ*mbaA*Δ*potD1* Mutant and the Effects on Biofilm Formation in *Vibrio cholerae*

by

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Abstract

Vibrio cholerae is a bacterial species identified as the causative agent of the disease cholera. Similar to other aquatic bacteria, *V. cholerae* forms complex communities of microorganisms bounded by a polysaccharide matrix, known as biofilms. Biofilms allow planktonic bacteria to become immobilized within environments of favorable conditions by permitting adherence to surfaces. This matrix-encased microbial community provides protection against environmental stressors to ensure survival. Polyamines, or small cationic molecules essential to cell processes, are among the many environmental signals that act in regulating biofilm formation.

V. cholerae cells contain two systems, the NspS/MbaA signaling complex and the PotABCD1 ATP-binding cassette transporter, that function to detect and import these polyamines. The objective of this project was to determine the interplay between these two systems in regulating biofilm formation by investigating whether PotD1 and MbaA inhibit biofilm formation additively. To accomplish this, I explored the effects of knocking out the MbaA and PotD1 proteins. I constructed a Δ*mbaA*Δ*potD1* double mutant bacterial strain. Through colony PCR and gel electrophoresis, I was able to confirm the construction of this mutant. I then performed biofilm assays with the newly constructed mutant, the wild-type, and Δ*mbaA* and Δ*potD1* single mutants. The results showed that the Δ*mbaA*Δ*potD1* mutant did not form more biofilm than the single mutants, contradicting the hypothesized effects. Performing further biofilm assays with this double mutant and collecting data over various time points will allow our lab to further characterize the role of the PotABCD1 transporter system and the NspS/MbaA complex in the regulation of biofilm formation.

Introduction

Vibrio cholerae (*V. chol*erae) is a Gram-negative bacterial species identified as the causative agent of the disease cholera (8). Cholera is an acute diarrheal disease that arises from a virulent amount of *V. cholerae* colonizing the lower intestine (4). Transmission to the human body occurs through the ingestion of contaminated food or water (9). Colonization occurs as the aquatic bacteria transition from a motile to a sessile state and aggregate inside of the gut (9).

Similar to other aquatic bacteria, *V. cholerae* forms complex communities of microorganisms bounded by a self-produced matrix, known as biofilms (8). The matrix is composed of polysaccharides, proteins, and DNA (8). Biofilms allow planktonic or free swimming bacteria to adhere to surfaces, immobilizing colonies of cells within environments of favorable conditions (8). *V. cholerae* cells have been shown to adhere to surfaces within natural aquatic environments (5). This highly regulated, matrix-encased microbe community not only provides protection against chemical and physical stressors to ensure survival, but also aids in virulence (2,6).

Polyamines are among the many environmental signals that act in regulating biofilm formation (4). These small cationic molecules are found within eukaryotic and prokaryotic cells and are essential to cell processes (7). Polyamines are composed of short hydrocarbon chains and amine groups that are protonated at physiological pH (7). Putrescine, diaminopropane, cadaverine, and norspermidine are the four polyamines synthesized by *V. cholerae*, while spermidine can be imported by this bacterium (8). Of these polyamines, spermidine and norspermidine, influence biofilm formation by targeting multiple transport and signaling pathways (2).

The NspS/MbaA signaling complex is among the signaling pathways found in *V. cholerae* cells that has been shown to regulate biofilm formation (1). NspS is a member of the periplasmic solute binding protein superfamily and is involved in signal transduction (1). MbaA is an integral membrane protein with c-di-GMP phosphodiesterase activity that acts in a complex with NspS (6). Together, NspS and MbaA detect and process the norspermidine signal, allowing for the regulation of biofilm formation (5,6). Studies have shown that NspS inhibits the repressing activity of MbaA, especially when bound to norspermidine (2,5). For this reason, norspermidine is known to up regulate biofilm formation through this signal transduction pathway (2).

Norspermidine, along with spermidine, can be imported into the cell via the PotABCD1 ATP-binding cassette transporter (1). This type of transporter is composed of a substrate-binding protein, two cytoplasmic proteins that form a channel in the membrane, and two cytoplasmic proteins that hydrolyze ATP (3). The *potD1* gene encodes the ligand-binding protein of PotABCD1 that imports spermidine and norspermidine into the cells (3). Although these polyamines only differ structurally by one methyl group, norspermidine and spermidine somehow act antagonistically to up-regulate and down-regulate biofilm formation (2).

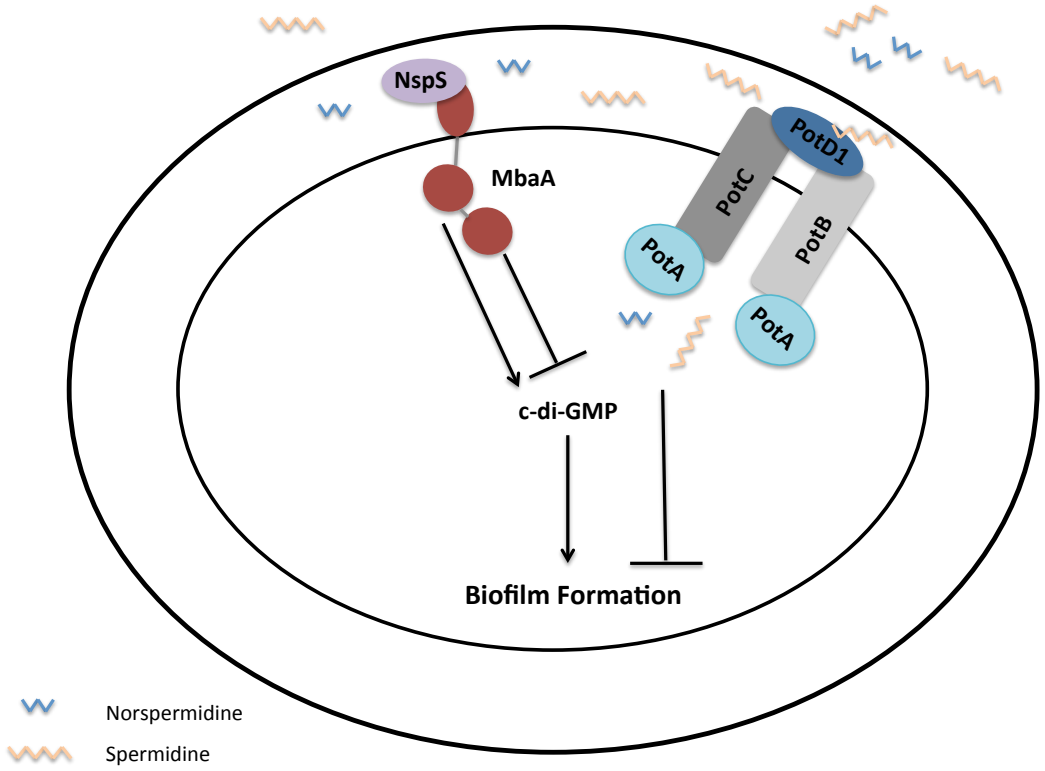


Figure 1. NspS/MbaA signaling complex and the PotABCD1 ATP-binding cassette transporter in *V. cholerae.*

