Construction of a Δ*vpsL*Δ*potB* Mutant and the Effects on Biofilm Formation in *Vibrio cholerae*

by

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ABSTRACT

Vibrio cholerae is an aquatic bacterium known as the causative agent of the intestinal disease cholera. *V. cholerae* uses its ability to form biofilms, an aggregation of microorganisms, to survive extreme conditions in both the environment and human host. Biofilm formation requires the expression of *vps* genes that encode proteins required for synthesis and transport of VPS. *V. cholerae* contains a PotABCD1 transport system that functions to transport polyamines, signals aiding in biofilm regulation, into the bacterium.

The objective of this study was to construct a bacterial strain that would allow further examination of the role of PotB on transport of polyamines into the cell. PotB constitutes one of the transmembrane proteins of the PotABCD1 transporter. Previously, mutations in the Pot proteins resulted in a significant increase in biofilm formation. To study the role of PotB, I worked to eliminate the bacterium's biofilm phenotype. A Δ*vpsL*Δ*potB* double mutant was generated to deplete the bacterium's ability to form biofilms. *vpsL* and *vpsA* represent the two *vps* clusters required for expression of the biofilm phenotype. Exclusion of both the *vpsL* and *potB* genes was performed to deplete the biofilm phenotype and allow examination of the transport system in the absence of PotB. The construction of the double mutant was confirmed through colony PCR and gel electrophoresis. Biofilm assays were performed using wild-type, Δ*vpsL*, Δ*vpsA*, Δ*potB*, and constructed Δ*vpsL*Δ*potB* mutants. The biofilm assay results showed that the constructed Δ*vpsL*Δ*potB* double mutant resulted in greater biofilm formation when compared with the other mutants, rejecting the initial hypothesis. Construction of other double mutants using different genetic backgrounds, specifically those confirmed to deplete the biofilm phenotype, as well as the use of more defined media, will help our lab further characterize the role of PotB on the transport of polyamines.

INTRODUCTION

Vibrio cholerae is a Gram-negative, aquatic bacterium found in diverse bodies of water, and is identified as the causative agent of the intestinal disease cholera (6). Cholera is characterized by excessive diarrhea, leading to increased levels of dehydration and death in major cases (3). In past centuries, cholera presented a constant threat to human health in highly developed countries. Today, however, its threats are limited to underdeveloped countries where water conditions are less hygienic (5). Cholera infection typically begins with the oral ingestion of contaminated water (5). To infect the human host, the bacterium must survive the unique conditions of the human body, including the gastric acid barrier of the stomach and the mucus lining covering the intestinal epithelial (5).

To survive the harsh conditions of its human host and other environmental conditions such as in the presence of ultraviolet radiation, pH extremes, and/or a range of temperatures, *V. cholerae* arranges itself into an aggregation of microorganisms called a biofilm, which links its ability to survive to seasonal epidemics (1,6). The exopolymeric matrix that constitutes the biofilm is composed of an array of proteins, DNA, and polysaccharides, that surround aggregated bacteria in favorable conditions (6). The developed aggregation of microorganisms assembles on both abiotic and biotic surfaces including crustaceans, aquatic plants, and the epithelial cells of the human body (6). *V. cholerae* utilizes its ability to form biofilms for transmission and infectivity occurring in the small intestine (1). Adherence of the surviving bacteria to the intestinal epithelial cells releases a cholera toxin (CT), which leads to the first symptoms of cholera infection (5). Stool samples of infected cholera patients show the presence of both biofilm-like aggregations and planktonic forms of *V. cholerae* (1).

V. cholerae biofilm development and attachment to abiotic surfaces has been

observed to occur in two stages throughout the first 24 hours of growth (9). The first stage of growth, occurring within the first 4-5 hours, shows brief attachment to a surface, while permanent attachment occurs in the second stage (9,2). Permanent immobilization of the microorganisms accompanies *vps* gene expression. *vps* genes encode for the proteins required for the synthesis and transport of VPS, a *V. cholerae* produced exopolysaccharide, commonly known as *Vibrio* polysaccharide (9, 10, 1). VPS has been deemed essential for the bacterium's development of a three-dimensional biofilm structure (3,1). Previous studies have also suggested a role of VPS in the infection abilities and *in vivo* colonization of the bacterium (1). Within the bacterium, *vps* genes are clustered amongst two regions, the *vps*-I operon and the *vps*-II operon. The *vps*-I operon contains *vps* genes A-K, while the *vps*-II operon is composed of *vps* genes L-Q (1). Each *vps* cluster is commonly referred to by the first gene within the operon, hence the *vps*-I operon is noted as *vpsA*, while the *vps*-II operon is noted as *vpsL*.

V. cholerae, and numerous other bacteria, acquire molecules from its surrounding environment for proliferation and developmental mechanisms. Polyamines constitute one of the major signaling molecules and have demonstrated a role in the bacterium's regulation of transcription and translation, as well as biofilm formation (6). *V. cholerae* has shown the ability to synthesize four polyamines, putrescine, diaminopropoane, cadaverine, and norspermidine, while it relies on the transportation of spermidine and spermine into its cells (4). Polyamines work to regulate the expression of genes to maintain optimal growth conditions, while extreme levels can disrupt cell growth and functions (8). Ideal polyamine concentrations within the bacterium are generated through processes such as import, biosynthesis, degradation, and excretion (8).

With a composition of a short hydrocarbon backbone and two or more amine groups, polyamines arrange themselves in a charge-structure conformation with regularly spaced positive charges along the chain. These distributed positive charges leave the polyamines protonated at physiological pH, requiring an active transporter to move the molecules across the membrane of the Gram-negative bacterium (6). *V. cholerae*, similar to *Escherichia coli* (*E. coli*), employs polyamine importers belonging to the ABC-type transporter superfamily, PotABCD and PotFGHI (8). The PotABCD transporter is determined to be a spermidine-preferential ABC-type importer, with each protein aiding in a different function of cell regulation and nutrient uptake (8). PotA is an ATPase that works to supply the essential energy to drive polyamines into the cell. PotB and PotC work as transmembrane proteins to develop a channel through the inner membrane, which allow the passage of polyamines. PotD functions as a substrate binding protein, binding to spermidine and putrescine and

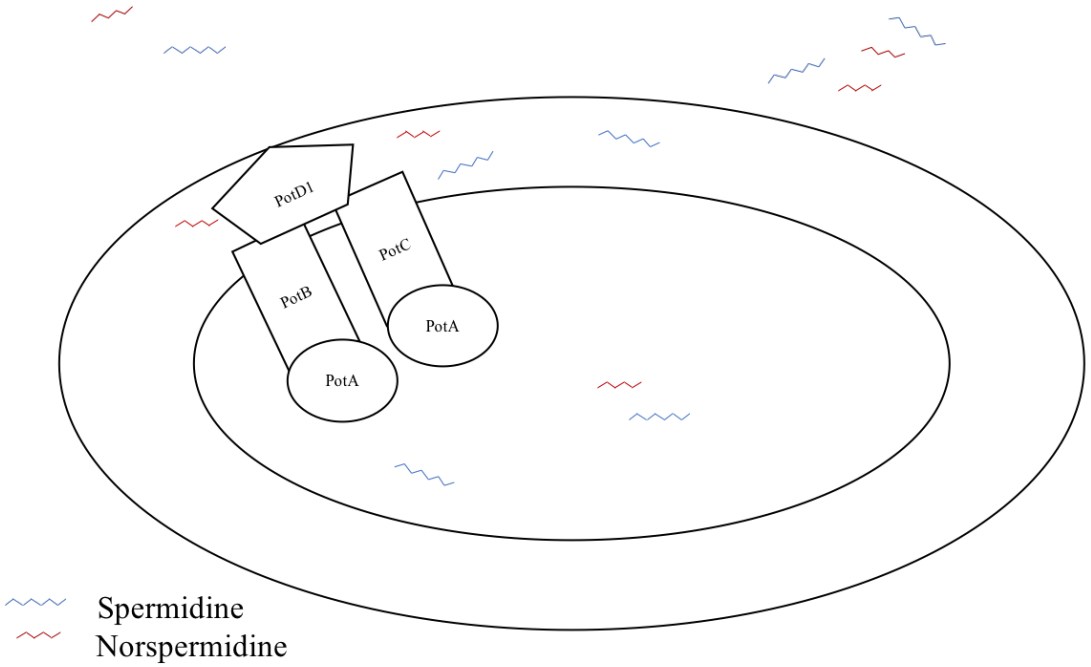

Figure 1. PotABCD1 polyamine transport system in *V. cholerae*. The system is composed of PotA (ATPase) PotB and PotC (channel proteins), and PotD1 a substrate binding protein.

introducing them to the system (8).

