CHARACTERIZATION OF THE NORSPERMIDINE/SPERMIDINE ABC-TYPE TRANSPORTER, POTABCD1, IN *VIBRIO CHOLERAE*

A Thesis
by
BLAKE EDWARD SANDERS

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Abstract

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Vibrio cholerae is an intestinal pathogen that also thrives in aquatic environments through forming biofilms, matrix-bound aggregation of cells offering physical protection from many environmental stressors and allowing the bacterium to survive harmful conditions. Formation of biofilm by *V. cholerae* can be regulated by various environmental signals including small molecules called polyamines. Polyamines are a ubiquitous class of molecules that are involved in cell growth and regulate a wide variety of biological functions through various mechanisms including transport. Bacteria contain multiple polyamine transport systems, generally ABC transporters composed of a substrate binding protein in the periplasm, two channel forming proteins, and a membrane associated ATPase involved in energy supply. *V. cholerae* has a putative ABC transporter, PotABCD1. Previous research in our lab has investigated the role of the substrate binding protein of this system, PotD1 and has shown that PotD1 is responsible for transport of the polyamines spermidine and norspermidine into *V. cholerae*. Moreover, PotD1 mutants displayed an increased biofilm

phenotype when compared with wild type. The roles of the other components of the polyamine transport system still need further investigation. The objectives of this study were to more thoroughly characterize the norspermidine/spermidine uptake system PotABCD2D1, and determine whether imported polyamines or the components of the transporter system affect biofilm formation. Also, due to the capability of PotD1 to facilitate transport of both norspermidine and spermidine, the preference of this protein for one polyamine over the other was investigated. My results to date indicate that PotB and PotC, the transmembrane channel forming proteins, are involved in spermidine and norspermidine uptake. In addition, PotD1 does not appear to have a large preference for norspermidine over spermidine under the conditions tested. PotA, PotB, PotC, and PotD mutants display increased biofilm growth compared to wild type, suggesting that the components of the transport system do not seem to play a role in biofilm formation, but rather spermidine import into the cell mediates this phenotype. The role in polyamine uptake of PotA, the ATPase component of the uptake system is still under investigation. Because ABC transporters have a profound impact on bacterial physiology, a better understanding of these systems is crucial. This work not only establishes PotABCD1 as the first norspermidine transporter ever reported in any species, but also further elucidates the role polyamines play in *V. cholerae* biofilm formation, which may aid in survival in aquatic conditions.

Acknowledgments

I would like to acknowledge the Appalachian State University Office of Student Research and Graduate Student Association Senate for contributing to the funding in support of this research. This research was also supported by the Grant #AI096358 from the National Institute of Allergy and Infectious Diseases awarded to Dr. Ece Karatan. I would like to thank the Karatan lab, past and present, which has been influential in completion of this research. Thanks to the members on my committee for offering helpful suggestions, and special thanks to my advisor and mentor for her support, encouragement, and guidance which has further driven my passion for microbiology.

Dedication

This work is dedicated to my family, especially my parents Jud and Phyllis Sanders who have always supported me in all my endeavors.

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Introduction

Polyamines are a ubiquitous class of small polycationic molecules with a hydrocarbon backbone and multiple amine groups. Unlike other cations, polyamines are arranged in a unique charge-structure conformation with the positive charges being found at regularly spaced intervals along the hydrocarbon chains. This arrangement allows for these molecules to serve as electrostatic bridges between negative phosphate charges on nucleic acid and other negatively charged polymers (Shah & Swiatlo, 2008). Polyamines within the cell are predominantly found as complexes with RNA and work together with cations like Mg^{2+} to stabilize higher orders of structure (Shah & Swiatlo, 2008). Polyamines such as putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane) are diamines with two amino groups each, while spermidine and spermine contain three and four amino groups respectively (Shah & Swiatlo, 2008). Putrescine, spermidine, spermine, and cadaverine are the most widely distributed cellular polyamines and are essential for normal cellular growth and multiplication of both prokaryotic and eukaryotic cells, with the intracellular concentration of spermidine being higher than that of putrescine in almost all bacteria (Cohen, 1998) (Fig. 1).

Polyamines are vital for cell proliferation, growth, and development in both prokaryotes and eukaryotes. These molecules have also been shown to regulate a wide variety of cellular processes like regulation of transcription and translation, virulence, and biofilm formation (Goytia & Shafer, 2010; Igarashi & Kashiwagi, 2000; Jelsbak *et al.*,

2012; Kurihara *et al.*, 2013; Ware *et al.*, 2006; Yoshida *et al.*, 2004). Lately, polyamines have been reported to be linked to microbial carcinogenesis, host cell apoptosis, escape from phagolysosomes, bacteriocin production, toxin activity, and protection from oxidative and acid stress (Shah & Swiatlo, 2008). Because of these multifaceted roles polyamines facilitate in bacteria, the modulation of these molecules is crucial and regulated by biosynthesis, degradation, and transport (Igarashi & Kashiwagi, 1999).

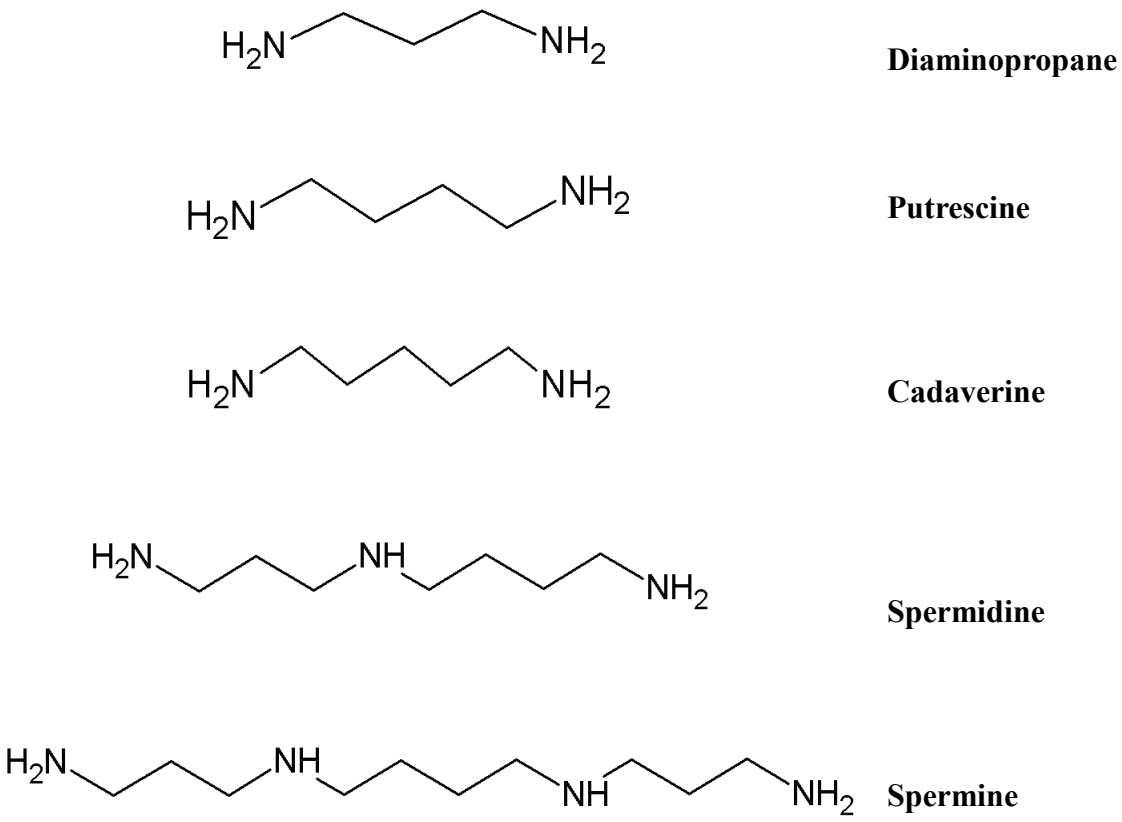


Fig. 1. Structures of common microbial polyamines. Chemical structures of common polyamines found in bacteria. Structures were drawn using ChemSketch software.

At physiological pH, polyamines are positively charged; therefore, an active transporter is essential for these molecules to pass through the inner membrane of Gram-

negative bacteria. Polyamine uptake has been extensively studied and characterized in the bacterium *Escherichia coli*, which includes two ABC transporters that are involved in spermidine and putrescine import. ABC transporters encompass one of the largest of all paralogous protein families with diverse functions. In fact almost 5% of the entire *E. coli* genome encodes components of ABC transporters (Linton & Higgins, 1998). These transporters convert the energy gained from ATP hydrolysis into trans-bilayer movement of substrates either into the cytoplasm or out of the cytoplasm (Higgins, 2001). These systems are widespread among living organisms, being detected in all genera of the three domains of life, and contribute to a wide variety of physiological roles.

Although widespread among organisms, the number of ABC transporters differs among species with organisms like *E. coli*, which live in diverse environments and adapt to a wide variety of conditions containing around 70 ABC transporters, and other organisms with more restrictive lifestyles having fewer transporters (Higgins, 2001). ABC transporters have been characterized with specificity for a wide range of substrates including sugars and other carbohydrates, amino acids, peptides, polyamines, metal ions, sulfate, iron, and complex polysaccharides (Garmory & Titball, 2004; Higgins, 2001). Even though most reported transporters show tight substrate specificity, some are multispecific such as the oligopeptide transporter which handles all di- and tripeptides and others have broad spectrum specificity for hydrophobic compounds like the LmrA multidrug transporter in *Lactococcis lactis* (Higgins, 2001).