Many studies have generated mutants lacking proteins necessary for the transport and signaling networks targeted by polyamines. These mutants allow researchers to characterize the specific roles of these proteins in polyamine regulation. For example, the Δ*mbaA* and Δ*potD1* single mutants have been studied, and the results suggest these mutants increased biofilm formation when compared to wild type cells (5,8). A Δ*mbaA*Δ*potD1* double mutant is not among the mutants that have been studied. Construction of this double mutant will allow for the determination of whether MbaA and PotD1 act to regulate biofilm formation through additive inhibition. We hypothesized these proteins act to inhibit biofilm formation in an additive manner, so the Δ*mbaA*Δ*potD1* double mutant should produce more biofilm than the Δ*mbaA* and Δ*potD1* single mutants. Construction of this double mutant will allow

our lab to further characterize the role of the PotABCD1 transporter system and the NspS/MbaA complex in the regulation of biofilm formation.

Methods

Bacterial strains and media

The bacterial strains and plasmids used to construct the double mutant included a *V. cholerae* strain, PW444, and an *E. coli* strain, AK058. Bacterial strains and plasmids were plated onto Luria-Bertani (LB) plates or sucrose plates. To make approximately twenty plates with LB media, 5.0 g of NaCl, 7.5 g of agar, 2.5 g of yeast extract, and 5.0 g of tryptone were weighed out and added to a 1000-mL Erlenmyer flask containing approximately 250 mL of deionized (DI) water. Another 250 mL of DI water was added to the flask and the contents were stirred on a stir plate using a stir bar for five minutes. The top of the flask was covered with aluminum foil and the media was sterilized in an autoclave using a liquid cycle with a sterilization time of twenty minutes at 121°C. Following sterilization, the broth was cooled and stirred. For LB plates with no antibiotics, the media was poured approximately halfway up each plate. The plates were stored inside of a sleeve in the inverted position once solidified. Plates with media containing ampicillin (amp), streptomycin (sm), or ampicillin and streptomycin (sm/amp) were also made. Antibiotics were added to the broth once cooled. LB-amp or LB-sm plates were prepared by inoculating the media with 100 µg/mL of the antibiotic. LB-sm/amp plates were inoculated with 50 µg/mL of ampicillin and 100 µg/mL of streptomycin. The media was poured into plates and the plates were stored using the same process as plates with strictly LB media.

Sucrose plates were prepared with 333.3 mL of DI water, 2.5 g of yeast, 2.5 g of tryptone, 7.5 g of agar, and autoclaved for sterilization. While the media was being

autoclaved, sucrose solution (30% sucrose) was prepared. A 1000-mL beaker containing 500 mL of sterile water was obtained. As the water was heated and spun, 300 g of sucrose was weighed out and added to the beaker. Sterilized water was added to the beaker until the bottom of the meniscus was around 900 mL, and the solution was spun over slight heat until it became translucent. The solution was transferred to a 1000-mL graduated cylinder and sterilized water was added until the bottom of the meniscus hit the 1000-mL mark. The solution was transferred back to the beaker and the contents were stirred. The sucrose was filter sterilized into a clean 1-L glass bottle, which was placed into a 50°C water bath. Once sterilized, the media was cooled and stirred. Following the addition of 166.7 mL of filter sterilized sucrose solution, the media was poured into plates and stored using the process previously mentioned for LB plates.

Construction of the Δ*mbaA*Δ*potD1* mutant

An *E. coli* strain (AK058) was streaked for isolation on an LB-amp plate and incubated overnight at 37°C. AK058 contains the pWM91 plasmid with a *ΔpotD1* mutation. A *V. cholerae* strain (PW444) was streaked for isolation on an LB-sm plate and incubated overnight at 37°C. PW444 contains a Δ*mbaA* mutation. To streak for isolation, aseptic techniques were always used. The bacteria were selected for using a sterile wooden stick over a flame. The wooden stick was used to streak the bacteria across one quadrant of the plate. A new sterile wooden stick was used to carry bacteria over from the first quadrant to a second quadrant, and the transferred bacteria were also streaked across the quadrant. The process was repeated two more times, totaling four quadrants. The following day, these two strains were mated. Colonies of *E. coli* were removed with a sterile wooden stick and spread

across an LB plate. On the same LB plate, the process was repeated using colonies of *V. cholerae*. The plate was incubated overnight at 37°C.

To select for a single cross over, half of the growth on the LB plate was removed with a sterile wooden stick and streaked for isolation onto an LB-sm/amp plate. The process was repeated for the second half of the growth. The plates were incubated overnight at 37°C. A single colony was removed from the LB-sm/amp plates, streaked for isolation onto an LB-sm/amp plate, and incubated overnight at 37°C. This process was repeated three times. To cross-out on LB, four isolated colonies were collected from the LB-sm/amp plates and streaked for isolation onto an LB plate. During this step, recombination occurs, resulting in the deletion of the ampicillin resistance and *SacB* genes. Four colonies from the LB plates were selected and streaked for isolation on sucrose plates. Sucrose plates were incubated at 37°C to rid of moisture prior to being streaked. The process was repeated seven more times. Sucrose plates were incubated at room temperature for forty-eight hours. Sucrose allows for the selection of bacteria where the plasmid was eliminated because the *SacB* gene on the plasmid prevents bacteria from growing on sucrose.