V. cholerae contains a PotABCD2D1 polyamine importer (**Figure 1**)(6). Previous studies have shown the role of PotD1 in transport of spermidine and norspermidine, while PotD2 remains absent in the process (6,4).

Characterization of the varying proteins constituting *V. cholerae* and their independent roles in polyamine transport and biofilm synthesis is generated through the construction of mutants lacking a specific protein. The absence of these proteins allows the observation of their direct impact on *V. cholerae*'s biofilm phenotype and transport system. Previous studies have addressed the impacts of VPS on *V. cholerae*'s ability to form biofilms. In an analysis of the impact of all *vps* genes on the biofilm phenotype, studies revealed the role of *vpsA* and *vpsL* clusters in biofilm formation as mutant strains exhibited a reduction in biofilm abilities accompanied with a decrease in VPS secretion (1). *V. cholerae* strains that exhibited enhanced *vps* gene transcription and subsequently increased VPS production also demonstrated increased biofilm formation abilities (1).

Studies aimed to understand the role of the PotABCD2D1 transport system on *V. cholerae* biofilm formation showed that mutations in the genes encoding PotA, PotB, PotC, and PotD lead to an increase in biofilm formation when compared to the wild-type strain (6,8). Biofilm formation is commonly influenced by the polyamines spermidine and norspermidine (6). As a spermidine-preferential transporter, these studies have suggested that the PotABCD transport system supplies adequate transport of the polyamines that aid in the biofilm phenotype (6,8). However, in order to thoroughly characterize the transporter, contribution of each gene to the transport of norspermidine and spermidine must be determined. Further studies in *V. cholerae* demonstrated that deletion of genes encoding each

Pot protein led to higher biofilm formation and an accompanied increase in *vps* gene transcription (7).

In order to further characterize the PotABCD1 transport system and the role of each Pot protein in the transport of spermidine and into the bacterial cells, we must be able to extract and quantify the polyamines. The high levels of VPS production experienced in the *pot* mutants has led to difficulties in employing these experiments. The objective of the study was to delete the *vpsL* operon, a known requirement for biofilm formation, from the *potB* mutant to deplete its biofilm formation abilities. Construction of a Δ*vpsL*Δ*potB* double mutant was employed for further examination of PotB's independent impact on polyamine transport following depletion of the biofilm phenotype. The double mutant was hypothesized to decrease biofilm formation when compared with wild type, resulting from the absence of the *vpsL* genes.

METHODS

Bacterial strains and media

A recipient *V. cholerae* strain, *AK396 (*VC 357Δ*potB*), with streptomycin (Sm) resistance and a donor *E. coli* strain *AK721* (SMλpir w/ pWM91::Δ*vpsL*) with ampicillin (Amp) resistance were utilized to construct the Δ*vpsL*Δ*potB* double mutant. Throughout the experiment, the bacterial strains were plated on Luria-Bertani (LB) plates with various antibiotics or sucrose plates. Approximately, twenty LB plates were utilized consisting of 5.0 g of NaCl, 7.5 g of agar, 2.5 g of yeast extract, and 5.0 g of tryptone. The ingredients, as well as 250 mL of deionized (DI) water, were measured prior to addition to a 1000-mL Erlenmeyer flask. The flask was placed on a stir plate and stirred using a Teflon stir bar for five minutes as an additional 250-mL of DI water was added. Following adequate stirring,

the flask was topped with aluminum foil and placed into an autoclave. A liquid cycle with a sterilization time of twenty minutes at 121°C was utilized to sterilize the LB media. After proper sterilization, additional stirring of the broth occurred as it was allowed to cool. An array of LB plates containing no antibiotic, ampicillin (Amp), streptomycin (Sm), and a combination of ampicillin and streptomycin (Sm/Amp) were created. Media for the LB plates with no antibiotics were poured approximately halfway up each plate. Additional antibiotics were added to the broth once it reached room temperature. LB-Amp and LB-Sm plates were prepared through addition of the respective antibiotic to a final concentration of 100 μg/mL. LB-Sm/Amp plates were prepared with 50 μg/mL of ampicillin and 100 μg/mL of streptomycin. The plates were allowed to cool and stored in an inverted position in a cold room at a temperature of 4°C.

Sucrose plates were made from 7.5 g agar, 5 g tryptone, 2.5 g yeast extract and 333.3 mL of DI water added to a 1L beaker and placed in an autoclave for sterilization. While the media was autoclaved, a 30% sucrose solution was prepared. A 1000-mL beaker was obtained and filled with 500 mL of sterile DI water and 300 g of sucrose under heating and mixture on a hot plate. Further addition of sterile DI water filled the beaker to 900 mL where it was mixed until the solution became translucent. Following color change, sterile DI water was added to bring the solution to 1000 mL. The sucrose solution was then filtered into a clean 1-L glass bottle. One hundred and sixty-six mL of the 30% filtered-sterilized sucrose was added to the prepared media and poured into plates using the same method described for LB plates.

Overnight and day cultures were prepared using Lysogeny broth (LB) and YT broth One-L of LB was prepared utilizing a 2000-mL Erlenmeyer flask. The flask was placed on a

stir plate as 500 mL of DI water was added in addition to 10 g of tryptone, 5 g of yeast extract, and 10 g NaCl. The flask was then filled with DI water to the 1000 mL mark. YT was prepared the same way, but with 0.584 g NaCl, 10 g yeast extract, and 15 g tryptone. The broths were stored in 250 mL bottles at room temperature

Table 1. Bacterial strains used in the study

Bacterial strain	Descriptor	Source
PW357	MO10 *lacz*::*vpsLp* → *lacZ*, sm^R	(Haugo and Watnick, 2002)
PW396	MO10 Δ*vpsA*, sm^R	(Kierek and Watnick, 2003)
AK396	VC357 Δ*potB*	(Sanders, 2015)
AK721	SM 10λpir w/pWM91::Δv*psL*	This study, (Haugo and Watnick, 2002)
AK814	PW357 Δ*vpsL* operon	Karatan lab stock
AK820	AK396 Δ*vpsL* operon	This study

Construction of the Δ*vpsL*Δ*potB* mutant

V. cholerae strain, *AK396*, was streaked for isolation on an LB-Sm plate. *AK396* contained a Δ*potB* mutation. An *E. coli* strain, *AK721*, was streaked for isolation on an LB-amp plate. *AK721* housed the pWM91 plasmid with a Δ*vpsL* insertion. Aseptic technique and sterile wooden sticks were utilized for the streaking of both strains. A single wooden stick was selected for initial streaking of the plate with the respective bacterial strain. After streaking of the first quadrant, the wooden stick was disposed in a beaker for autoclave. An additional wooden stick was selected and utilized to streak from the first quadrant into the

second. The same process was continued, with a new sterile wooden stick, between each quadrant until the four quadrants were created. Following streaking, the plates were inverted and incubated overnight at 37°C. After overnight growth and the isolation of single colonies, the strains were mated. A single *E. coli* colony from its respective LB-Amp plate was selected and spread on a new non-selective LB plate using a sterile wooden stick for maximum surface area. A single *V. cholerae* colony was selected and spread over the non-selective LB plate containing the streaked *E. coli* colony. The non-selective LB plate was inverted and incubated overnight at 37°C.

Following overnight growth, the *AK721* x *AK396* plate used for mating was utilized for selection of a single cross over. The growth on the LB plate was divided and removed with a sterile wooden stick. Each half of growth on the LB plate was streaked for isolation on an individual LB-Sm/Amp plate, generating two new plates. The new plates were incubated overnight at 37°C. Utilizing a sterile wooden stick, a single colony was removed from the LB-Sm/Amp plate and streaked for isolation on a new LB-Sm/Amp plate. The process was repeated three additional times, each with a different single colony. The plates were incubated overnight at 37°C. Four single colonies were selected from the LB-Sm/Amp colony purification plates and streaked for isolation on non-selective LB plates to allow for cross-out. The plates were inverted and incubated overnight at 37°C.

Four colonies from the LB plates were selected using the previous methods and streaked for isolation on sucrose plates. Each original LB colony was plated on two independent sucrose plates, giving rise to eight plates total. The sucrose plates were inverted and incubated at room temperature for forty-eight hours.