The basic unit of an ABC transporter consists of four core domains (Hyde *et al.*, 1990), two membrane associated domains or transmembrane domains (TMDs) and two ATP-binding domains or nucleotide binding domains (NBDs) (Higgins, 2001). The two

TMDs span the membrane multiple times via α-helices, forming the channel through which the solute traverses the membrane. The NBD's catalyze ATP hydrolysis. These domains are hydrophilic and peripherally associated with the cytoplasmic face of the membrane (Higgins, 2001). The conservation of this entire domain is important in defining this superfamily of transporters (Higgins *et al.*, 1986). In many ABC transporters, especially importers, supporting domains have been recruited for function (Fig. 2) (Garmory & Titball, 2004). The substrate binding proteins (SBPs) bind the substrate external to the cell and deliver it to the membrane-associated complex (Higgins, 2001). Import across the outer membrane may involve outer membrane proteins (OMPs), indeed most small substrates, like polyamine cations cross the outer membrane through the non-specific porins such as OmpF or OmpC of *Enterobacteriacea* (Nikaido, 2003).

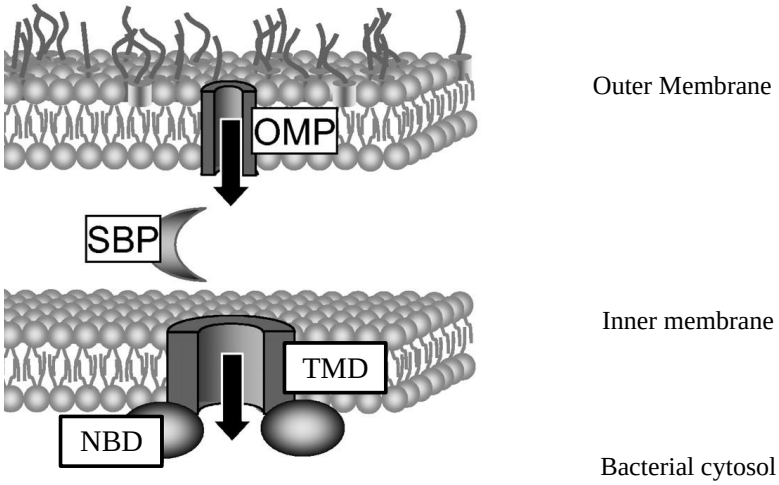


Fig. 2. Schematic diagram of typical organization of ABC importers in bacteria. ABC transporters utilize additional domains for transport. OMP, outer membrane protein. SBP, substrate binding protein. TMD, transmembrane domain. NBD, nucleotide binding domain or ATP-binding domain. Modified from Garmory and Titball. 2004.

The first step in transport involves substrate recognition by the binding protein. Once bound, a conformational change occurs generally engulfing the ligand. The binding protein is then in a closed position, which exposes potential binding sites for interaction with the TMDs. The ligand is then released from the binding protein to interact with the channel-forming transmembrane domain. Substrate binding leads to a conformational change in the TMDs which is transmitted to the NBDs to initiate ATP hydrolysis (Higgins, 2001) Binding and hydrolysis of ATP induces conformational changes in the NBDs that are transmitted to the TMDs via non-covalent interactions.

At this interface are conserved short α-helices, located in cytoplasmic loops between transmembrane segments; these helices comprise the majority of contact between the interface of these separate domains, accordingly these are termed coupling helices (Dawson *et al.*, 2007) (Fig. 3). Upon binding of ATP, the gap between the NBD closes, bringing the coupling helices closer together. The TMDs flip from facing inward to facing outward, exposing binding and extrusion sites to opposite sides of the membrane, consequently moving the substrate across the bilayer (Locher, 2009) (Fig. 4).

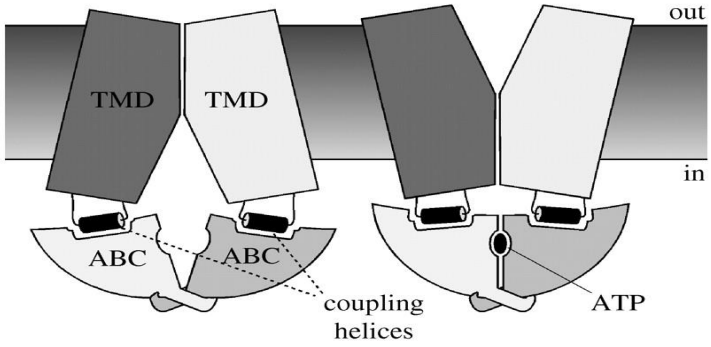


Fig. 3. Diagram depicting coupling mechanism of ABC transporters for import. Binding of ATP triggers the closing of a gap between the ABCs, which moves the coupling helices closer together changing the conformation of the transmembrane domains (TMDs). Hydrolysis of ATP and release of products change the TMDs back to an inward conformation. Modified from Locher, 2009.

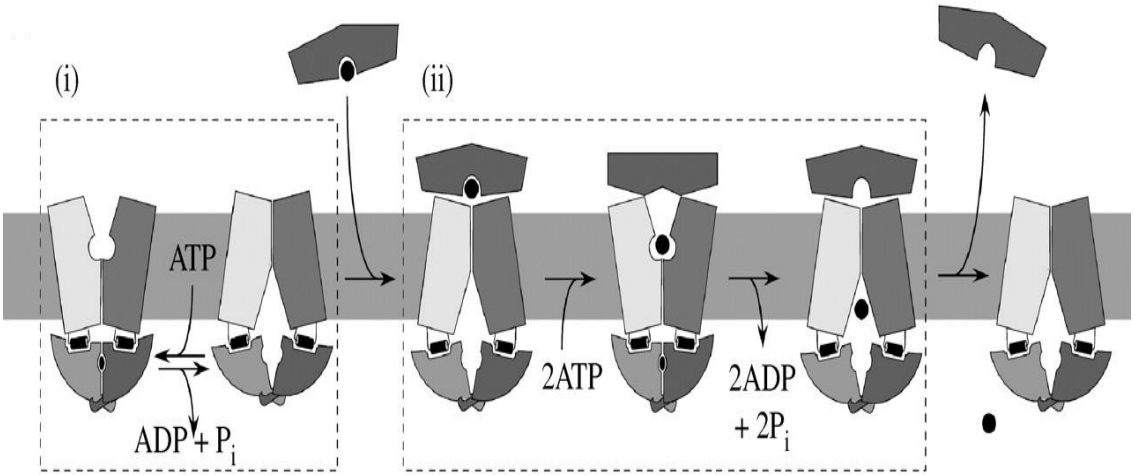


Fig. 4. Schematic of the structure and mechanism of ABC importers. (i) Coupling mechanism in the absence of substrate or binding protein. (ii) Two conformations are essential for successful transport of substrate by ABC transporter. Substrate binding protein attaches to ligand and delivers to the transport system, where ATP is then bound by ABC to change conformation of transmembrane domains. After hydrolyzing ATP, the conformation of the TMDs changes inward moving substrate through. Modifed from Locher, 2009.

Polyamine ABC transporters in *E. coli* and many other bacterial species are organized as four gene operons and designated as *potABCD*, a spermidine preferential system, and *potFGHI*, a putrescine specific uptake system (Igarashi & Kashiwagi, 1999) (Fig. 5). PotD and PotF are the periplasmic substrate binding proteins that bind the extracellular polyamines, while PotA and PotG proteins are the membrane-associated cytosolic-ATPases involved in the energy supply. The remaining proteins of these systems have membrane spanning α-helices and form transmembrane channels for polyamine transport. PotB and PotC of the spermidine uptake system contain six putative transmembrane segments linked by hydrophilic segments of variable length.

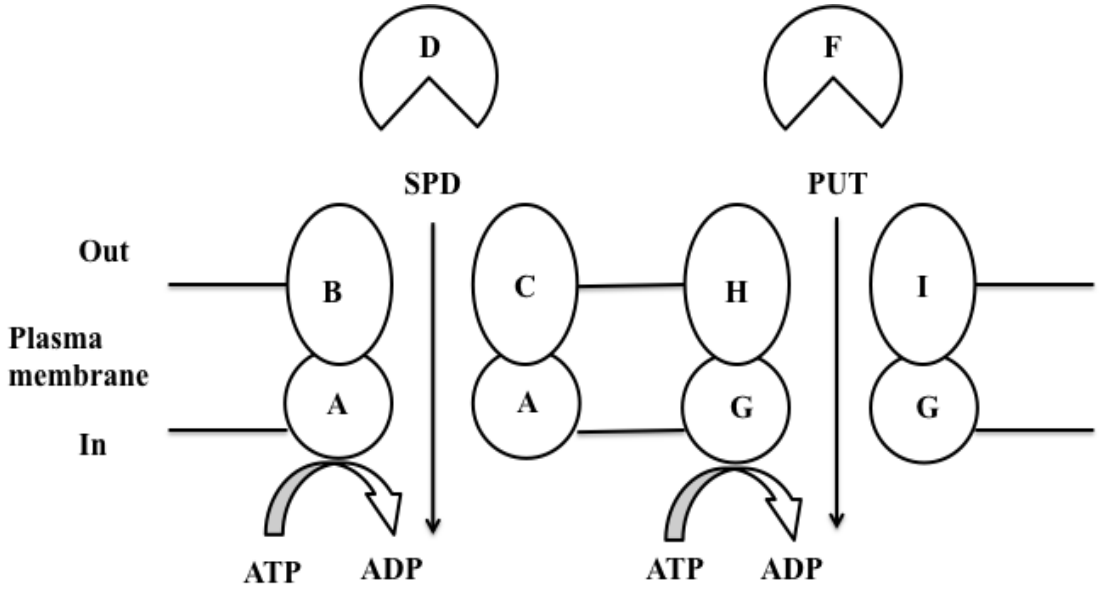


Fig. 5. Polyamine ABC importers in *E. coli*. *E. coli* contains two polyamine transporters that have been well characterized. PotABCD is a spermidine preferential importer, while PotFGHI is a putrescine specific importer. Adapted from Igarashi and Kashiwagi, 1999.