Checking for the Δ*mbaA*Δ*potD1* mutant

After forty-eight hours, the strains were checked. The back of an LB-sm/amp plate was divided into thirty equally sized boxes. The process was repeated on an LB-sm plate. The squares were labeled one through thirty. Thirty single colonies from the sucrose plates were patched onto the labeled squares. The corresponding numbers on both the LB-sm and LB-sm/amp plate were patched with the same isolated colonies. The plates were incubated overnight at 37°C. Colonies that grew exclusively on the LB-sm plates were streaked for isolation onto fresh LB-sm plates and incubated overnight at 37°C. These colonies were

chosen because they no longer contained the ampicillin resistance gene, which was supposed to be deleted following recombination. The following day, a single colony from an LB-sm plate was selected with a toothpick and deposited into a PCR tube containing 100 µL of DI water. This process was repeated eight times. The tubes were vortexed, placed in a thermocycler, and incubated at 95°C for five minutes to lyse the cells, and then spun for five minutes at 8K to spin down cellular debris. A mastermix was prepared with 164.0 µL of nuclease free water, 50.0 µL of OneTaq Buffer, 5.0 µL of dNTP, 5.0 µL of primer 1 (P332), 5.0 µL of primer 2 (P335), and 1.25 µL of OneTaq enzyme in a 200-mL Erlenmeyer flask and mixed. P332 and P335 were the primers used to amplify a region approximately 800 bps in length. One primer anneals 400 bps upstream of *potD1* and the other anneals approximately 400 bps downstream of *potD1*. An 800 bp fragment was expected if the *potD1* gene was deleted and an 1800 bp fragment was expected if the deletion did not occur. Nine new PCR tubes were obtained, marked with the same labels as the PCR tubes containing the samples, and filled with 23 µL of mastermix. Supernatant from the PCR tubes containing the lysed cells was also added to each respective new PCR tube in 2 µL quantities. The solution was pulsed and a colony PCR was run for ninety minutes and thirty-five cycles. In each cycle, DNA fragments were denatured at 98°C for ten seconds, the primers annealed with the fragments at 55.5°C for twenty seconds, and elongation of the primers occurred at 68°C for one minute. The initial denaturation occurred over thirty seconds and the final extension occurred over six minutes.

Following colony PCR, the samples were run on a gel composed of 0.5 g of agarose and 50 mL of 0.5X TBE. The ingredients to make 5X TBE include 20 mL of 0.5 M EDTA, 54 g of trizma base, and 27.5 g of boric acid. The gel ingredients were poured into an

Erlenmeyer flask and covered with a Kimwipe. The flask was microwaved for thirty seconds, swirled to mix, microwaved for twenty more seconds, swirled again, and cooled for ten minutes. Once cooled, 2.5 µL of ethidium bromide was added and the solution was poured into a gel box. A comb with ten wells was added and removed once the gel solidified. The gel was covered with 0.5X TBE (900 mL of water and 100 mL of 5X TBE). To begin loading the gel, 5 µL of loading dye was first added to each PCR tube. The first well of the gel was loaded with 5 µL of the molecular weight marker and the following wells were loaded with 12 µL of the samples. The gel was run at 155 volts and 0.1 amperes for forty minutes. The gel was imaged using AlphaImager HP.

Preparation of stock cultures

Three newly constructed double mutants containing both the Δ*mbaA* and the Δ*potD1* mutations were frozen by first preparing overnight cultures. A single colony was selected from the LB-sm plates streaked with these strains and placed into a borosilicate tube containing 2 mL of LB broth and 2 µL of sm. This process was repeated two times. The tubes were incubated over-night in a shaking incubator at 37°C. The following day, 2 mL of LB+30% glycerol was added to the overnight cultures. From each culture tube, 1 mL was pipetted into its respective labeled cryogenic vial, totaling three cryogenic vials. The vials were frozen.

Verification of the strains

The frozen Δ*mbaA*Δ*potD1* double mutants were streaked onto fresh LB-sm plates, along with Δ*potD* and Δ*mbaA* mutants. The Δ*potD1* mutant in wild-type background was the positive control. The Δ*mbaA* mutant served as the negative control because the *potD1* gene was still present in the genome of this mutant. The entire colony PCR and imaging process

previously mentioned was repeated using these five strains to check the strains against positive and negative controls. The mastermix contained 98.4 µL of nuclease free water, 30.0 µL of OneTaq Buffer, 3.0 µL of dNTP, 3.0 µL of primer 1 (P332), 3.0 µL of primer 2 (P335), and 0.75 µL of OneTaq enzyme.

Biofilm Assay

Wild-type, the Δ*potD1* mutant, the Δ*mbaA* mutant, and one newly constructed double mutant containing both the Δ*mbaA and* the Δ*potD1* mutations were streaked for isolation on LB-sm plates and incubated overnight at 27°C. Four microfuge tubes were labeled with these strain's numbers and names and filled with 300 µL of LB broth. A sterile wooden stick was used to remove bacteria from the LB-sm plates to deliver the respective strain into the labeled microfuge tube. A 1:10 dilution of each sample was pipetted into four new microfuge tubes. In duplicates, 100 µL of each diluted sample was pipetted into the wells of a microtiter plate and the optical density of each sample was measured at an absorbance of 655 nanometeres (nm) after shaking the plate for ten seconds. The two readings from each diluted sample were averaged, and the optical density measured for a well containing no sample (0.033) was subtracted from the average. This number was to be used as M2 in the M1V1=M2V2 equation, which was then used to calculate V2. M1 was 0.02, which was the desired final optical density in the tubes, and V1 was the final volume of the sample, which was 300 µL. A mastermix containing 5 mL of LB broth and 1 µL/mL of streptomycin was prepared. Three borosilicate glass tubes were obtained per strain and filled with 300 µL of mastermix. The previously calculated amounts of each respective sample (V2) was added to each borosilicate tube and the tubes were incubated over night at 37°C. The next day, 150 µL of the planktonic cells from each borosilicate tube was removed and placed into the wells of

a microtiter plate. The remaining planktonic cells were discarded. Each borosilicate tube was washed with 300 µL of 1X PBS (1 L of water, 8.0 g of NaCL, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4) without disturbing the biofilm. The process was repeated, but instead of removing the PBS, glass beads were added to each tube. The tubes were vortexed for approximately one minute until the samples no longer contained large, solid particulates of biofilm. One-hundred and fifty µL of each sample was added into a new well on the microtiter plate. The optical density of each sample was once again read at an absorbance of 655 nm. Microsoft Excel was used to construct bar graphs of the averages and standard deviations for each sample. The entire biofilm assay procedure was performed twice to determine whether the double mutant formed more biofilm than the Δ*mbaA* and Δ*potD1* mutants.