Colony PCR to screen for the Δ*vpsL*Δ*potB* mutant

The sucrose plates were analyzed for isolated single colonies after incubation for forty-eight hours. An LB-Sm/Amp plate was marked and divided into 47 equally sized boxes. The same boxes were marked on an LB-Sm plate. The boxes were numbered one through forty-seven. Forty-seven individual colonies from the sucrose plates were patched on the LB-Sm plate and the LB-Sm/Amp plate. Corresponding numbers on the LB-Sm and LB-Sm/Amp plates represented the same isolated colony. The plates were incubated overnight at 37°C.

Colonies that grew exclusively on the LB-Sm plate were utilized for colony PCR. Sm resistant and Amp-sensitive colonies represented incorporation of the Δ*vpsL* insertion into the *V.cholerae* chromosome and removal of the rest of the plasmid from the chromosome. A single colony from the LB-Sm plate was selected using a toothpick and deposited into a sterile PCR tube containing 100μL of DI water. The colony was equally dispersed throughout the water through adequate mixing with a pipette. The process was repeated eight more times, with seven additional LB-Sm colonies and a positive control. *AK721* was used as the positive control. The tubes were incubated in a thermocycler for five-minute at 95°C to lyse the cells. Following proper lysing of the cells, the tubes were centrifuged for five minutes at 8000 rpm. A mastermix was prepared in a microcentrifuge tube consisting of 164.0 μL of DI water, 50.0 μL of 5x OneTaq Standard Reaction Buffer, 5.0 μL of dNTP, 5.0 μL of 100 μM primer 1 (P358), 5.0 μL of 100 μMprimer 2 (P359), and 1.25μL of OneTaq enzyme. A 750 bp fragment was expected if the *vpsL* gene was deleted. If the *vpsL* gene was not properly deleted, no band would be detected due to lower extension intervals set for the PCR cycling conditions. Nine new PCR tubes were obtained and marked with corresponding numbers to those of the sample containing PCR tubes. Twenty-three μL of mastermix was

added to each new PCR tube. 2 μL of supernatant from the PCR tubes containing the lysed bacteria were added to their respective mastermix PCR tube. The new solutions were mixed. Colony PCR was run for ninety-minutes and thirty cycles. An initial denaturation was run at 94°C for 30 seconds. Each cycle contained a denaturation phase at 94°C for 15 seconds, an annealing phase at 55°C for 15 seconds, and an extension phase at 68°C for one minute. A final extension phase was run at 68°C for five minutes.

Following PCR, the samples were loaded into an agarose gel composed of 0.5 g agarose powder and 50 mL of 1X Tris acetic acid EDTA buffer (TAE). One hundred mL of 10X TAE was diluted with 900 mL of water to make a 1X TAE solution. Ten X TAE was prepared with 900 mL DI water, 48.4 g Tris base, 11.4 mL glacial acetic acid, 3.72 g EDTA. Additional DI water was utilized to adjust the volume to 1 L. An Erlenmeyer flask was utilized for the mixing of the gel ingredients and microwaved for one minute to melt the agarose. Upon proper heating and mixture, 2.5 μL of ethidium bromide was added and the solution was poured into a gel box prepared with a ten well comb. Once solidified, the comb was removed from the gel. One X TAE was added to the gel box, covering the entirety of the gel. Five μL of purple 2-log ladder was added to the first well, standing as a molecular size marker. Five μL of purple loading dye was added to each PCR tubes. The dye was thoroughly mixed with each sample using a pipette. 15 μL of each sample was loaded into a well. The gel was run at 0.1 Ampess for forty-five minutes. The gel was imaged using AlphaImager HP.

Preparation of stock cultures

One newly constructed double mutant strain containing both the Δ*vpsL* and the Δ*potB* mutations from colony twenty-two was frozen. Colony twenty-two, which was identified to

contain the double mutant through colony PCR, was streaked for isolation on an LB-Sm plate. A single colony was inoculated into a borosilicate tube containing 2 mL of LB broth and 2 μL with 100 mg/mL streptomycin. The tubes were incubated over-night in a 37°C shaking incubator. Following over-night incubation, 2 mL of LB containing 30% glycerol was added to the overnight cultures. 1 mL of this culture was pipetted into a cryogenic vial and frozen. The strain was numbered as *AK820*.

Biofilm assay with LB

The Δ*vpsL*Δ*potB* double mutant, wild-type, Δ*vpsL*, and the Δ*potB* mutant were streaked for isolation on LB-Sm plates. The plates were incubated at 27 °C. Following overnight incubation, a mastermix was prepared consisting of 6 mL of LB and 6 μL of 100 mg/mL streptomycin. Four borosilicate tubes were obtained, one for each *V. cholerae* strain. 2 mL of the mastermix was loaded into each borosilicate tube. Each borosilicate tube was inoculated with a single colony from its respective LB-Sm plate through the use of a sterile wooden toothpick. The tubes were placed in a shake incubator overnight at 27 °C. The next day, day cultures were prepared. Four new borosilicate tubes were obtained and loaded with 2 mL of LB and 2 μl of 100 mg/mL streptomycin each. Each day culture tube was inoculated with 40 μL of its respective overnight culture. The tubes were incubated at 27°C for four hours. The cells were grown to mid-log phase. A microplate was obtained and loaded with 100 μL of each strain to measure the optical density of the samples at an absorbance of 655 nm following a ten second mixing period. Two readings were performed for each strain to determine an average optical density for the strain. An LB background measurement of 0.040 was subtracted from each average. The value of the average optical density minus the LB background was used as C2 in the C1V1=C2V2 equation. A value of 0.340 was the desired

final optical density (C1). A value of 300 μL was the desired final volume (V1). The V2 value of each strain was calculated. Three borosilicate tubes were gathered for each original strain. A mastermix containing 10 mL LB and 10 μL of 100 mg/mL streptomycin was prepared. The tubes were prepared with the calculated amount of sample and mastermix and incubated overnight at 27°C.

Quantification of the biofilm and planktonic cells was performed the next day. One-hundred and fifty μL of planktonic cells from each borosilicate tube was removed and added to a microplate. The remaining planktonic cells were removed. The borosilicate tubes were washed with 300 μL of PBS. PBS was constructed from 1 L D1 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. The PBS was removed and discarded into a waste container. Three-hundred μL of PBS, as well as glass beads were added to the washed tubes. The tubes were vortexed for approximately 30 seconds until the samples no longer contained visible particulates of biofilm. One-hundred and fifty μL of each sample was added to the microplate. The optical densities of planktonic and biofilm cells were observed at an absorbance of 655 nm. Microsoft Excel was utilized to construct bar graphs for each sample showing average optical densities and standard deviations amongst replicates.

Biofilm assay with YT broth

The frozen Δ*vpsL*Δ*potB* double mutant (*AK820*), as well the wild-type, Δ*vpsA* mutant, Δ*potB* mutant, and Δ*vpsL* mutant were streaked for isolation on LB-Sm plates and incubated overnight at 27°C. The following day, a mastermix containing 32 mL of YT and 32 μL of 100 mg/mL streptomycin was prepared. Fifteen borosilicate tubes were obtained with three labeled for each strain. Each tube was prepared with 2 mL of YT and 2 μL of 100 mg/mL

streptomycin. A sterile toothpick was utilized for selection of a single colony from the respective LB-Sm plates and inoculated into the borosilicate tubes. The process was repeated for all 15 tubes. The tubes were incubated overnight in a shake-incubator set at 27°C. The next day, 40 μL of each overnight culture was inoculated into a new day-culture tube containing 2 mL of YT and 2 μL of 100 mg/mL streptomycin. The tubes were incubated at 27°C for four hours. After growth to mid-log phase, 100 μL of each strain was loaded into a microplate well and the optical density of each sample was measured at an absorbance of 655 nm following a ten second mixing period. Two readings were obtained for each strain to measure the average optical density of each sample. YT was utilized for subtraction of background (0.035) from the average. This number was used as the C2 in the C1V1=C2V2 equation, which was used to calculate V2. A C1 value of 0.340 was the desired final optical density number. A V1 value of 300 μL signified the final sample volume. A mastermix containing 16 mL of YT and 16 μL of 100 mg/mL streptomycin was prepared. Three borosilicate tubes were obtained per replicate and inoculated with the calculated amount of sample and YT mastermix. The tubes were incubated overnight at 27°C.