Research has shown that the substrate binding proteins of the ABC importer not only function in polyamine transport, but also are involved in other important phenotypes. The substrate binding protein of the spermidine uptake system, PotD, plays a role as a transcriptional inhibitor of the *potABCD* operon in *E. coli* when excess PotD is present (Antognoni *et al.*, 1999). In addition, *E. coli* PotD has been shown to influence swarming motility, a bacterial process that allows cells to move in a coordinated manner on surfaces and expand populations (Kurihara *et al.*, 2009). Furthermore, *E. coli* PotD is speculated to be involved in initiation of an SOS response. Bacteria respond to stress and DNA damage by stimulation of the SOS response, leading to arrest of cell division and facilitation of DNA repair (Manasherob *et al.*, 2012). In *Streptococcus pneumoniae*, a Gram-positive human pathogen and one of the causes of bacterial pneumonia, PotD was shown to influence both systemic and pulmonary infection, where attenuation of

virulence was observed in a mouse model when *potD* was disrupted (Ware *et al.*, 2006). While there have been studies on the polyamine uptake systems in *E. coli* and *S. pneumoniae*, very little is known about the uptake system in *Vibrio cholerae*.

V. cholerae is a Gram negative, aquatic bacterium that resides in brackish waters, rivers, and estuaries, and is the causative agent of the intestinal disease cholera. Cholera is a major public health problem antagonizing developing countries where outbreaks occur due to poverty and poor sanitation. The disease is characterized by severe dehydration and voluminous diarrhea, which can lead to death in 50-70% of untreated patients (Faruque *et al.*, 1998). *V. cholerae* contains a putative polyamine importer PotABCD2D1 (Fig. 6). The genomic locus encodes homologues of the PotABCD transporter proteins of *E. coli*, however, it contains two genes that are annotated as *potD*, namely, *potD1* and *potD2*. Mutational analysis of these genes encoding the two substrate binding proteins has shown that PotD1 plays a role in the transport of both spermidine and norspermidine, while PotD2 is not involved in the uptake of these two polyamines (Cockerell *et al.*, 2014; Mcginnis *et al.*, 2009). Furthermore, PotD1 mutants display an increased biofilm phenotype when compared with wildtype (Mcginnis *et al.*, 2009).

V. cholerae forms biofilms to increase survival capability, aiding in protection from environment stressors like pH, temperature, UV radiation, osmotic shock, and desiccation (De Carvalho, 2007; Donlan & Costerton, 2002; Flemming, 1993). A biofilm is an aggregation of microorganisms formed on environmental abiotic surfaces. In addition, biofilms can form on biotic surfaces in the natural environment such as epithelial cells within the human body (Karatan & Watnick, 2009). Biofilms are bound together by an extrapolymeric substance (EPS) that creates a matrix encapsulating the

bacteria with many other molecules such as polysaccharides, extracellular DNA, and proteins. The development of biofilm is a dynamic event that can be regulated and influenced by various environmental signals such as osmolarity, nucleosides, sugars, quorum sensing, and polyamines (Hammer & Bassler, 2003; Haugo & Watnick, 2002; Kapfhammer *et al.*, 2005; Karatan *et al.*, 2005).

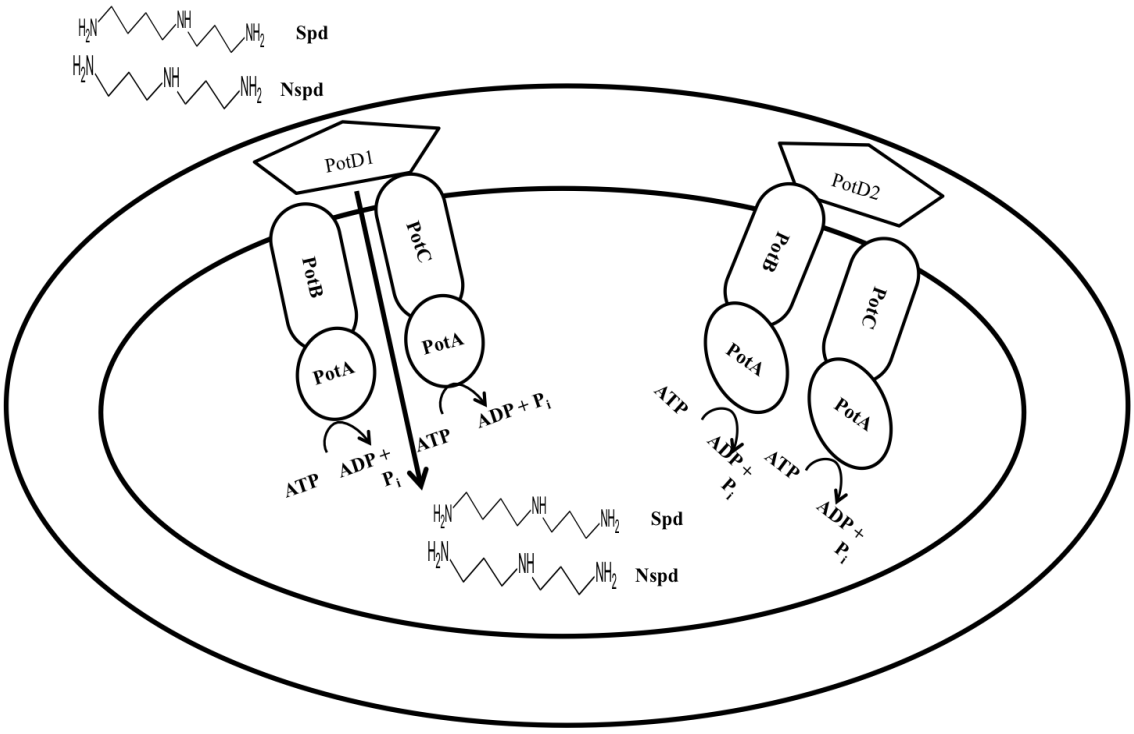


Fig. 6. Schematic diagram of putative polyamine transport system PotABCD2D1 in *V. cholerae.* This system consists of PotA (an ATPase), PotB and PotC (channel forming proteins), and two substrate binding proteins PotD1 and PotD2.

The polyamines spermidine and norspermidine are some of the environmental signals regulating biofilm formation in *V. cholerae.* The polyamine distribution of *Vibrio* species is unique in that norspermidine (Fig. 7), a polyamine similar to spermidine with two C_3 chains, is one of the dominant polyamines along with putrescine, while spermidine may be found only in small amounts under normal growth settings

(Yamamoto *et al.*, 1983). Furthermore, norspermidine has been found to be a major polyamine in numerous aquatic plants and aquatic organisms (Hamana *et al*., 1998; Hamana *et al.*, 1991). Norspermidine is synthesized by decarboxylation of carboxynorspermidine by carboxynorspermidine decarboxylase encoded by the *nspC* gene (Lee *et al.*, 2009). Deletion of this gene, results in inhibition of biofilm due to the lack of norspermidine in *V. cholerae* (Lee *et al.*, 2009). Norspermidine has also been shown to act as an intercellular signaling molecule modulating biofilm formation through the NspS-MbaA signal transduction pathway, where NspS interacts with the periplasmic region of MbaA to influence its enzymatic activity (Karatan *et al.*, 2005). Binding of NspS to norspermidine is thought to increase the inhibitory effect of NspS on MbaA, increasing levels of the bacterial secondary messenger, c-di-GMP, necessary for EPS production, thus increasing biofilm formation (Karatan *et al.*, 2005). Additionally binding of NspS to spermidine has the opposite effect (Mcginnis *et al.*, 2009).

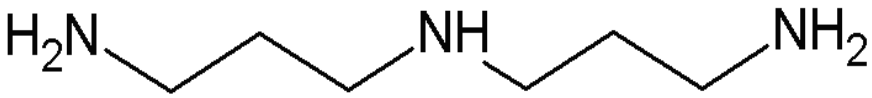


Fig. 7. Structure of the triamine, norspermidine. Chemical structure of norspermidine, one of the dominant polyamines in *Vibrio* species. Similar to spermidine with one methylene group difference. Structure was drawn using ChemSketch program.

While the effects of external polyamines on biofilm formation in *V. cholerae* have been elucidated, the mechanism by which intracellular polyamines influence this phenotype is still unknown in this bacterium. The effect of internal polyamines on biofilm formation has been studied in *E. coli* and *Yersinia pestis*. In *E. coli*, a set of genes whose expression is enhanced by polyamines at the translational level are defined

as the "polyamine modulon" (Igarashi & Kashiwagi, 2006). There are several mechanisms fundamental to polyamine stimulation of the production of the members of the modulon. One of the major ways that polyamines stimulate synthesis is when a Shine-Dalgarno (SD) sequence in the mRNA is distant from the initiation codon AUG. In this case the polyamine aids in the formation of the initiation complex by relaxing the structure by narrowing the distance between the SD sequence and initiation codon AUG (Igarashi & Kashiwagi, 2006). Recently, it has been shown that the polyamine modulon is not only involved in cell growth and viability, but also plays a role in biofilm formation through polyamines stimulating production of CpxR and UvrY, response regulator proteins in two component signal transducing systems (Sakamoto *et al.*, 2012). The Cpx system controls expression of genes encoding proteins required for formation of *E. coli* curli fimbriae, the essential biofilm constituent that plays a key role in initial adhesion and cell to cell interactions (Beloin *et al.*, 2008; Jubelin *et al.*, 2005). UvrY controls activity of the carbon storage regulator in *E. coli*, inhibiting glycogen biosynthesis and catabolism, gluconeogenesis, and biofilm formation (Romeo, 1998).