Results

Construction of the Δ*mbaA*Δ*potD1* mutant

The Δ*mbaA*Δ*potD1* mutants were made by mating an *E. coli* strain, AK058, containing the pWM91 plasmid with a Δ*potD1* deletion with a *V. cholerae* strain, PW444, with an Δ*mbaA* deletion. To check eight of the strains for the *potD1* deletion, colony PCR was performed to determine whether the 800 bp fragment resulting from a deletion of the *potD1* gene was present. The PCR products were run on an agarose gel and the gel was imaged using an AlphaImager. Lane 1 contained the Quick-Load 2-Log DNA Ladder (0.1 – 10.0 kb), lanes 2 through 9 contained the double mutant candidates, and lane 10 contained the Δ*potD1* mutant. Of the eight double mutant strains checked, eight exhibited bands around the 800 bp marker, indicating that the *potD1* gene was deleted (Figure 2). The fragment being amplified was 1800 bps in length, so had the deletion been unsuccessful we would

expect a band near the 2000 bp marker. If the *potD1* gene was successfully deleted, then the deletion would remove majority of the gene, resulting in an 800 bp fragment.

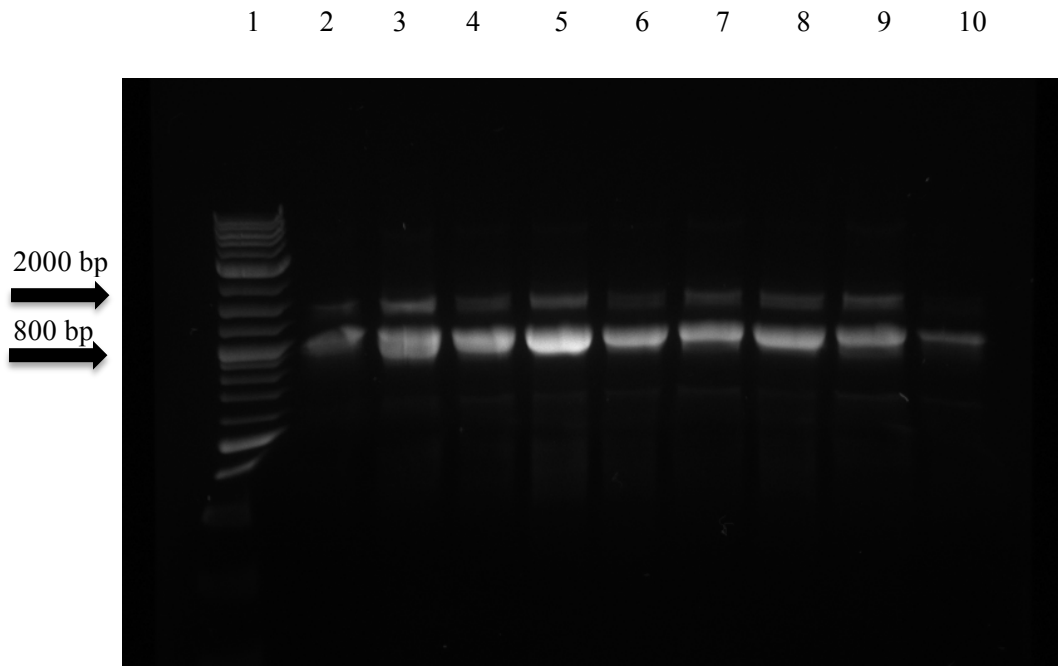


Figure 2. Gel image of the colony PCR products from the amplification of the Δ*potD1* gene for eight Δ*mbaA*Δ*potD1* mutant candidates and the Δ*potD1* mutant. Lanes 2 through 9 contained double mutant candidates. Lane 10 contained the Δ*potD1* mutant.

Three of the strains that were used in the colony PCR and contained 800 bp bands were selected and checked again, this time against a positive and negative control. The positive control was the Δ*potD1* mutant run in lane three (Figure 3). The negative control was the Δ*mbaA* mutant run in lane seven (Figure 3). Colony PCR was performed, the products were run on an agarose gel, and the results were imaged using an AlphaImager. The sample in lane six exhibited an 800 bp-band, similar to the Δ*potD1* mutant (Figure 3). The other two samples in lanes four and five exhibited a band created by a fragment 1800 bps in length, similar to the Δ*mbaA* mutant (Figure 3). The 1800 bp band indicates the *potD1* deletion was unsuccessful. The double mutants that were checked twice and displayed a 800

bp fragment following the first colony PCR, but an 1800 bp fragment following the second colony PCR may have become contaminated upon freezing of the strains.