The following day, 150 μL of planktonic cells from each borosilicate tube was removed and placed into the wells of the microplate. The remaining planktonic cells were discarded, and the borosilicate tubes were washed with 300 μL PBS. The initial PBS was removed and the addition of 300 μL PBS was repeated. Glass beads were added to the tubes in addition to PBS. The tubes were vortexed for approximately 30 seconds. One-hundred and fifty μL of each sample was added to a new well on the microplate. The optical density of all planktonic and biofilm cells was read at an absorbance of 655 nm. Bar graphs showing the average and standard deviations of each sample were constructed using Microsoft Excel.

Sequence analysis of pWM91::Δ*vpsL*

Two borosilicate tubes were utilized to prepare overnight cultures. Each borosilicate tube was filled with 2 mL LB and 2 μL of 100 mg/mL ampicillin. A sterile wooden toothpick was utilized to select a single colony from a previously streaked LB-Amp plate containing *AK721* (SMλpir w/ pWM91:Δ*vpsL*). The process was repeated for an additional *AK721* colony. The tubes were incubated overnight in a shaking incubator at 37° C.

Plasmids were isolated from the individual colonies using a Wizard Plus SV Miniprep DNA Purification System (Promega). Four 1.5 mL microcentrifuge tubes were collected and labeled for the respective colony, allowing two microcentrifuge tubes per colony. Eight hundrend mL of each overnight was dispensed into its representative microcentrifuge tube and centrifuged for five minutes at 10.0 rpm. Following centrifugation, a vacuum was used to remove the supernatant from each microcentrifuge tube. Two hundred and fifty μL of Cell Resuspension Solution was added and the cell pellet was re-suspended by pipetting. After complete re-suspension, 250 μL of Cell Lysis Solution was added and each tube was inverted four times for proper mixing. The tubes were incubated at room temperature for four minutes to allow complete lysis of the cells. Ten μL of Alkaline Protease Solution was added to each tube and inverted four times. The tubes were incubated for an additional four minutes at room temperature. The Alkaline Protease Solution allowed for inactivation of endonucleases and other protein products released during the cell lysis phase. Three hundred and fifty μL of Neutralization Solution was immediately added to each tube with four inversions following its addition. The bacterial lysates were centrifuged for ten minutes at 14.0 rpm at room temperature.

The lysates were then transferred to spin column by decanting. The spin column was

placed in a clean 1.5 microcentrifuge tube centrifuged at 14.0 rpm for one minute. Following centrifugation, flowthrough was discarded and 750 μL of diluted Column Wash Solution was added to the spin column. The column/microcentrifuge complex was centrifuged for one minute at 14.0 rpm. The flowthrough was discarded as previously described. The wash procedure was repeated with 250 μL of Column Wash Solution and centrifugation occurred for two minutes at 14.0 rpm. The spin column was transferred to a new 1.5 mL microcentrifuge tube. Fifty μL of Nuclease-Free Water was added to the column and centrifuged for one minute to elute the plasmid of interest, pWM91::Δ*vpsL*. The duplicate representative microcentrifuge tubes were combined into one and stored in a freezer while the spin column was discarded.

Nanodrop readings were performed on both *AK721* purified plasmids. The first plasmid resulted in a 260/280 reading of 1.87 and a 260/230 reading of 1.15 with a concentration of 33.5 ng/μL. The second plasmid resulted in a 260/230 reading of 2.08 and a 260/230 reading of 1.28 with a concentration of 43.9 ng/μL.

A 50 μL PCR mastermix was constructed of 97.5 μL of DI water, 3 μL dNTP, 7.5 μL of the M13 Forward primer (PA360), 7.5 μL of the M13 Reverse primer (PA361), 30 μL 5X Q5 Standard Reaction Buffer, and 1.5 μL Q5 polymerase. One μL of the concentrated plasmid was added to PCR tube 1, with 1 μL of a diluted (10:1 with DI water) plasmid was added to PCR tube 2. The samples were loaded into a PCR machine and run for thirty cycles. An initial denaturation was run at 98°C for 30 seconds. Each cycle contained a denaturation phase at 98°C for 10 seconds, an annealing phase at 50°C for 30 seconds, and an extension phase at 72°C for 30 seconds. A final extension phase was run at 72°C for two minutes.

Following PCR, the samples were loaded onto a gel composed of the same

ingredients above. Five μL of each respective sample was added to a new PCR tube with 2 μL of purple loading dye. Five μL of 2 log ladder was loaded to lane 1, while 7 μL of sample from PCR tube 1 was added to lane 2 and 7 μL of sample from PCR tube 2 was added to lane 3. The gel was covered with TAE and run for 30 minutes at 0.1 amperes.

The purified plasmid was sent to Eurofins Genomics for sequencing. The received sequences were copied into Vector NTI. Sections of the forward and reverse sequence were copied into the Integrated Microbial Genomes and Microbiomes database BLASTn (https://img.jgi.doe.gov/). BLASTn is an algorithm designed to identify similar nucleotide sequences within a database. The sequences were compared against the MO10 *V. cholerae* genome to determine the presence of the *vpsL* deletion in the plasmid.

RESULTS

Construction of the Δ*vpsL*Δ*potB* mutant

The Δ*vpsL*Δ*potB* double mutant candidates were created through mating of an *E. coli* strain, *AK721*, which contained the pWM91 plasmid with a Δ*vpsL* insertion and a *V. cholerae* strain, *AK396*, containing a Δ*potB* deletion. Proper insertion of Δ*vpsL* within the newly constructed double mutants was validated through colony PCR. The Δ*vpsL* insertion was identified by a 750 bp fragment. Colony PCR was performed on eight double mutant candidates and a single *AK721* (pWM91::Δ*vpsL*) colony. The resulting PCR products were loaded onto an agarose gel and imaged using an AlphaImager. Lane 1 contained the 2-Log DNA Ladder for fragment size comparison. Lane 2 contained the PCR product of the *AK721* colony (**Figure 2**). The single *AK721* colony was utilized as a positive control. Lanes 3 through 10 contained the PCR products of Δ*vpsL*Δ*potB* double mutant candidates (**Figure 2**). Only one of the possible eight double mutant candidates exhibited a band at 750 bp,

indicating proper deletion of the *vpsL* gene (**Figure 2**). The absence of bands was indicative of unsuccessful deletions as the resulting fragment would be too large for amplification using the extension time selected for the PCR cycles. A band was not visualized in lane 2 where the AK721 positive control was loaded (**Figure 2**).

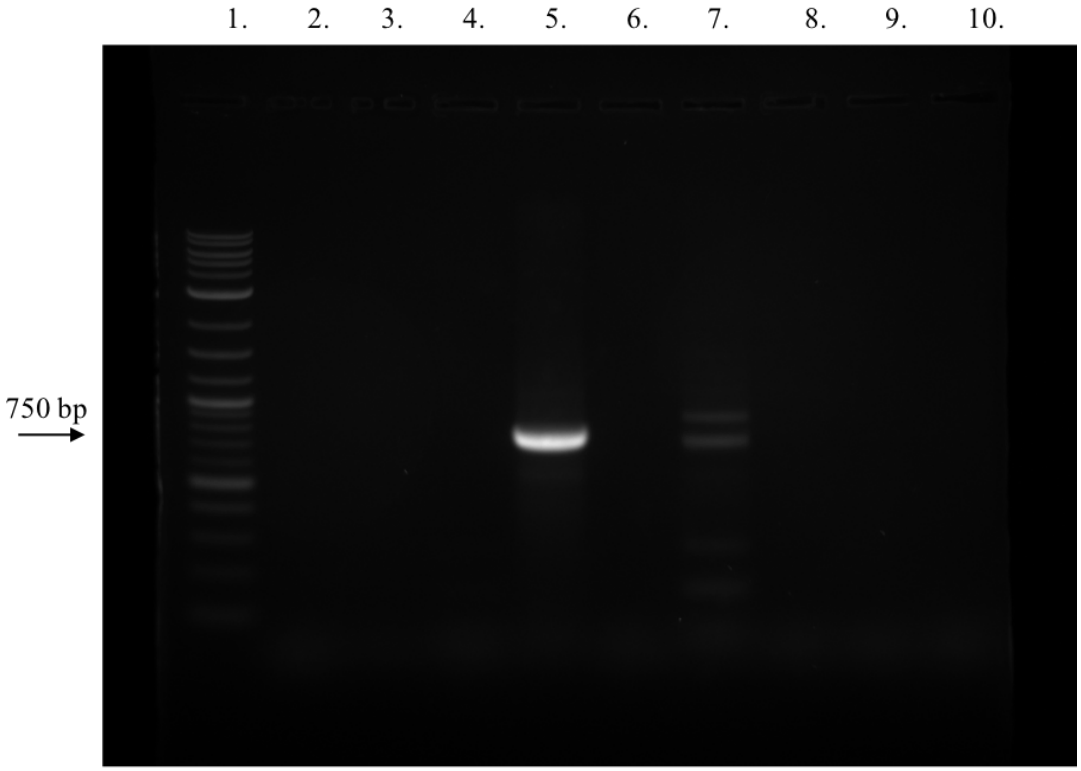

Figure 2. A gel image of the PCR reaction products run to determine the presence of a Δ*vpsL* insertion within eight Δ*vpsL*Δ*potB* double mutant candidates. Lane 2 contained the PCR reaction product of a single *AK721* colony. Lanes 3 through 10 contained the PCR product of Δ*vpsL*Δ*potB* candidates.