Y. pestis has a rodent-flea life cycle with transmission depending heavily upon a set of genes termed the hemin storage locus (*hms*). These genes facilitate blockage, eventually leading to colonization of the proventricular valve that divides the midgut from the esophagus (Patel *et al.*, 2006). HmsHFRS are necessary for the biosynthesis of the biofilm polysaccharide poly-β-1,6-N-acetyl-D-glucosamine in *Y. pestis* (Wortham *et al.*, 2010). Polyamines have been suggested to play a role in *Y. pestis* biofilm formation by serving as signaling molecules affecting gene or protein expression, an intermediate in biofilm synthesis, or a structural component of biofilm (Patel *et al.*, 2006). Further

research on *Y. pestis* biofilm formation has provided evidence that polyamines, specifically putrescine, affect the level of key Hms proteins. Transcriptional reporters and qRT-PCR have also determined that polyamines are not affecting transcription of the *hms* genes or their mRNA stability (Wortham *et al.*, 2010). But rather, polyamines are enhancing the translation of key Hms mRNAs (Wortham *et al.*, 2010).

The goal of this study was to more thoroughly characterize the norspermidine/spermidine uptake system PotABCD2D1 and to further understand the effect polyamines have on *V. cholerae* biofilm formation. In this study, I found that PotB and PotC, the transmembrane channel forming proteins, are involved in spermidine and norspermidine uptake. PotA, PotB, PotC, and PotD mutants display increased biofilm growth compared to wild type, suggesting that the components of the transport system do not seem to play a role in biofilm formation, but rather spermidine import into the cell mediates this phenotype. In addition, PotD1 does appear to have a slight preference for norspermidine over spermidine under the conditions tested. Because ABC transporters have a profound impact on bacterial physiology, a better understanding of the mechanism and abilities of ABC transporters is crucial. This work not only establishes PotABCD2D1 as the first norspermidine transporter ever reported in any species, but also further elucidates the role polyamines play in *V. cholerae* biofilm formation.

Materials and Methods

Bacterial strains, plasmids, primers, media, and reagents

The bacterial strains, plasmids, and primers used throughout this study are listed in Table 1, Table 2, and Table 3, respectively. Primer synthesis and DNA sequencing was performed by Eurofins MWG Operon (Huntsville, AL) and Cornell University (Ithaca, NY), respectively. Strains were grown on Luria-Bertani broth (LB) (1% Tryptone, 0.5% Yeast Extract, 85 mM NaCl) agar with relevant antibiotics for 24 hours at 27°C before being incubated in tryptone broth. Tryptone broth (1% Tryptone, 85 mM NaCl) contains approximately 3 μM spermidine. Cultures were grown in tryptone broth unless otherwise stated. The polyamines, putrescine, cadaverine, diaminopropane, spermidine and norspermidine, were purchased from Sigma-Aldrich (St. Louis, MO). The restriction enzymes, *EcoR*I, *Nco*I, *Xho*I, *Spe*I, *Apa*I , and Phusion and OneTaq polyamerases were purchased from New England Biolabs (Beverly, MA). Chemicals and reagents were purchased from Alfa Aesar (Ward Hill, MA), Amresco (Solon, OH), Fischer-Scientific (Fairlawn, NJ), or Sigma-Aldrich (St. Louis, MI) unless otherwise stated.

Table 1. Bacterial strains

Strains	Genotype	Reference
E. coli		
DH5α	F- Φ80*lacZ*ΔM15, Δ(*lac*ZYA-*arg*F) U169 *deoR*, *recA1*, phoA, *end*A1 *hsd*R17 (rk2, mk+) *sup*E44, *thi*-1 *gyr*A96, *rel*A1, λ-	Invitrogen
DH5αλpir	supE44, ΔlacU169 hsdR17, recA1 endA1 gyrA96 thi-1 relA1, λpir	(Hanahan, 1983)
SM10λpir	*thi thr leu tonA lacY supE recA::RP4-2-Tc::MuλpirR6K; Km*^{R}	(Miller & Mekalanos, 1988)
V.cholerae		
PW357	MO10 *lacz::vpsLp* → *lacZ*, Sm^R	(Karatan *et al.,* 2005)
AK160	PW357 Δ*potD1*, Sm^R	(Cockerell *et al.,* 2014)
AK314	PW357 *nspC*::*kan*, Kan^R, Sm^R	(Cockerell *et al.,* 2014)
AK317	PW357 *nspC*::*kan*, Δ*potD1*, Kan^R, Sm^R	(Cockerell *et al.,* 2014)
AK333	PW357 *nspC*::*kan*, Δ*potB*, Kan^R, Sm^R w/ *potD1*-V5	Rutkovsky and Karatan (unpublished)
AK334	PW357 *nspC*::*kan*, Kan^R, Sm^R w/ pACYC184	Rutkovsky and Karatan (unpublished)
AK395	PW357 Δ*potB*, Sm^R	This study
AK397	PW357 Δ*potC*, Sm^R	This study
AK399	PW357 *nspC*::*kan*, Δ*potB*, Kan^R, Sm^R	This study
AK404	PW357 *nspC*::*kan*, Δ*potC*, Kan^R, Sm^R	This study
AK429	PW357 Δ*potA*, Sm^R	Villa and Karatan (unpublished)
AK437	PW357 Δ*potB*, Sm^R w/ pBS3	This study
AK446	PW357 *nspC*::*kan*, Δ*potB*, Kan^R, Sm^R w/ pBS3	This study
AK461	PW357 Δ*potC*, Sm^R w/ pBS4	This study
AK464	PW357 *nspC*::*kan*, Δ*potC*, Kan^R, Sm^R w/pBS4	This study

Deletion of the potB and potC genes

To determine the role of the transmembrane permeases of the putative ABC-type transporter PotABCD1, *potB* and *potC* genes were deleted using double homologous recombination. Previous studies in the Karatan lab have already constructed in-frame

deletions of *potD1* and *potD2* (Mcginnis *et al.*, 2009). Briefly, fragments containing approximately 400 bp upstream and downstream of the *potB* and *potC* genes were amplified by PCR from *V. cholerae* chromosomal DNA using Phusion polymerase (Fig. 8). In order to fuse the two fragments using splicing by overlap extension PCR, internal primers were engineered with complementary sequences allowing for the generation of a recombinant molecule ultimately lacking the gene of interest. Once these fragments were denatured, mixed and reannelaed, the 3'-end of the top strand of one fragment annealed onto the 3'-end of the bottom strand of the other fragment, which facilitated an overlap that generated the recombinant product (Horton *et al.*, 1990). Splicing of these fragments produced in-frame deletions that removed 791 bp of the 861 bp *potB* gene and 753 bp of the 790 bp *potC* gene.

Table 2. Plasmids

Plasmids	**Genotype**	**Reference/source**
pCR2.1-TOPO	plasmid for TOPO cloning, Ap^R	Invitrogen
pWM91	*oriR*6k, *lacAα*, *sacB*, homologous recombination plasmid, Ap^R	(Metcalf *et al.,* 1996)
pACYC184	cloning plasmid, low copy, Tet^R	New England Biolabs
pBS1	pWM91 carrying an internal in-frame deletion of potB	This study
pBS2	pWM91 carrying an internal in-frame deletion of potC	This study
pBS3	pACYC184::*potB V5*	This study
pBS4	pACYC184::*potC V5*	This study

Table 3. Primers

Primer	Description	Sequence
Construction of *V. cholerae potB* deletion		
PA84	Forward primer for upstream fragment	5'-CGAAATCAACGTCTTCCAAGC-3'
PA85	Reverse primer for upstream fragment	5'-TTACGAGCGGCCGCACATCATTGAGGACTACCTCCC-3'
PA86	Forward primer for the downstream fragment	5'-TGCGGCCGCTCGTAAGTGGAGCTAGACTAATGGGAC-3'
PA87	Reverse primer for the downstream fragment	5'-GAGTGAAGAAGCCCAGTTTCG-3'
Construction of *V. cholerae potC* deletion		
PA88	Forward primer for upstream fragment	5-CCATTTATGATCCTTCCGCTC-3'
PA89	Reverse primer for downstream fragment	5'-TTACGAGCGGCCGCATCCCATTAGTCTAGCTCCAC-3'
PA90	Forward primer for the downstream fragment	5'-TGCGGCCGCTCGTAAGTCGCTTCACAGTTGTTAGCAAG-3'
PA91	Reverse primer for the downstream fragment	5'-TGXXACGAAGTAGGTAGAAGG-3'
Cloning *potB* into pACYC184 plasmid for complementation		
PA247	Forward primer for *potB* gene	5'-CGTCACTTGGGTTGAAAGCT-3'
PA248	Reverse primer for *potB* gene with a V5 tag	5'-CCATGGCTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCGCCGCTGCCGCTGCCATCGTTCACTTTTAGCTTTTGG-3'
Cloning *potC* into pACYC184 plasmid for complementation		
PA249	Forward primer for *potC* gene	5'-GCCATGGCGATCATGCTGTAC-3'
PA250	Reverse primer for *potC* gene with a V5 tag	5'-CCATGGCTACGTAGAATCGAGACCGAGGAGAGGGTTAGGGATAGGCTTACCGCCGCTGCCGCTGCCCTTCACTTTTTCTCTTGCTAAC-3'

After purification of PCR products, adenines were added to the 3' ends of the blunt ended fragments for TA cloning. Following manufacturer's instructions, 10 μL of PCR product was mixed with 33.5 μL of water, 5 μL *OneTaq* polymerase buffer, 1 μL dATP, and 0.5 μL *OneTaq* polymerase and incubated at 72°C for 10 minutes. This PCR product with adenines on 3'ends was then used to clone into a linearized pCR2.1-TOPO vector with single thymine overhangs following instructions from manufacturer (Invitrogen, Carlsbad, CA) and electroporated into electrocompetent DH5α using a BIO-RAD MicroPulser (Hercules, CA) at 1.8 kV. These transformed cells were incubated in SOC medium to recover at 37 °C at 200 rpm for 1 hour, and then plated on LB-ampicillin (100 μg/mL) agar plates with 20 μL of X-gal (20 mg/mL in dimethyl sulfoxide).