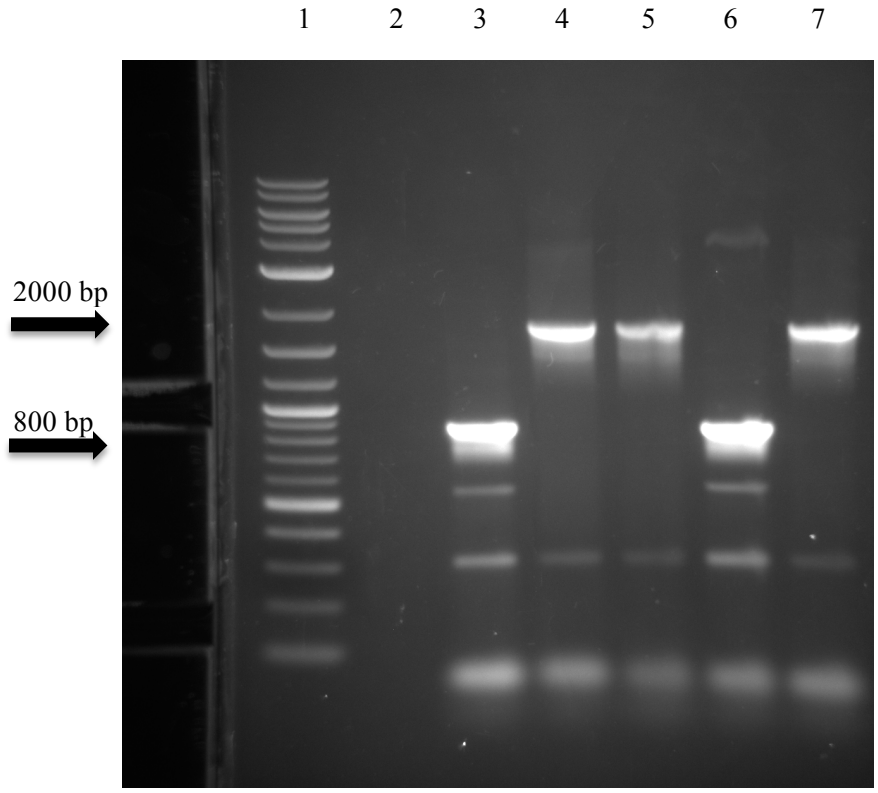


Figure 3. A gel image of the colony PCR products from the amplification of the Δ*potD1* gene for the Δ*mbaA*Δ*potD1*, Δ*mbaA*, and Δ*potD1* mutants. Lane 3 contained the Δ*potD1* mutant. Lanes 4 and 5 contained mutants where the *potD1* deletion was unsuccessful. Lane 6 contained a double mutant candidate. Lane 7 contained the Δ*mbaA* mutant.

Effects of Δ*mbaA* and Δ*potD1* on biofilm formation

The purpose of looking at the effects of *mbaA* and *potD1* on biofilm formation was to determine whether these proteins inhibit biofilm formation in an additive manner. To ensure any effects observed on biofilms were not an effect on cellular growth, the planktonic cell counts for wild-type and the Δ*mbaA,* Δ*potD1,* and Δ*mbaA*Δ*potD1* mutants were determined by extracting the planktonic cells and measuring the optical density of each sample at an absorbance of 655 nm. The average cell count for the wild-type was significantly greater than the average cell count for the Δ*mbaA,* Δ*potD1, and* Δ*mbaA*Δ*potD1* mutants (Figures 4 and

6). Wild-type had an average absorbance of approximately 0.739 (Figure 4). The Δ*mbaA*Δ*potD1* mutant had an average absorbance reading of 0.325 (Figure 4). The planktonic cell sample of the Δ*mbaA* mutant had an average absorbance of approximately 0.320 and the Δ*potD1* mutant sample had an average absorbance of approximately 0.224 (Figure 4).

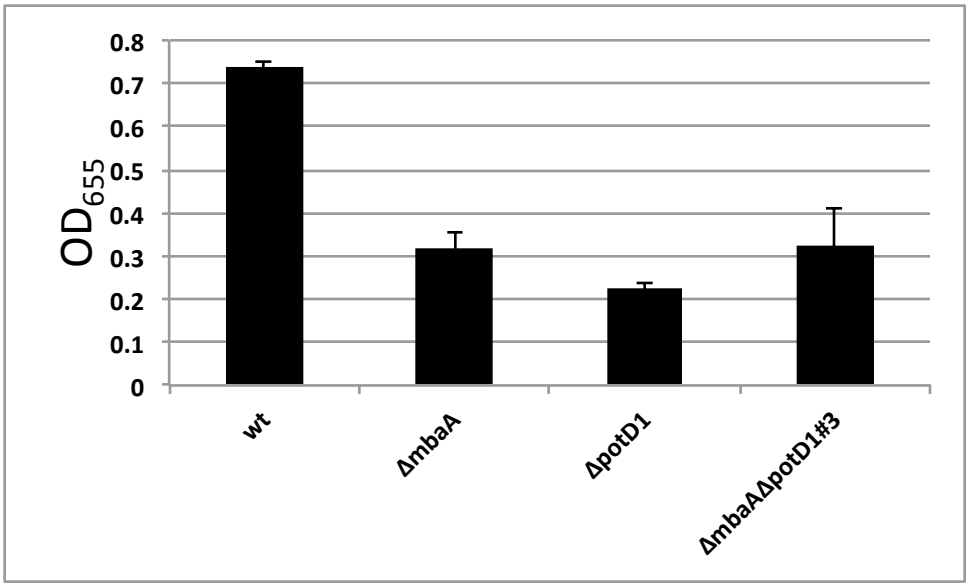


Figure 4. Planktonic cell density of wild-type and mutant *V. cholerae* cells. Biofilm assays were performed on wild-type (WT) and the Δ*mbaA,* Δ*potD1,* and Δ*mbaA*Δ*potD1* (#3) mutants in culture tubes and planktonic cells were extracted and quantified as described in Materials and Methods. The average of three replicates was recorded and error bars were used to show standard deviations.

The biofilm cell counts for the wild-type and the Δ*mbaA,* Δ*potD1,* and Δ*mbaA*Δ*potD1* mutants were determined by measuring the optical density of each sample at an absorbance of 655 nm using a spectrophotometer. Wild-type biofilm cells exhibited a significantly less average absorbance than the Δ*mbaA,* Δ*potD1,* and Δ*mbaA*Δ*potD1* mutants. The average cell count for the wild-type was approximately 0.067 (Figure 5). The Δ*mbaA*Δ*potD1* mutant had an average absorbance reading of 0.432 (Figure 5). The average absorbance reading for the Δ*mbaA* mutant was 0.421 and the average absorbance reading for the Δ*potD1* mutant was 0.444 (Figure 5).