The absence of a 750 bp fragment in lane 2 was unexpected. To determine if the plasmid used for conjugation was constructed correctly, it was isolated from the cells and the relevant regions were sequenced. Following sequence analysis and identification of the Δ*vpsL* insertion from alignment with the MO10 *V. cholerae* genome as described in Methods, an additional PCR reaction was performed to confirm the deletion in the

Δ*vpsL*Δ*potB* double mutant candidate identified (**Figure 3**). The PCR reaction product of the double mutant candidate from lane 5 (**Figure 2**) was run on an agarose gel with a positive and negative control (**Figure 3**). The PCR product of the isolated pWM91::Δ*vpsL* plasmid was utilized as a positive control, while the PCR product of a wild-type *V. cholerae* colony stood as the negative control. The resulting PCR samples were separated by running on an agarose gel and imaged with an AlphaImager.

Lane 2 contained the PCR reaction product of the isolated pWM91::Δ*vpsL* plasmid. (**Figure 3**). Lane 3 contained the PCR reaction product of the wild-type *V. cholerae* colony, while lane 4 contained the PCR reaction product of the Δ*vpsL*Δ*potB* candidate colony (**Figure 3**).

The isolated pWM91::Δ*vpsL* PCR sample in lane 2 exhibited a 750 bp-band, expected from the absence of the *vpsL* gene. The Δ*vpsL*Δ*potB* double mutant candidate PCR sample in lane 4 exhibited an almost identical band at 750 bps, indicating a successful deletion of the *vpsL* gene. The wild-type *V. cholerae* PCR product in lane 3 showed no indication of a band at 750 bp, which was expected as it still contained the *vpsL* gene (**Figure 3**).

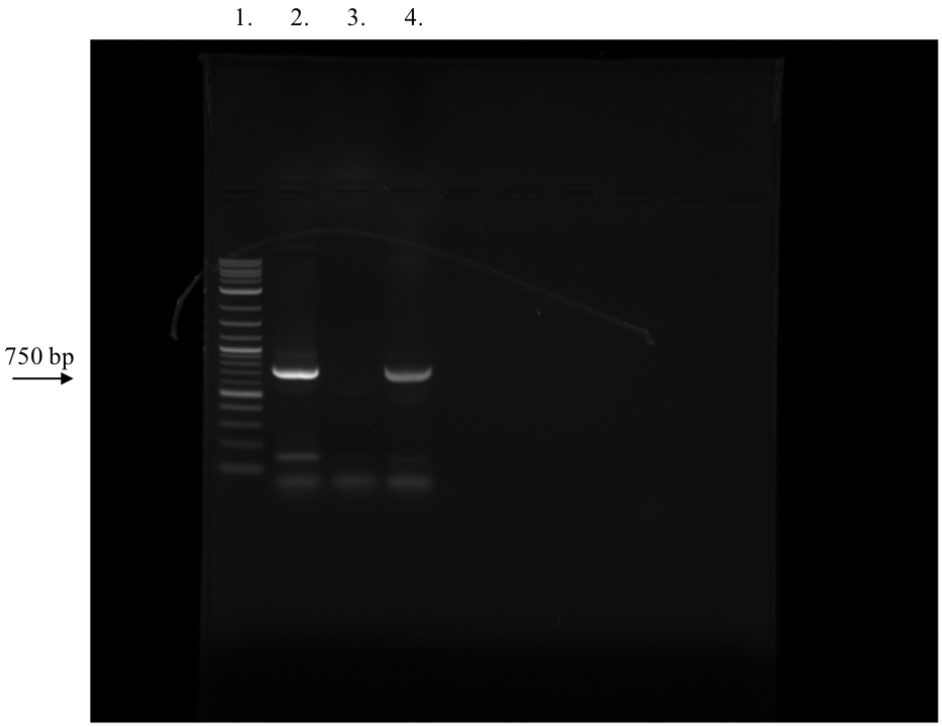

Figure 3. A gel image of the PCR reaction products run to determine the presence of the Δ*vpsL* insertion. Lane 2 contained the PCR product of the isolated pWM91::Δ*vpsL* plasmid. Lane 3 contained the PCR product of wild-type *V. cholerae*. Lane 4 contained the PCR product of a Δ*vpsL*Δ*potB* double mutant candidate.

Effects of Δ*vpsL*Δ*potB* deletion on biofilm formation

To determine if deletion of the *vpsL* operon in a Δ*potB* mutant inhibited biofilm formation, I conducted biofilm assays. The mutation's impact on biofilm formation was quantified through analysis of planktonic and biofilm cell densities. The planktonic cells were measured to quantify cell growth. The planktonic cell density of wild-type *V.cholerae*, Δ*potB*, *ΔvpsL*, and Δ*vpsL*Δ*potB* mutants were determined through measurement of the optical density at an absorbance of 655 nm (OD_{655}). The OD_{655} values of wild-type and the Δ*vpsL* mutant were greater than the values observed for the Δ*potB* and Δ*vpsL*Δ*potB* mutants (**Figure 4**). Wild-type had an OD_{655} of approximately 0.30. The Δ*potB* mutant showed an OD_{655} of roughly 0.18. The Δ*vpsL* mutant had an OD_{655} value near 0.30, while the

Δ*vpsL*Δ*potB* double mutant had an OD_{655} reading of 0.20 (**Figure 4**).

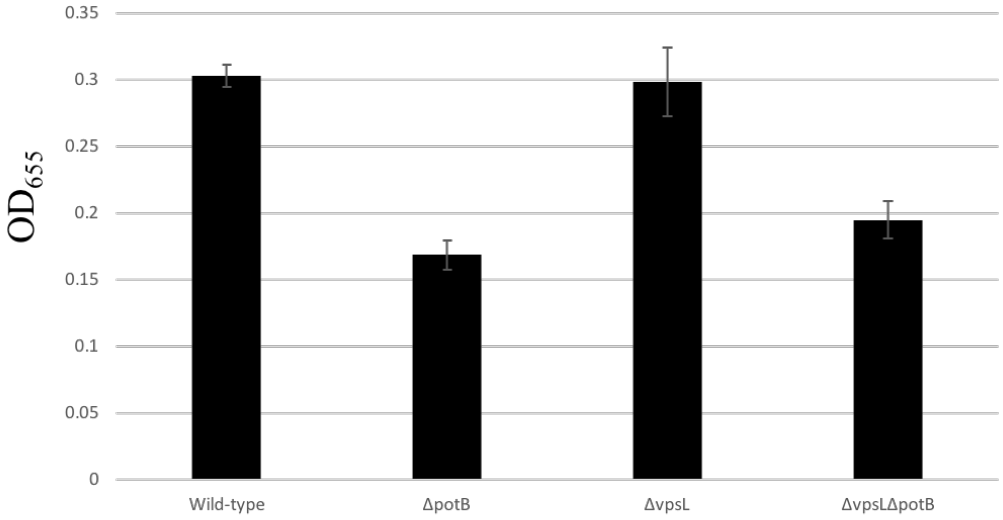

Figure 4. Planktonic cell density of wild-type and mutant *V. cholerae* cells. Biofilm assays were performed on wild-type and the Δ*potB*, Δ*vpsL*, and Δ*vpsL*Δ*potB* mutants in borosilicate culture tubes containing LB and 100 mg/mL streptomycin. Biofilm assays were performed and quantified as described in Methods. The average of three technical replicates was recorded with error bars signifying the standard deviation for each biological replicate.