Blue white screening was used to select for plasmids carrying the insert and colony PCR was used to confirm presence of insert. Briefly, single colonies were resuspened in 100 μL of water and heated at 95°C for 5 minutes to lyse the cells. Two μL of this lysate containing the template DNA were used in a PCR reaction with 16.4 μL of water, 5 μL 5X *OneTaq* Standard Reaction Buffer, 0.5 μL dNTPS, 0.5 μL of each outside primer, and 0.125 μL *OneTaq* DNA polymerase. Cycling conditions were as follows: initial denaturation at 98°C for 30 seconds, 35 cycles of 98°C for 10 seconds, 56°C for 20 seconds, 68°C for 60 seconds, and a final extension at 72°C for 6 minutes. Colonies testing positive for insert were grown overnight. Next, the plasmids were purified using the Promega Wizard Plus SV Minipreps DNA Purification System (Madison, WI) and sent out for sequencing.

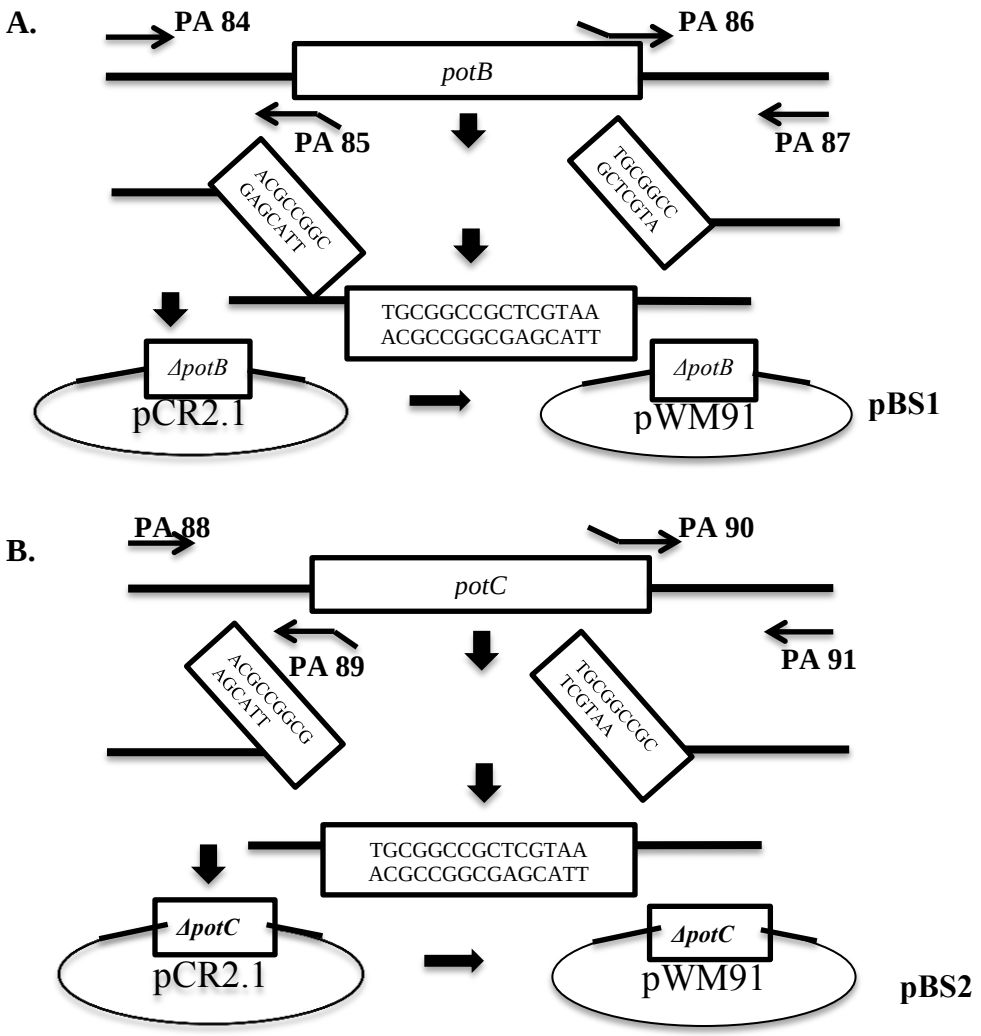


Fig. 8. Construction of the pBS1 and pBS2 plasmid containing a deletion of one of the transmembrane permeases of the polyamine transport system PotABCD1. (A) The pBS1 plasmid was used to delete the *potB* gene encoding one of the channel forming proteins **(B)** The pBS2 plasmid was used to delete the *potC* gene encoding for one of the other channel forming proteins of the polyamine transporter in *V. cholerae*.

Sequences were verified by services provided by Cornell University (Ithaca, NY). The Δ*potB* insert was then excised using *Xho*I and *Spe*I, while the Δ*potC* insert was excised using *Apa*I and *Spe*I. These were then purified using the GE Healthcare Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (Buckinghamshire, UK).

Next the inserts were ligated into the pWM91 plasmid linearized with the same enzymes, electroporated into electrocompetent DH5αλpir *E. coli* using a BIO-RAD MicroPulser (Hercules, CA) at 1.8 kV, and verified using colony PCR. Colonies testing positive for insert were grown overnight. Next, the plasmids were purified using the Promega Wizard Plus SV Minipreps DNA Purification System (Madison, WI) and electroportated into electrocompetent SM10λpir *E. coli* using a BIO-RAD MicroPulser (Hercules, CA) at 1.8 kV, and verified using colony PCR.

These strains were used for conjugation into *V. cholerae* PW357 and a *V. cholerae* PW357 *nspC::kan* mutant using SacB counter selectable mutagenesis (Metcalf *et al.*, 1996). PW357 is a *V. cholerae* O139 MO10 strain with a fusion of the *vpsL* promoter to the *lacZ* gene (Haugo & Watnick, 2002). Fresh plates for the recipient *V. cholerae* and donor SM10λpir *E. coli* containing the plasmid pWM91::Δ*potB* or pWM91::Δ*potC* were streaked on LB plates containing streptomycin (100 μg/mL) and ampicillin (100 μg/mL), respectively, and incubated overnight at 37°C. The next day, either of the *E. coli* strains was mixed with the recipient *V. cholerae* PW357 strain or the PW357 *nspC::kan* mutant on LB agar plates and incubated at 37°C overnight. For conjugating into the *V. cholerae* PW357, half of the growth was streaked for isolation onto LB agar plates containing streptomycin (100 μg/mL) and ampicillin (50 μg/mL), then incubated overnight at 37°C to select for single crossover events. Four single colony isolates were then streaked on LB agar containing streptomycin and ampicillin and incubated overnight at 37°C. Next, four single colony isolates were streaked on non-selective agar with no antibiotic allowing for a second recombination event to occur within the colony removing the ampicillin resistance gene and the *sacB* gene. Following

overnight incubation, multiple isolated colonies were selected and streaked for isolation on sucrose plates and incubated for two days at room temperature. Sucrose plates were made by combining 5 g yeast extract, 10 g tryptone, 15 g tryptone with 667 mL H_2O and autoclaving the mixture. After cooling to around 50°C, 333 mL of 30% filter-sterilized sucrose solution was added. Colonies were patched on LB agar containing either ampicillin (50 μg/mL) or streptomycin (100 μg/mL) to screen for streptomycin resistance and ampicillin sensitivity to confirm successful homologous recombination within *V. cholerae* PW357 strain. Colonies positive for the Δ*potB* insert were verified by colony PCR described above using primers PA84 and PA87. Colonies positive for the Δ*potC* insert were verified by colony PCR using primers PA88 and PA91.

For conjugating into *V. cholerae* PW357 *nspC::kan* mutant, half of the growth was streaked for isolation onto LB agar plates containing streptomycin (100 μg/mL), ampicillin (50 μg/mL), and kanamycin (30 μg/mL). Four single colony isolates were then streaked on LB agar containing streptomycin, ampicillin, and kanamycin and incubated overnight at 37°C. Next, four single colony isolates were streaked on non-selective agar with no antibiotic allowing for a second recombination event to occur within the colony removing the ampicillin resistance gene and the *sacB* gene. Following overnight incubation, multiple isolated colonies were selected and streaked for isolation on sucrose plates, which contained kanamycin (30 μg/mL), and incubated for two days at room temperature. Isolated colonies were then patched on LB agar containing either ampicillin (50 μg/mL) or streptomycin (100 μg/mL) plus kanamycin (30 μg/mL) to screen for streptomycin and kanamycin resistance and ampicillin sensitivity to confirm successful homologous recombination. Colonies positive for the Δ*potB* insert were

verified by colony PCR using primers PA84 and PA87. Colonies positive for the Δ*potC* insert were verified by colony PCR using primers PA88 and PA91.