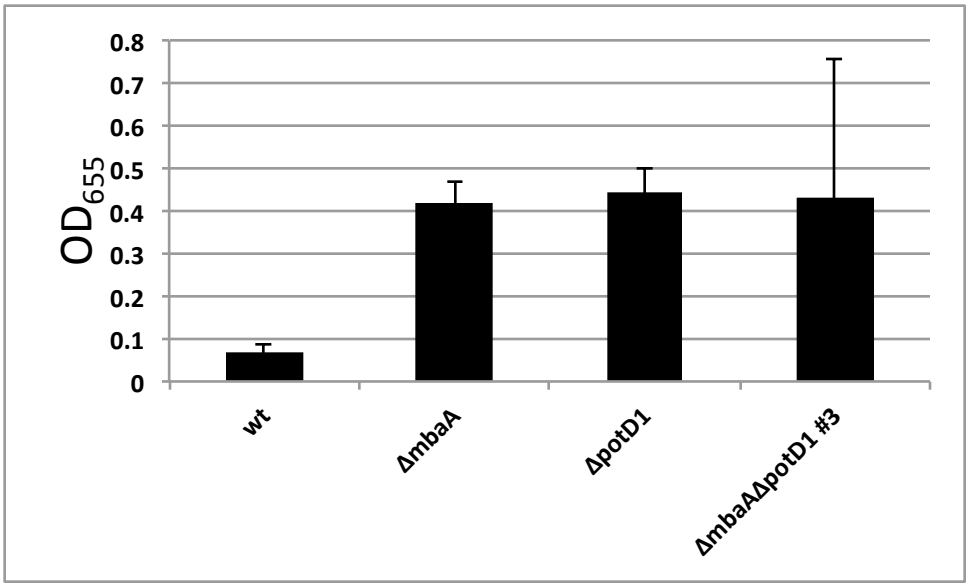


Figure 5. Biofilm cell density of wild-type and mutant *V. cholerae* cells. Biofilm assays were performed on wild-type (WT) and the Δ*mbaA,* Δ*potD1,* and Δ*mbaA*Δ*potD1* (#3) mutants in culture tubes and biofilms developed overnight and were quantified the next day as described in Materials and Methods. The average of three replicates was recorded and error bars were used to show standard deviations.

Assessment of biofilm formation at 16 hours

A second biofilm assay was performed in an attempt to decrease the large error bars seen in the average optical density of the double mutant. The large error bars could be the result of taking measurements after twenty-four hours. Biofilms become thicker and thicker as more time is given to allow them to form. Thick biofilms are difficult to grind up, and when optical density measurements are taken on biofilms that are not completely ground up, the readings are inaccurate. To avoid this issue, the second biofilm assay biofilm cell optical density readings were taken after sixteen hours instead of twenty-four. The optical densities were unintentionally measured using a 595 filter instead of a 655 filter.

Quantification of the planktonic cells for the wild-type and Δ*mbaA*, Δ*potD1*, and Δ*mbaA*Δ*potD1* mutants after the second biofilm assay yielded similar results to those of the first biofilm assay. Wild-type had an average absorbance of approximately 0.714 (Figure 6). The Δ*mbaA*Δ*potD1* mutant had average absorbance reading of 0.361 (Figure 6). The Δ*mbaA*

mutant planktonic cell sample had an average absorbance of approximately 0.330 and the Δ*potD1* mutant had an average absorbance of approximately 0.307 (Figure 6).

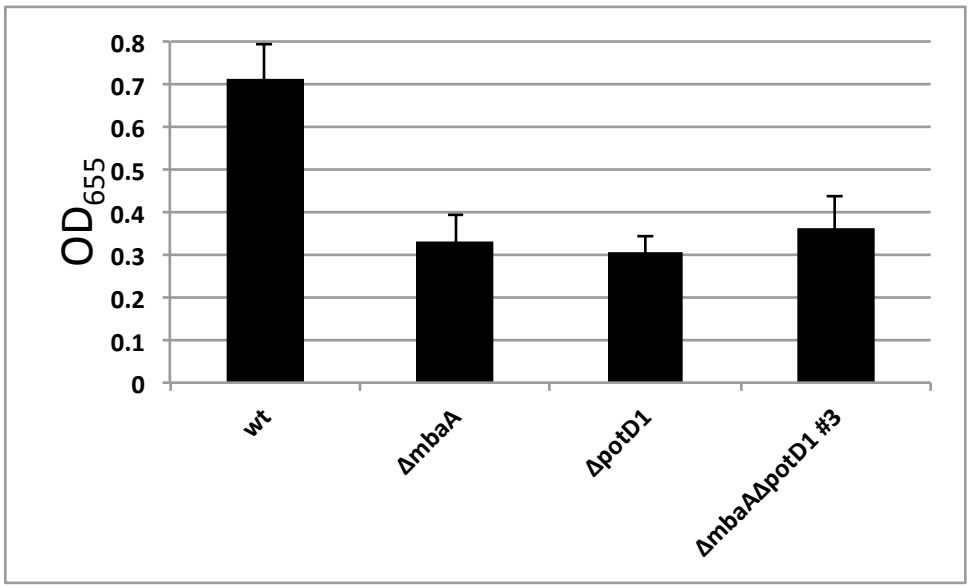


Figure 6. Planktonic cell density of wild-type and mutant *V. cholerae* cells. Biofilm assays were performed on wild-type (WT) and the Δ*mbaA,* Δ*potD1,* and Δ*mbaA*Δ*potD1* (#3) mutants in culture tubes and planktonic cells were extracted and quantified as described in Materials and Methods, except that they were read using a 595 filter instead of a 655 filter. The average of three replicates was recorded and error bars were used to show standard deviations.

Quantification of biofilm formed by the wild-type and Δ*mbaA*, Δ*potD1*, and Δ*mbaA*Δ*potD1* mutants after the second biofilm assay yielded similar results to those of the first biofilm assay. Wild-type biofilm cells exhibited a significantly less average optical density at 655 nm than the Δ*mbaA,* Δ*potD1,* and Δ*mbaA*Δ*potD1* mutants. The average cell count for the wild-type was approximately 0.082 (Figure 7). The Δ*mbaA*Δ*potD1* mutant had an average absorbance reading of 0.420 (Figure 7). The average absorbance reading for the Δ*mbaA* mutant was approximately 0.291 and the Δ*potD1* mutant's average absorbance reading was approximately 0.417 (Figure 7).