The biofilm cell density of wild-type, Δ*vpsL*, Δ*potB*, and Δ*vpsL*Δ*potB* mutants were determined through measurement of the optical density of biofilm cells at 655 nm (OD_{655}). Wild-type, the Δ*potB*, Δ*vpsL*, and Δ*vpsL*Δ*potB* mutants all showed similar average biofilm cell densities. Wild-type had an approximate OD_{655} of 0.41. The Δ*potB* mutant had an OD_{655} value of roughly 0.51. The Δ*vpsL* mutant had an OD_{655} reading of 0.40, while the Δ*vpsL*Δ*potB* double mutant had a reading of 0.49 (**Figure 5**).

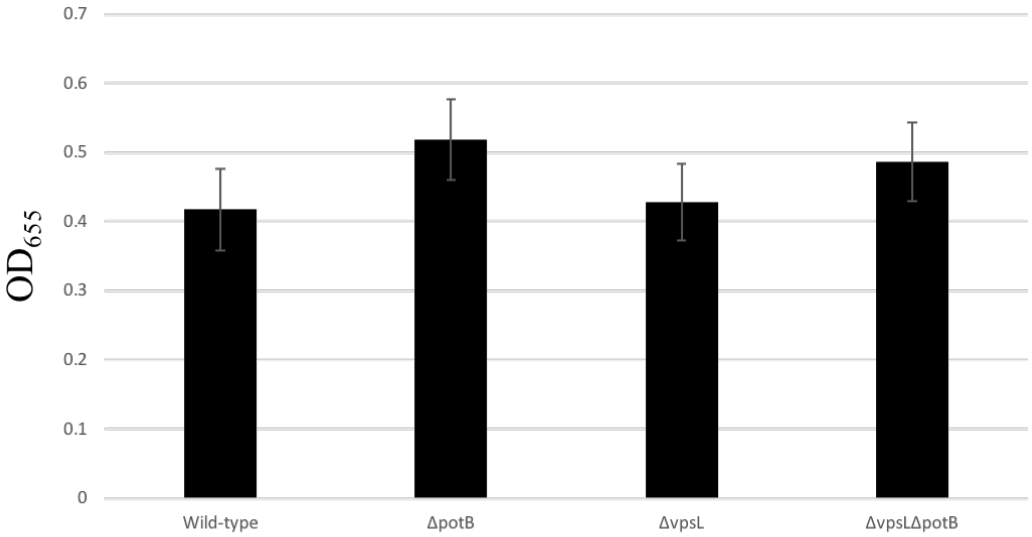

Figure 5. Biofilm cell density of wild-type and mutant *V. cholerae* cells. Biofilm assays were performed on wild-type and the Δ*potB*, Δ*vpsL*, and Δ*vpsL*Δ*potB* mutants in borosilicate culture tubes containing LB and 100 mg/mL streptomycin. Biofilm assays were performed and quantified as described in Methods. The average of three technical replicates was recorded with error bars signifying the standard deviation for each biological replicate.

Assessment of biofilm formation with YT broth

Biofilm assays were performed using YT broth to observe the changes in average planktonic and biofilm cell densities in comparison to those performed in LB. Results of the biofilm assay performed with LB showed unexpected results for the biofilm phenotype of the Δ*vpsL* and Δ*vpsL*Δ*potB* mutants. Previous studies have shown that in Δ*vpsL* mutants, biofilm formation was depleted (1). However, in the LB biofilm assay, the Δ*vpsL* mutant showed an OD_{655} value of 0.40 (**Figure 5**). Media containing higher concentrations of Na^+ and Ca^{2+} have been linked with increased biofilm phenotype (10). The LB media contained 171 mM of NaCl, while the YT broth contained 10 mM NaCl. The decreased salt concentration of YT broth was utilized to observe if the high NaCl concentration of LB was responsible for the unexpected biofilm formation of the Δ*vpsL* mutant or if the resulting phenotype was constant for the Δ*vpsL* mutant across varying media.

Average cell densities of three biological replicates of each strain for YT biofilm phenotype were determined using Excel and used for comparison amongst the mutants. The average planktonic OD_{655} values for wild-type and the Δ*vpsL* mutant were greater than the values observed for the Δ*vpsA*, Δ*potB*, and Δ*vpsL*Δ*potB* mutants (**Figure 6**). Wild-type had an approximate OD_{655} of 0.73. The Δ*potB* mutant had an OD_{655} at 0.41. The Δ*vpsL* mutant has an OD_{655} reading of 0.74, while the Δ*vpsL*Δ*potB* double mutant had a value of 0.37 (**Figure 6**).

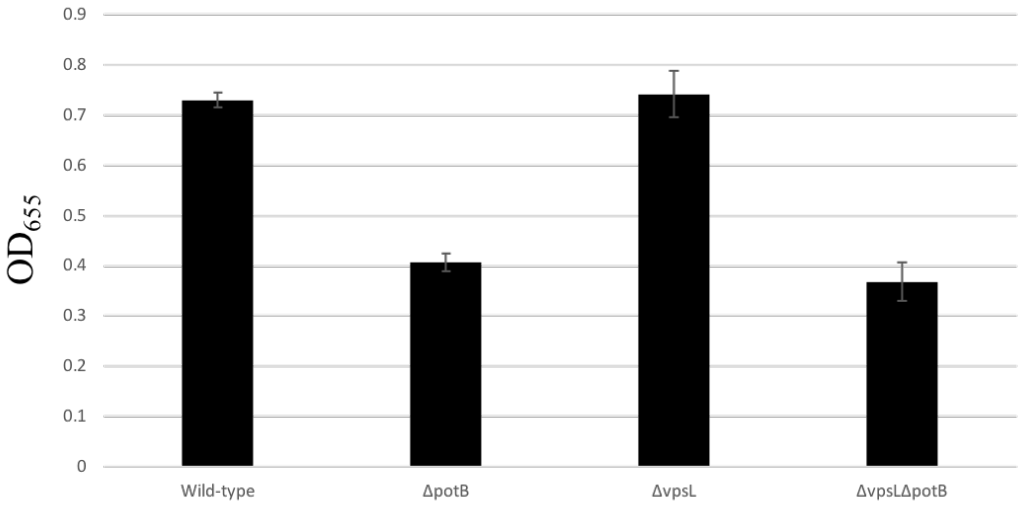

Figure 6. Planktonic cell density of wild-type and mutant *V. cholerae* cells. Biofilm assays were performed on wild-type and the Δ*potB*, Δ*vpsL*, and Δ*vpsL*Δ*potB* mutants in borosilicate culture tubes containing YT broth and 100 mg/mL streptomycin. Biofilm assays were performed and quantified as described in Methods. The average of nine technical replicates, three from each biological replicate, was recorded with error bars signifying the standard deviation.

The biofilm cell density of wild-type, Δ*potB*, Δ*vpsL*, and Δ*vpsL*Δ*potB* were determined through measurement of the optical density of the biofilm cells of each sample at 655 nm. The Δ*potB* and Δ*vpsL*Δ*potB* double mutant showed higher biofilm OD_{655} values in comparison to wildtype and Δ*vpsL* (**Figure 7**). Wildtype showed a relative OD_{655} of 0.10. The Δ*potB* mutant demonstrated an OD_{655} value 0.57. The approximate OD_{655} value of the

Δ*vpsL* mutant was 0.14, while the Δ*vpsL*Δ*potB* double mutant was 0.53 (**Figure 7**).

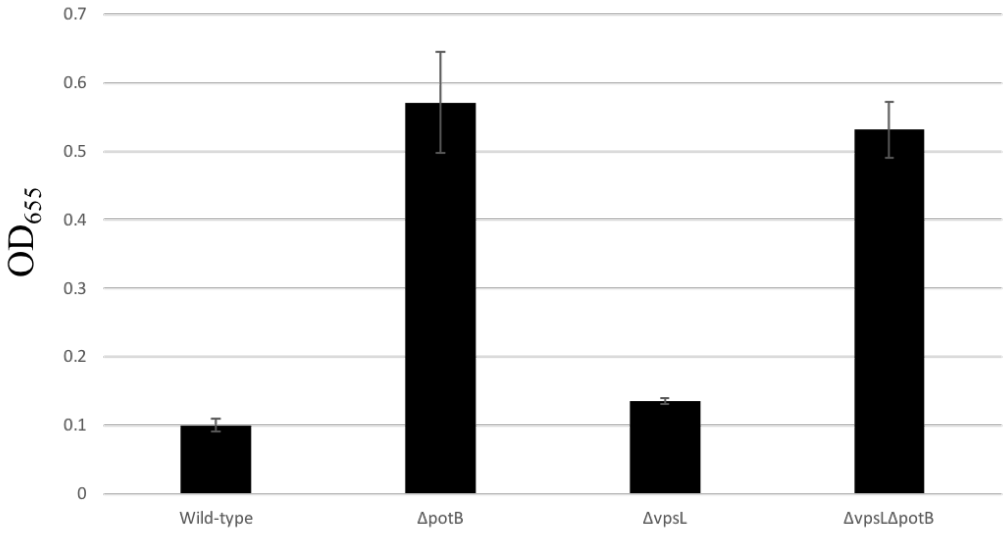

Figure 7. Biofilm cell density of wild-type and mutant *V. cholerae* cells. Biofilm assays were performed on wild-type and the Δ*potB*, Δ*vpsL*, and Δ*vpsL*Δ*potB* mutants in borosilicate culture tubes containing YT broth and 100 mg/mL streptomycin. Biofilms assays were performed and quantified as described in Methods. The average of nine technical replicate, three from each biological replicate, was recorded with error bars signifying the standard deviation.