Complementation of pot mutants

To further validate and confirm the role of the transmembrane permeases of the putative ABC-type transporter PotABCD1, the *pot* mutants were complemented with plasmids carrying each of these genes (Fig. 9, 10). Primers were used to amplify the entire *potB* and *potC* genes. Forward primers annealed to a region 20 basepairs upstream of the predicted ribosome binding site of each gene and the reverse primers annealed to a region 22 basepairs upstream of the stop codon. The reverse primers also encoded a V5 epitope tag to be engineered upstream of the *potB* and *potC* annealing regions, because of this the stop codon was not encoded in the primers. Furthermore, the reverse primer PA250 contained an *Nco*I restriction enzyme site to facilitate cloning of the *potC* gene into the *V. cholerae* compatible pACYC184 plasmid used for complementation.

For complementation of *potB*, cycling conditions for PCR were as follows: initial denaturation at 98°C for 30 seconds, 30 cycles of denaturation at 98°C for 10 seconds, annealing at 55°C for 20 seconds, extension at 72°C for 60 seconds, and a final extension at 72°C for 6 minutes. For complementation of *potC* cycling conditions for PCR consisted of an initial denaturation at 94°C for 30 seconds, 30 cycles of denaturation at 94°C for 10 seconds, annealing at 60°C for 15 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 6 minutes. The PCR products were separated on a 1% agarose gel and correct size products were excised and purified using the GE Healthcare Illustra™ GFX™ PCR DNA and Gel Band Purification Kit (Buckinghamshire, UK).

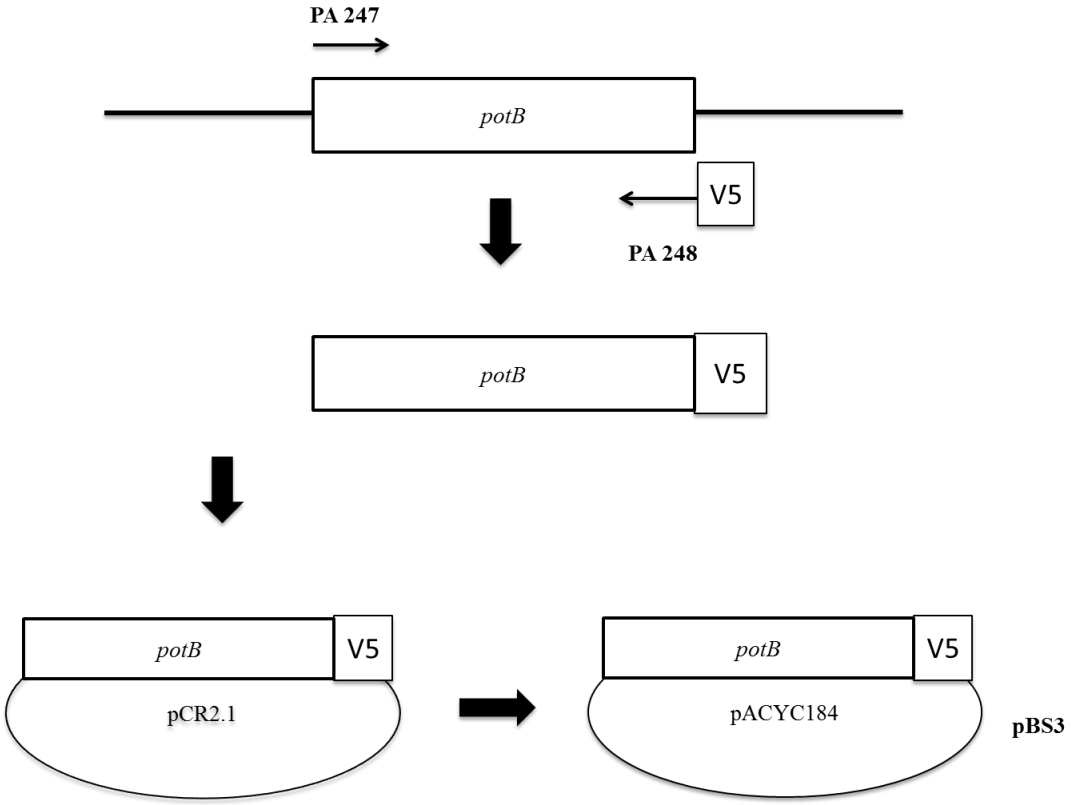


Fig. 9. Construction of the pBS3 plasmid carrying the *potB* gene for complementing deletion mutants. The pBS3 plasmid was used to complement the *V. cholerae ΔpotB* and *nspC::kan, ΔpotB* double mutant strain to further confirm role of transmembrane permease.

After adding adenines, these products were cloned in to a pCR2.1-TOPO vector following instructions from manufacturer (Invitrogen, Carlsbad, CA) and electroporated into electrocompetent DH5α using a BIO-RAD MicroPulser (Hercules, CA) at 1.8 kV. These transformed cells were incubated in SOC medium to recover at 37°C at 200 rpm, and then plated on LB-ampicillin (100 μg/mL) with 20μL of X-gal (20 mg/mL in dimethyl sulfoxide). Blue white screening was used to select for plasmids carrying the

insert and colony PCR, as described previously, was used to confirm if insert was present. Colonies testing positive for insert were grown overnight. The next day, the plasmids were purified using the Promega Wizard Plus SV Minipreps DNA Purification System (Madison, WI) and sent out for sequencing. Sequence was verified by services provided by Cornell University (Ithaca, NY).

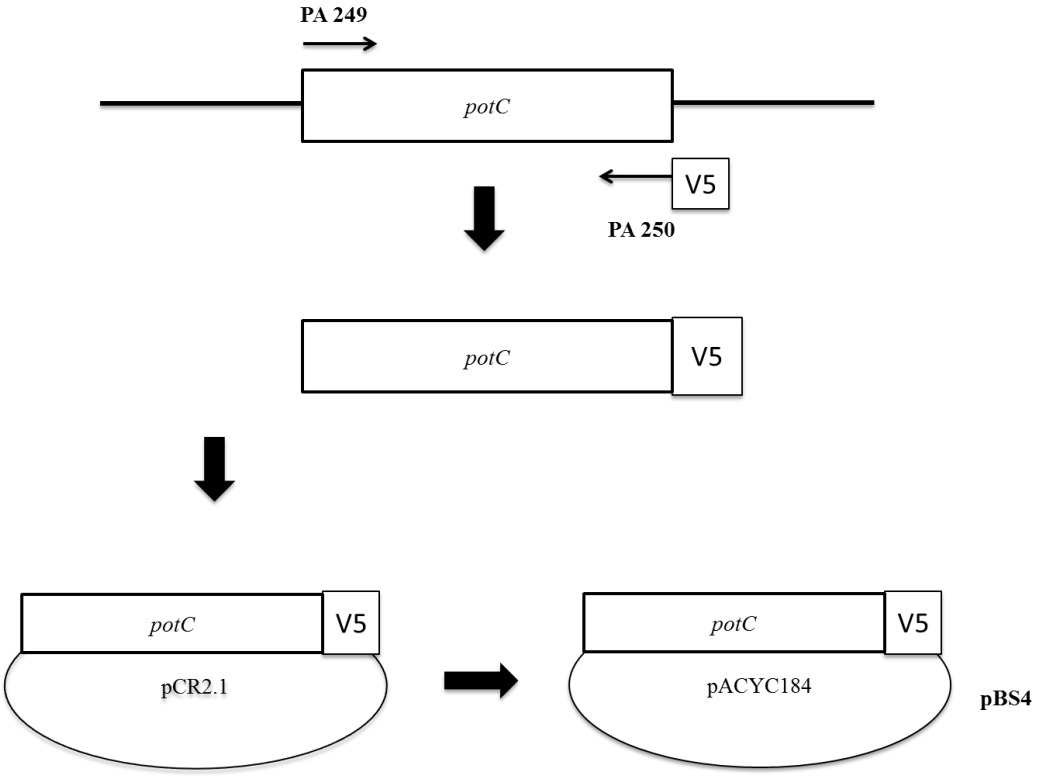


Fig. 10. Construction of the pBS4 plasmid carrying the *potC* gene for complementing deletion mutants. The pBS3 plasmid was used to complement the *V. cholerae* Δ*potC* and *nspC::kan,* Δ*potC* double mutant strain to further confirm role of transmembrane permease.