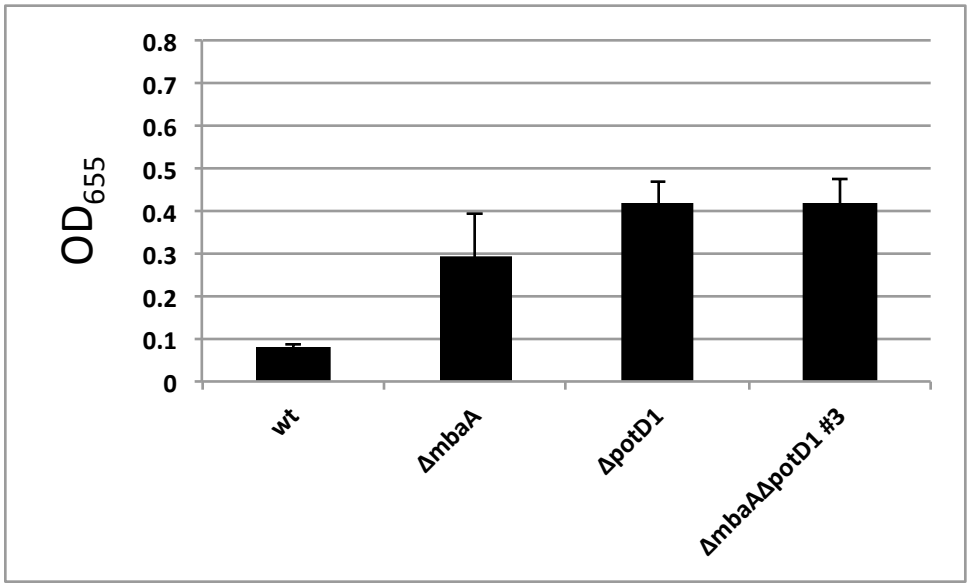
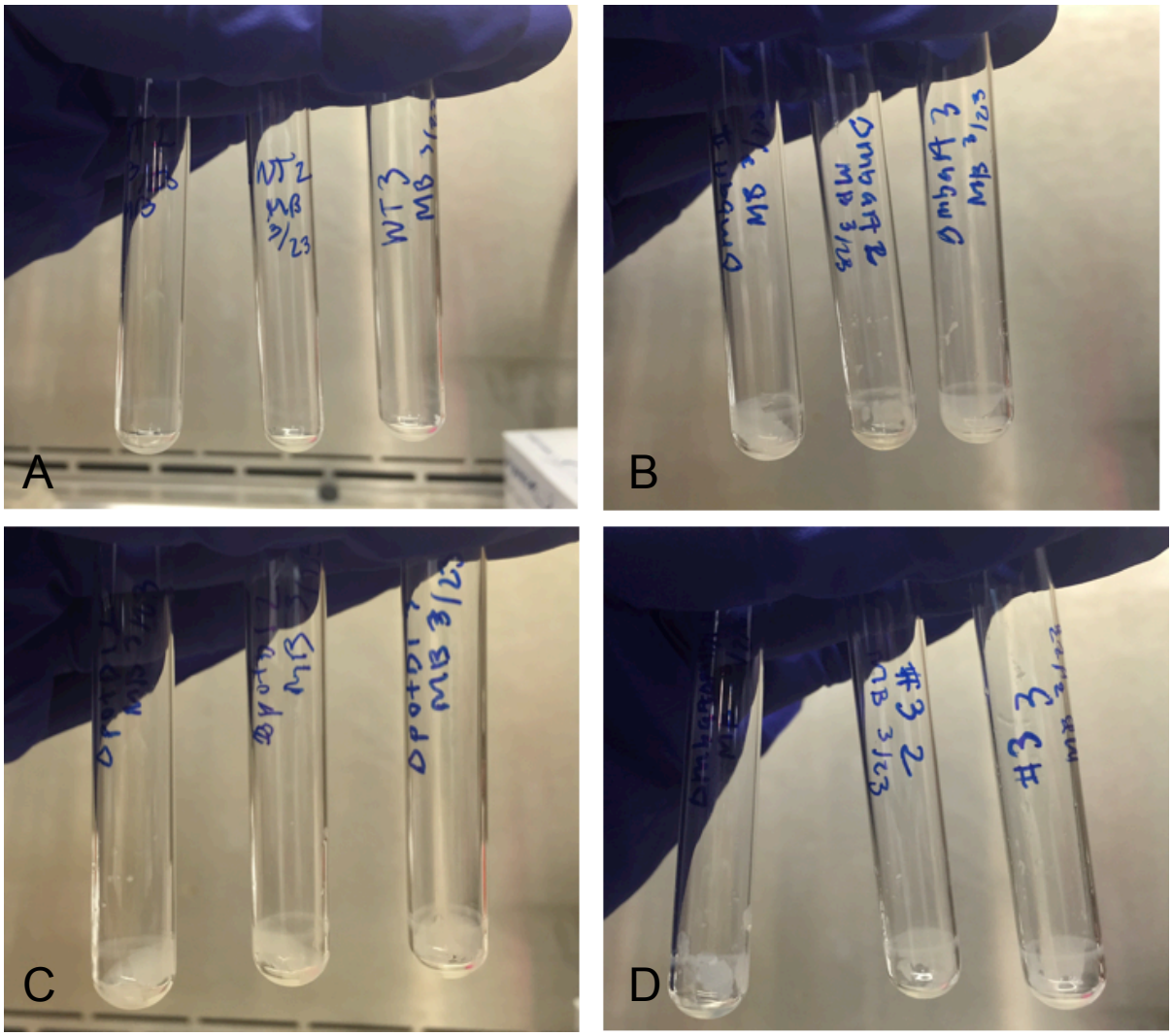


Figure 7. Biofilm cell density of wild-type and mutant *V. cholerae* cells. Biofilm assays were performed on wild-type (WT) and the Δ*mbaA,* Δ*potD1,* and Δ*mbaA*Δ*potD1* (#3) mutants in culture tubes and biofilms developed overnight and were quantified the next day as described in Materials and Methods, except they were allowed to form for 16 hours instead of 24 hours and were read using a 595 filter instead of a 655 filter. The average of three replicates was recorded and error bars were used to show standard deviations.

Pictures were taken of the culture tubes containing the biofilms produced from the second biofilm assay for visual comparisons. The wild-type still produced the least amount of biofilm after sixteen hours (Figure 8).



F**igure 8.** Biofilms formed by WT (A), and the Δ*mbaA* mutant (B), Δ*potD1* mutant (C), and Δ*mbaA*Δ*potD1* mutant (D).