Incorporation of the Δ*vpsA* mutant in biofilm assay

Previous studies have suggested that VPS genes are essential for formation of a biofilm structure (1). These studies have also demonstrated the effects of a Δ*vpsL* mutation on biofilm phenotype, suggesting a complete depletion in biofilm cell densities (1). These results were not observed in the biofilm assays performed in this study. Regardless of the broth type used, the Δ*vpsL* mutant showed average biofilm cell densities similar to that of the wild-type *V. cholerae*.

The studies showing the effects of a Δ*vpsL* mutant on the biofilm phenotype employed similar experiments showing the effects of a Δ*vpsA* mutant (1). The Δ*vpsA* mutants showed a depletion in biofilm formation (1). An additional biofilm assay was performed with the inclusion of a Δ*vpsA* mutant. The incorporation of the Δ*vpsA* mutant into the second

biofilm assay was done as a control to determine if its expected phenotype was achieved and for possible explanation of the unexpected Δ*vpsL* mutant phenotype observed. If the biofilm phenotype associated with the Δ*vpsA* mutant was consistent with that observed for the Δ*vpsL* mutant in the previous biofilm assays, then it would be confirmed that something different was occurring within our assays. If the Δ*vpsA* mutant showed the expected decrease in biofilm formation, it would be confirmed that something occurred within the construction of the Δ*vpsL* mutant, aiding in its ability to form biofilms.

Calculated planktonic cell densities after the second biofilm assay showed fairly consistent OD_{655} values for wild-type, Δ*potB*, Δ*vpsL*, and Δ*vpsL*Δ*potB* mutants in comparison to those of the first biofilm assay. Wild-type had an OD_{655} relative value of 0.79. The Δ*potB* mutant showed an approximate OD_{655} value of 0.44 (**Figure 8**). The Δ*vpsL* mutant had an OD_{655} reading of 0.79, while the Δ*vpsL*Δ*potB* double mutant had of 0.46. The average planktonic cell density of the Δ*vpsA* mutant was similar to that of the Δ*potB* and Δ*vpsL* mutants, with an approximate OD_{655} of 0.50 (**Figure 8**).

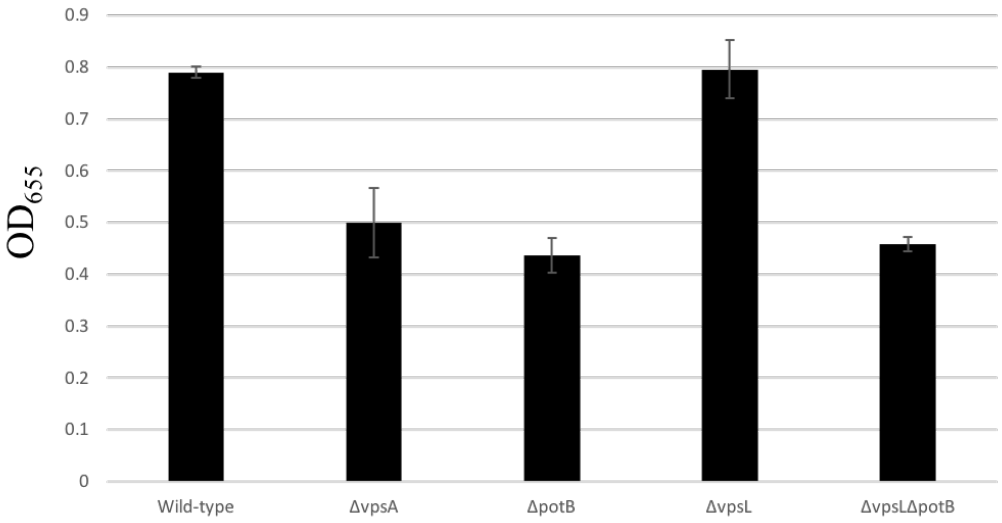

Figure 8. Planktonic cell density of wild-type and mutant *V. cholerae* cells. Biofilm assays were performed on wild-type and the Δ*potB*, Δ*vpsL*, and Δ*vpsL*Δ*potB* mutants in borosilicate culture tubes containing YT broth and 100 mg/mL streptomycin. Biofilm assays were performed and quantified as described in Methods. The average of nine technical replicates, three from each biological replicate, was recorded with error bars signifying the standard deviation.

Biofilm cell densities after the second biofilm assay showed similar trends as those observed in the first biofilm assay but had overall lower OD_{655} values. Once again, wild-type and the Δ*vpsL* mutant showed significantly less average optical densities than the Δ*potB* and the Δ*vpsL*Δ*potB* mutants. Wild-type showed a relative OD_{655} value of 0.12. The Δ*potB* mutant had an approximate OD_{655} of 0.56. The Δ*vpsL* mutant had an OD_{655} of 0.13, while the Δ*vpsL*Δ*potB* double mutant had a reading of 0.47. The Δ*vpsA* mutant demonstrated an average optical density below that of wild-type and the Δ*vpsL* mutant with a relative OD_{655} of 0.02 (**Figure 9**).

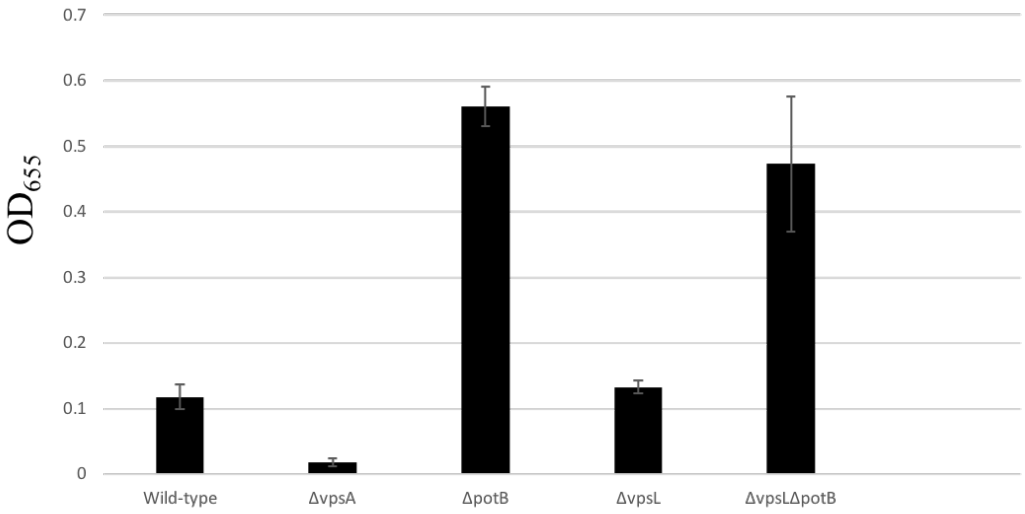

Figure 9. Biofilm cell density of wild-type and mutant *V. cholerae* cells. Biofilm assays were performed on wild-type and the Δ*potB*, Δ*vpsL*, and Δ*vpsL*Δ*potB* mutants in borosilicate culture tubes containing YT broth and 100 mg/mL streptomycin. Biofilms assays were performed and quantified as described in Methods. The average of nine technical replicate, three from each biological replicate, was recorded with error bars signifying the standard deviation.

Picture documentation allowed visual observation of biofilm formation and comparison after 18 hours. The Δ*vpsA* mutant showed no biofilm production after 18 hours, while the Δ*potB* and Δ*vpsL*Δ*potB* double mutant seemed to be visually similar with the highest amount of biofilm production. (**Figure 10**).