The *potB*-V5 insert was excised from the pCR2.1 vector using *Eco*RI, while the *potC*-V5 insert was excised using *Nco*I. Next, these were purified using the GE Healthcare Illustra™ GFX™ PCR DNA and Gel Band Purification Kit

(Buckinghamshire, UK). The inserts were then ligated into the medium copy pACYC184 plasmid linearized with the same enzyme, and transformed into DH5α. Alkaline phosphatase, Calf intestinal, purchased from New England Biolabs (Beverly, MA) was used to catalyze the dephosphorylation of the 5' and 3' ends of the pACYC184 plasmid to prevent recircularization of plasmid during ligation. These colonies were screened for the insert using colony PCR, as previously described, and colonies testing positive for insert were grown overnight. Next, the pBS3 plasmid was purified using the Promega Wizard Plus SV Minipreps DNA Purification System (Madison, WI) and transformed into a *V. cholerae* Δ*potB* mutant strain, as well as *nspC::kan,* Δ*potB* double mutant strain. The pBS4 plasmid was purified and transformed into a *V. cholerae* Δ*potC* mutant strain, as well as *nspC::kan,* Δ*potC.*

Western Blotting to confirm the presence of PotB and PotC proteins

Cells were grown overnight in Luria-Bertani broth (LB) (1% Tryptone, 0.5% yeast extract, 85 mM NaCl), pelleted, resuspended in 1X phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4), lysed using sonication, and then centrifuged for 3 min at 16,000 X g. The supernatant was removed and diluted 1:1 with 1X Laemmli sample buffer containing β-Mercaptoethanol and placed in a hot water bath at 65°C for 10 minutes. Seventeen μL of sample was then loaded on a polyacrylamide denaturing gel with a 12% acrylamide resolving gel and 5% stacking gel and ran at 150V for 1 hour and 15 minutes. The gel and blotting paper were equilibrated in 1X Transfer Buffer containing 50 mM TRIS, 40 mM Glycine, 1.5 mM SDS, and 20% Methanol. A PVDF membrane was briefly immersed in 100% methanol and then incubated in 1X Transfer Buffer. The gel was

transferred to the membrane using a BIO-RAD Mini Trans-Blot (Hercules, CA) for 60 min at 100V. The membrane was then blocked overnight with 5% skim milk in 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) at 4°C. The next day, the membrane was washed three times in PBS with 0.05% Tween 20 and then incubated with a monoclonal V5 antibody conjugated to horseradish peroxidase (AbD Serotec, Raleigh, NC), diluted 1:20,000 in PBS containing 3% skim milk, for 1 hour at room temperature. After incubating, the membrane was washed again three times in PBS with 0.05% Tween 20. Next, the membrane was incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) for 5 minutes, then imaged using a BIO-RAD Molecular Imager® Gel Doc™ XR System (Hercules, CA).

Polyamine extraction

To define the role of PotB and PotC in the uptake of norspermidine and spermidine, polyamines were extracted as previously described (Mcginnis *et al.*, 2009; Morgan, 1998; Parker *et al.*, 2012). Tryptone media was used due to LB medium containing approximately 15 μM spermidine and reports showing that polyamines extracted from *V. cholerae* grown in minimal media contain only putrescine, diaminopropane, cadaverine, and norspermidine (Lee *et al.*, 2009). All strains were grown at 27°C to mid-log phase, pelleted, washed with 1X PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4), and resuspended in water at 10 μL per mg wet cell weight. Cells were lysed using sonication, and debris removed by centrifugation. Cellular proteins and DNA were precipitated by a 50% tricholoracetic acid solution (TCA) and centrifuged, leaving the supernatant containing the polyamines.

The supernatant was removed and then benzoylated. In order to clarify the binding preference of the substrate binding protein, PotD1, polyamines were added to the media in varying concentrations and cell extracts were analyzed.

Benzoylation

Benzoylation was performed as described previously (Mcginnis *et al.*, 2009; Morgan, 1998; Parker *et al.*, 2012). Samples were extracted by 1mL of chloroform twice, evaporated to dryness, and dissolved in 100 μL mobile phase used for HPLC (60% methanol/40% water). Along with the benzoylation of the polyamine extractions, a standard mix containing 0.1 mM of each polyamine was prepared each time.

HPLC analysis

High performance liquid chromatography employed a Waters 1525 Binary Pump with a 2487 Dual Wavelength Absorbance Detector set at 254 nm and a Phenomenex Spherclone 5u ODS column (5 μm, 250 X 4.6 mm) that was fitted with a 4.0 X 30 mm guard cartridge (Phenomenex, Torrance, CA). The sample runs were implemented using a gradient of 45-60% methanol in water for 30 minutes, with a 10-minute isocratic equilibration of 45% methanol in water. For other sample runs another method was executed using a gradient of 45-60% methanol in water for 45 minutes, with a 10-minute isocratic equilibration of 45% methanol in water. For each run, 40 μL of each sample was injected.

Biofilm assays

Biofilm assays were performed as previously described (Karatan *et al.*, 2005) in triplicate and repeated several times to ensure reproducibility. Cells were scraped from

LB agar plates and resuspended in 300 µl of tryptone broth. This culture was then diluted 1:10 into fresh medium and the optical cell density was measured at 655 nm to calculate volume of inoculum for preparation of biofilm. Borosilicate tubes were filled with 300 µl of tryptone broth and inoculated with the strains of interest to yield an OD of 0.04. Cultures were grown at 27°C for 24 hours, planktonic cells were removed, and remaining biofilm was washed with 1X PBS. Glass beads were used to vortex and homogenize biofilm and a microplate reader (Bio-Rad, Hercules, CA) was used to quantify optical cell density of planktonic and biofilm cells at 655 nm.

Results

Construction of the ΔpotB and ΔpotC single mutants

To study the role of the transmembrane permeases of the ABC-type transporter, PotABCD2D1, I first constructed a single deletion mutant strain of each gene. A region approximately 400 bp upstream and downstream of the *potB* gene was amplified using primers PA84, PA85, PA86, and PA87. Primers PA84 and PA85 were used in a PCR reaction to generate the upstream fragment while primers PA86 and PA87 were used in a separate PCR reaction to construct the downstream fragment.

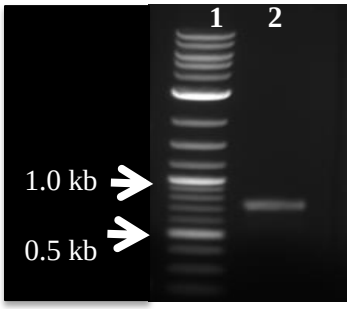


Fig. 11. Confirmation of successful splicing of Δ*potB* upstream and downstream fragments. This image represents the fused product of both upstream and downstream fragments in a PCR reaction using primers PA84 and PA87. Lane 1 represents the NEB 2-log ladder and lane 2 is the 791 bp spliced product, which was further purified and used for TA cloning.

In order to fuse the two fragments using splicing by overlap extension (SOE) PCR, primers PA85 and PA86 were engineered with complementary SOE tags. The upstream and downstream fragments were spliced together in another PCR reaction using primers PA84 and PA87 to form a recombinant molecule ultimately removing 791 bp of the 861 bp *potB* gene. Gel electrophoresis was used to assess the splicing of the two

fragments. Presence of an approximately 800 bp product confirmed that the two fragments were successfully spliced together (Fig. 11).

After purification of the PCR products, adenines were added to the 3' ends of the blunt ended fragments. The insert was then cloned into a pCR2.1 TOPO plasmid, the construct was electroporated into *E. coli* DH5α, and eight colonies were tested to detect the presence of the insert by using colony PCR (Fig. 12). Only one individual colony contained the insert. This plasmid was isolated and the sequence was verified. The plasmid pCR2.1::Δ*potB* was digested with *Xho*I and *Spe*I to release the insert, the reaction was ran on an agarose gel, and the insert was excised and gel-purified (Fig. 13). The insert was then ligated into a linearized pWM91 plasmid and electroporated into *E. coli* DH5αλpir. The plasmid containing the insert was verified by colony PCR of *E. coli* DH5αλpir, purified, and then electroporated into *E. coli* SM10λpir.

This strain was used for conjugation into *V. cholerae* using homologous recombination as described in the methods. The streptomycin resistant and ampicillin sensitive colonies were screened by colony PCR to confirm the presence of the Δ*potB* insert (Fig. 14). When observed on an agarose gel, two individual colonies were shown to contain the insert, due to the presence of a band around 800 bp indicative of the 791 bp spliced product of the Δ*potB* insert.

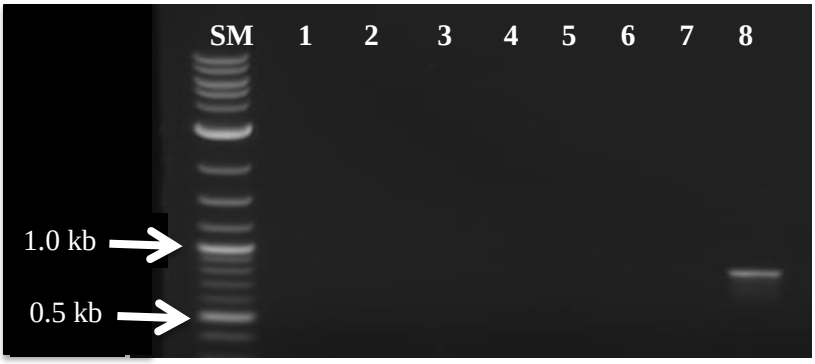


Fig. 12. Confirmation of the Δ*potB* insert in pCR2.1 . Eight isolated colonies were chosen and colony PCR was performed using primers PA84 and PA87 to verify presence of insert. SM (size marker): NEB 2-log DNA ladder; lane 1: colony 1; lane 2: colony 2; lane 2: colony 3; lane 4: colony 4; lane 5: colony 5; lane 6: colony 6; lane 7: colony 7; lane 8: colony 8. Colony 8 indicates the correct sized product around 791 bp and was chosen for further cloning procedures.