Discussion

Studying biofilms can provide an understanding for how and why particular bacteria are able to survive in the presence of environmental stressors (8). Biofilms have been recognized to protect *V. cholerae* in natural aquatic environments and in the human intestines (8). In aquatic environments, biofilms have been shown to protect *V. cholerae* cells from osmotic stress (8). In the intestines, biofilm protection has been associated with increased

virulence and pathogen count by providing mechanisms to defend against the human immune system (8).

Studies have shown that various polyamines regulate multiple signaling and transport systems involved in the regulation of biofilm formation in *V. cholerae* (8). The purpose of this experiment was to further characterize the roles of the polyamines norspermidine and spermidine and their regulatory pathways in the regulation of biofilm formation. To accomplish this, we studied biofilm formation in Δ*mbaA* and Δ*potD1* single mutants and in a Δ*mbaA*Δ*potD1* double mutant.

Previous studies have shown that the proteins MbaA and PotD1 repress biofilm formation (8). In these studies, Δ*mbaA* and Δ*potD1* single mutants produced more biofilm than wild type cells (5,8). Considering these findings, we hypothesized that the MbaA and PotD1 proteins inhibit biofilm formation in an additive manner in *V. cholerae*. We expected that the Δ*mbaA*Δ*potD1* double mutant would produce more biofilm than the Δ*mbaA* and Δ*potD1* single mutants. The results from the biofilm assays suggest that the double mutant produced similar amounts of biofilm as the Δ*potD1* mutant and the Δ*mbaA* mutant.

Wild type cells yielded the greatest amount of planktonic cells when compared to the double and single mutants (Figures 4 and 6). The average optical density measurements at 655 nm for planktonic wild type cells were 0.739 and 0.714 (Figures 4 and 6). The average optical density measurements for the Δ*mbaA* mutant were 0.320 and 0.330 (Figures 4 and 6). The average optical density measurements for the Δ*potD1* mutant were 0.224 and 0.307 (Figures 4 and 6). The Δ*mbaA*Δ*potD1* double mutant had an average optical density of 0.325 in the first biofilm assay (Figure 4) and the second biofilm assay yielded an average optical density of 0.361 (Figure 6). Planktonic cells are free-swimming cells that indicate bacteria

are not forming biofilms to attach to surfaces and are typically least abundant in high biofilm-forming bacteria (6). These results suggest wild type cells produced the most free-floating cells. In addition to this, we demonstrated that the Δ*mbaA*Δ*potD1* double mutant produced significantly more biofilm than wild type cells and similar amounts of biofilm compared to those of the Δ*mbaA* and Δ*potD1* mutants (Figures 5 and 7). The average optical density measurement at 655 nm for biofilm cells of the Δ*mbaA*Δ*potD1* double mutant was 0.432 in the first experiment and 0.420 in the second experiment (Figures 5 and 7). The average optical density measurements for the Δ*mbaA* and Δ*potD1* mutants and wild type cells were 0.421, 0.444, and 0.067, respectively, in the first experiment (Figure 5). In the second experiment, the average optical density measurements for the Δ*mbaA* and Δ*potD1* mutants and wild type cells were 0.291, 0.417, and 0.082, respectively (Figure 7). These results suggest the PotABCD1 transporter system and the NspS/MbaA complex do not act to inhibit biofilm formation in an additive manner.

It has been shown that the NspS/MbaA complex and the PotABCD1 transporter both respond to spermidine and norspermidine, so we predicted that the two systems would act additively in inhibiting biofilm formation (8). The NspS/MbaA complex binds exogenous spermidine and norspermidine via the NspS sensor and the PotABCD1 transporter imports both polyamines into the cell upon binding to PotD1 (1,8). Thus, polyamines can act as environmental signals capable of regulating biofilm formation through intracellular and extracellular mechanisms (8).

Norspermidine is recognized as a biofilm formation activator (8). The PotABCD1 transporter binds preferentially to norspermidine, which can competitively inhibit spermidine binding (8). The NspS/MbaA complex senses norspermidine in the environment to increase

biofilm formation (1). NspS binds to exogenous norspermidine and is hypothesized to inhibit the phosphodiesterase activity of MbaA by interacting with the protein's periplasmic domain (1). MbaA proteins contain GGDEF and EAL domains (1). GGDEF domains act as enzymes that catalyze the reaction converting guanosine triphosphate (GTP) to c-di-GMP (5). These enzymes are called guanylate cyclases (5). EAL domains degrade c-di-GMP by phosphodiesterase activity (5). Studies have demonstrated that a decrease in biofilm formation is associated with a decrease in c-di-GMP (5). NspS prevents MbaA from degrading c-di-GMP to increase biofilm formation, and norspermidine only furthers phosphodiesterase inhibition (1).

In contrast, spermidine is known to inhibit biofilm formation (8). Studies have shown that the binding of spermidine to PotD1 and importation into the cell decreases the amount of biofilm formed through intracellular means (8). However, even in the absence of PotD1, exogenous spermidine inhibits biofilm formation (8). Thus, an alternative method must respond to the presence of spermidine (8). It has been suggested that spermidine could bind NspS and regulate biofilm formation in a NspS/MbaA-dependment manner, similar to exogenous norspermidine (8). Spermidine could bind to NspS and prevent NspS from interacting with MbaA to inhibit MbaA's phosphodiesterase activity. Thus, increased phosphodiesterase activity would diminish c-di-GMP levels and lead to the production of less biofilm (1).

The results from this study suggest that the MbaA signal transducer and PotD1 solute binding protein do not additively inhibit biofilm formation through extracellular and intracellular mechanisms. It is possible that these results were due to the formation of the maximum amount of biofilm by the double mutant and single mutants when the optical

density measurements were taken. The double mutant may form biofilm at a quicker rate than the single mutants. To check for this, future experiments could include performing another biofilm assay and recording the optical densities every two hours over a sixteen-hour period. This could potentially yield differences that occur earlier on in biofilm formation before the cells consume the available resources.

In conclusion, while this study failed to demonstrate an additive relationship between MbaA and PotD1 in inhibiting biofilm formation, future studies could yield results suggesting otherwise by assessing the optical density of biofilms at earlier stages of biofilm development. Elucidation of these pathways would provide a means to understand how *V. cholerae* integrates multiple cellular pathways to regulate biofilm formation as a defense mechanism that allows these cells to persist in the intestines and in their natural environments.

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