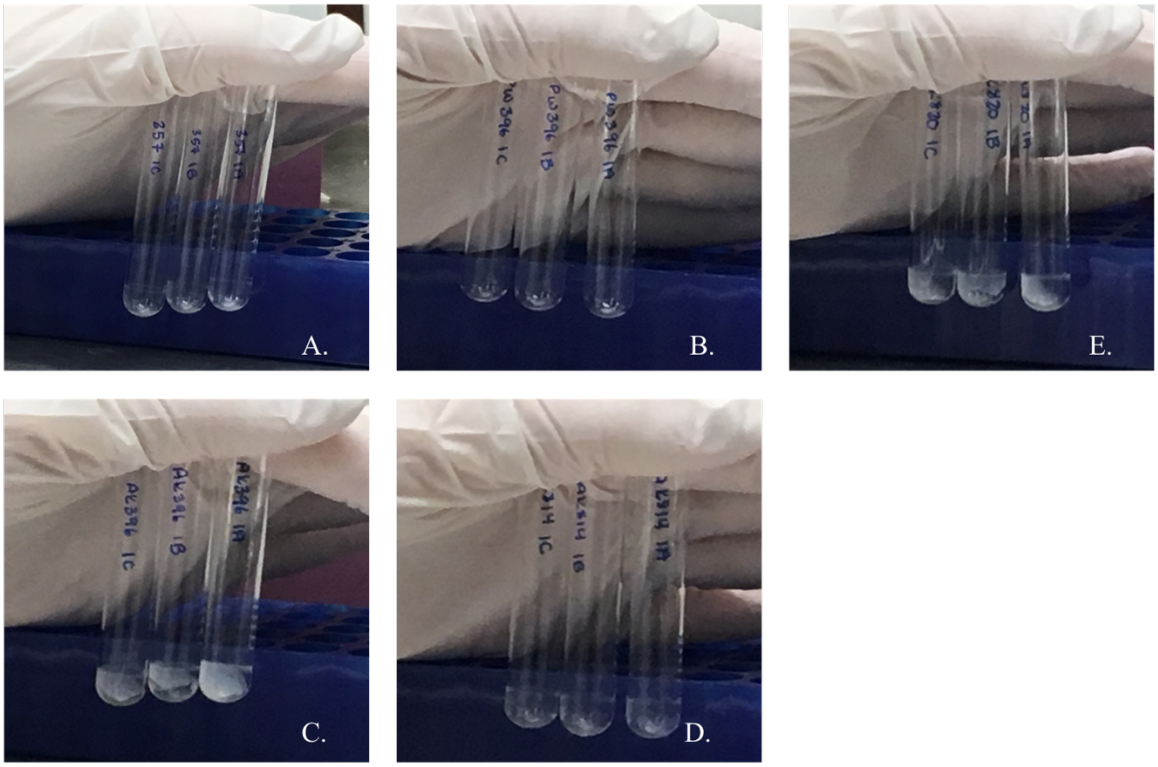

Figure 10. Biofilm formation in wild-type (A), Δ*vpsA* (B), Δ*potB* (C), Δ*vpsL* (D), and Δ*vpsL*Δ*potB* (E) following the second biofilm assay performed with YT broth.

DISCUSSION

ABC transporters are known to play a crucial role in bacterial physiology, specifically through their intake of polyamines, making their characterization significant in understanding of the bacterium (6). Previous studies aimed to investigate the role of *V. cholerae*'s ABC transporter, PotABCD1, have shown the effects of polyamines and the transport proteins on the biofilm phenotype, while the mechanism of transport is still unknown (6). The objective of this study was to further observe the impact of PotB on polyamine transport following depletion of its biofilm formation abilities of *V. cholerae*. This was employed through creation of a Δ*vpsL*Δ*potB* mutant.

Vibrio polysaccharide (VPS) is known to be essential for the formation of a mature biofilm structure (1). VPS production is controlled by an array of *vps* genes, which are

clustered into the *vps*-I and *vps*-II operons (1). The *vps*-I operon contains *vps* genes A-K, while the *vps*-II operon contains L-Q (1). Previous studies have observed the effects of in-frame deletion mutants of each *vps* gene to determine if all or which *vps* genes are required for biofilm formation (1). Both the Δ*vpsA* and Δ*vpsL* mutants demonstrated a reduction in the production of VPS and ability to form biofilms (1). Based on the known effects of a *vpsL* deletion on the biofilm phenotype, I hypothesized that my double mutant would show decreased biofilm formation due to the absence of the *vpsL* gene. The absence of *vpsL* and resulting diminished biofilm formation would then allow for better characterization of PotB's impact on transport.

Across the trials, the Δ*vpsL* mutant showed average planktonic cell densities similar to that of wild-type. The lack of distinct differences between the biofilm forming abilities of wildtype and Δ*vpsL* suggested the need for further analysis of possible factors influencing its biofilm phenotype.

The results of the biofilm assay performed with LB did not produce the results anticipated for the Δ*vpsL* and Δ*vpsL*Δ*potB* mutants as they did not show diminished biofilm formation. Previous studies have shown the enhancement of biofilm formation in the presence of high Na^+ and Ca^{2+} concentrations (3). I hypothesized that the 100 mM salt concentration present in LB might have led to the increase in biofilm formation for these mutants. This led to the utilization of YT broth which contained a lower salt concentration of 10mM. The similarities between wild-type and the Δ*vpsL* mutant remained when the biofilm assays were performed in the YT buffer.

The reduced salt concentration of YT broth showed decreased average biofilm optical densities in wild-type and mutant strains in comparison to those observed with LB. However,

the depletion was constant across all strains, and did not change the biofilm phenotype of the Δ*vpsL* and Δ*vpsL*Δ*potB* mutants to match the expected hypothesis. The inclusion of the Δ*vpsA* mutant in the second YT biofilm was employed to study the effectiveness of the YT broth and the validity of the biofilm assay. The Δ*vpsA* mutant showed an average planktonic optical density of 0.50 (**Figure 8**), and an average biofilm optical density of 0.02 (**Figure 9**). Biofilm formation was almost completely depleted in the Δ*vpsA* mutant, as expected based on reported studies (1). The effect of the Δ*vpsA* mutant on depletion of the biofilm phenotype validated the biofilm assay and the need for future studies involving *vpsL*.

The biofilm assay results for the generated Δ*vpsL*Δ*potB* double mutant did not support our hypothesis. The double mutant showed increased biofilm formation in comparison to the wild-type strain. These results were observed in all trials, regardless of the broth type used (**Figures 5,7,9**). These results suggest that there may be some unknown regulation aspects involving *vpsL*. In addition, I hypothesize that other *vps* genes could be compensating for the lack of *vpsL*. Further studies are being employed to study the effects of PotB on transport through its incorporation into a different genetic background. Δ*vpsA* has been selected as the new genetic background as its depletion of the biofilm phenotype was observed in the second YT biofilm assay (**Figure 9**). The differences in the effects of the Δ*vpsL* single mutant shown previously and those shown in this study may be the result of less defined media or differences in the media varying between companies.

CONCLUSION

As a pathogenic bacterium, *V. cholerae* remains a constant threat to society. In 2017, an epidemic struck Yemen leading to thousands testing positive for *V. cholerae*. While advanced treatment and early detection methods have led to a decline in the number of

associated deaths, many are still suffering from the cholera associated symptoms with death in serious cases where proper treatment is not in place. Yemen is just one example of a country recently impacted by this bacterium. The poorer hygienic and medical practices of underdeveloped countries leave them more prone to fatalities associated with the congestion of the bacterium (5).

Regardless of the improved understanding and medicinal treatments, *V. cholerae* continues to evolve and form new variant strains, aiding in the bacterium's ability to counter immunity and current treatment (5). As seen in the performed study, deletion of genes known to be required for biofilm formation, does not always lead to a depletion of the biofilm phenotype. *V. cholerae* may have the ability to compensate for the loss of specific proteins with the hyper expression of others. The study of the bacterium's biofilm formation abilities through the manipulation of properties such as genes and extracellular signals constituting the phenotype, can lead to better understanding of the bacterium's survival and infection mechanisms. Understanding the mechanisms and signals that aid in the bacterium's survival and infection within the human host can lead to the development of more targeting treatments to prevent proliferation of the matrix and travel of the bacterium to the site of infection, eventually leading to the depletion of cholera cases worldwide.

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