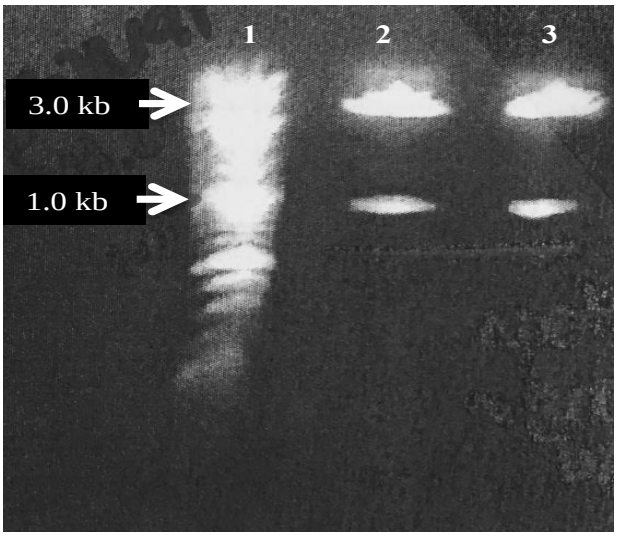


Fig. 13. Gel electrophoresis of digested pCR2.1:: Δ*potB*. In order to ligate this insert into pWM91, pCR2.1:: Δ*potB* was digested with *Xho*I and *Spe*I and ran on 1% agarose gel. Lane 1 contains the NEB 2-log DNA ladder, lanes 2 and 3 contain the cut pCR2.1 plasmid containing the insert.

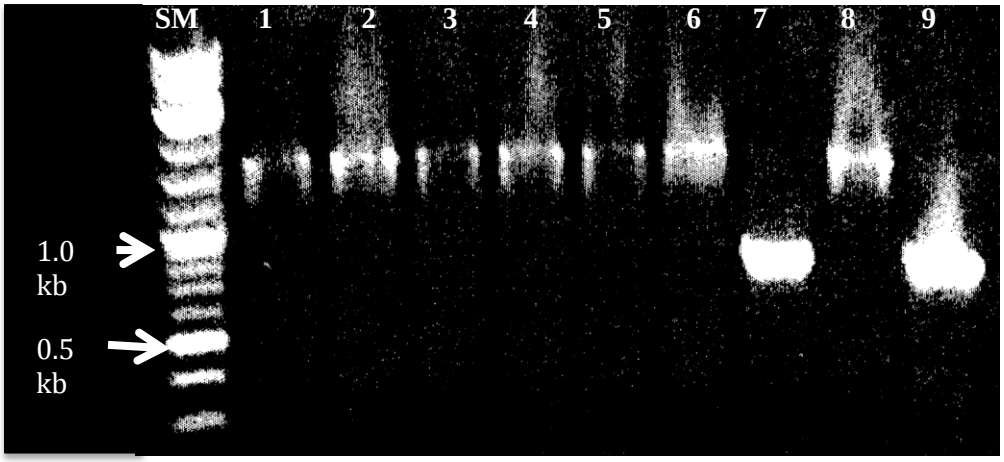


Fig. 14. Colony PCR depicting successful homologous recombination which confirms presence of Δ*potB* insert in *Vibrio cholerae* PW357. Nine ampicillin sensitive and streptomycin resistant colonies were screened using colony PCR. Primers PA84 and 87 were used to verify presence of the Δ*potB* insert. SM (size marker): NEB 2-log ladder; lane 1: colony 1; lane 2: colony 2; lane 3: colony 3; lane 4: colony 4; lane 5: colony 5; lane 6: colony 6; lane 7: colony 7; lane 8: colony 8; lane 9: colony 9. Colonies 7 and 9 indicate successful homologous recombination due to presence of an 800bp band indicative of the Δ*potB* insert.

This entire process was repeated for the construction of the *potC* mutant. A region approximately 400 bp upstream and downstream of the *potC* gene was amplified using primers PA88, PA89, PA90, and PA91. Primers PA88 and PA89 were used in a PCR reaction to generate the upstream fragment while primers PA90 and PA91 were used in a separate PCR reaction to construct the downstream fragment. In order to fuse the two fragments using splicing by overlap extension (SOE) PCR, primers PA89 and PA90 were engineered with complementary SOE tags. The upstream and downstream fragments were spliced together in another PCR reaction using primers PA84 and PA87 to form a recombinant molecule ultimately removing 753 bp of the 790 bp *potC* gene. Gel electrophoresis was used to confirm the splicing of the two fragments (Fig. 15). Presence of an approximately 800-bp product confirmed that the two fragments were successfully spliced together.

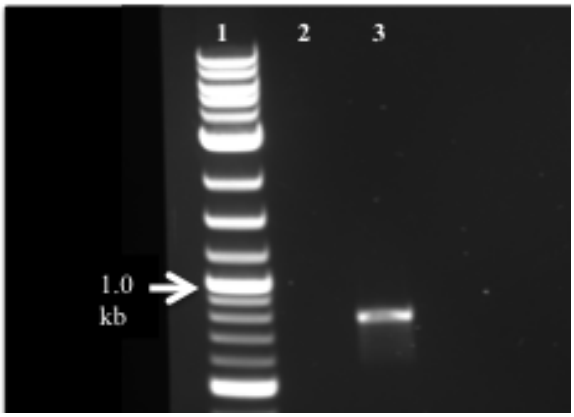


Fig. 15. Confirmation of successful splicing of Δ*potC* upstream and downstream fragments. This image represents the fused SOE product of both upstream and downstream fragments in a PCR reaction using primers PA88 and PA91. Lane 1 represents the NEB 2-log ladder and lane 3 is the 753 bp spliced product, which was further purified and used for TA cloning.

After purification of the PCR products, adenines were added to the 3' ends of the blunt ended fragments. The insert was then cloned into a pCR2.1 TOPO plasmid, the construct was electroporated into *E. coli* DH5α, and nineteen colonies were tested to detect the presence of the insert by using colony PCR (Fig. 16). Five colonies were found to contain the insert indicated by the band around 800 bp. This plasmid was isolated and the sequence was verified. The plasmid pCR2.1::Δ*potC* was digested with *Apa*I and *Spe*I to release the insert, the reaction was ran on an agarose gel, and the insert was cut out and gel-purified (Fig. 17). The insert was then ligated into a linearized pWM91 plasmid, and electroporated into *E. coli* DH5αλpir. The plasmid containing the insert was verified by colony PCR of *E. coli* DH5αλpir, purified, and then electroporated into *E. coli* SM10λpir. This strain was used for conjugation into *V. cholerae*, PW357, using homologous recombination as described in the methods. The streptomycin resistant and ampicillin sensitive colonies were screened by colony PCR to confirm the presence of the Δ*potC* insert (Fig. 18). When observed on an agarose gel, two individual

colonies were shown to contain the insert, due to the presence of a band around 800 bp indicative of the spliced product of the Δ*potC* insert.

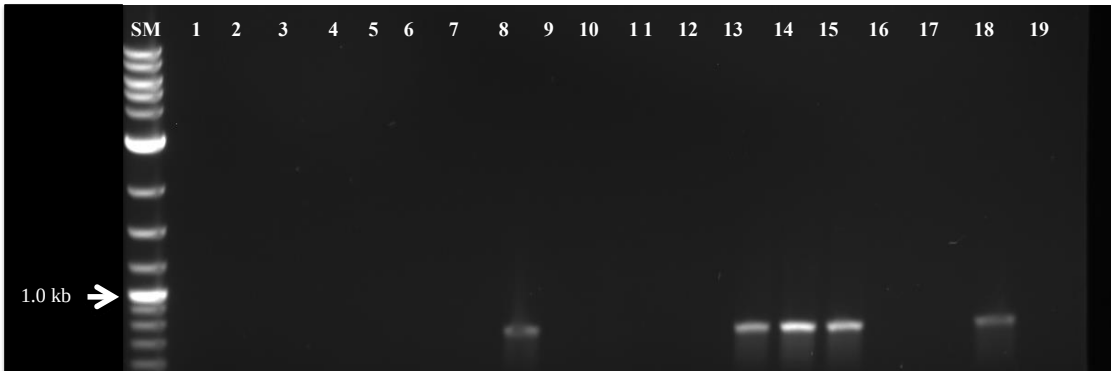


Fig. 16. Confirmation of the Δ*potC* insert in pCR2.1. Nineteen isolated colonies were chosen and colony PCR was performed using primers PA88 and PA91 to verify presence of insert. SM (size marker): NEB 2-log DNA ladder; lanes 1-19: colonies 1-19. Colonies 8, 13, 14, 15, and 18 indicates the correct sized product around 753 bp.

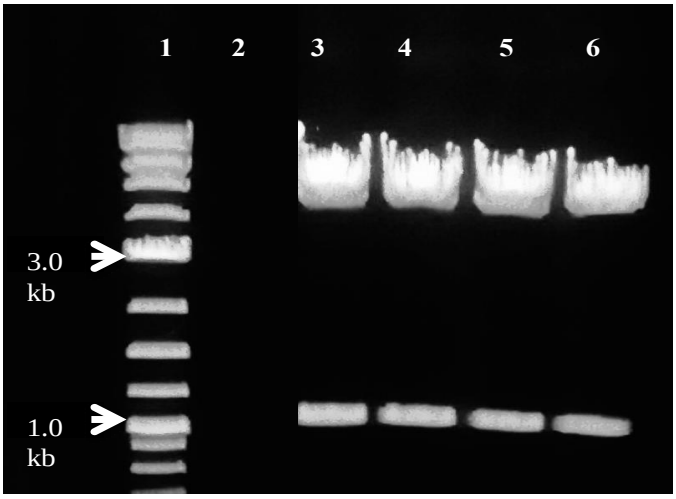


Fig. 17. Gel electrophoresis of digested pCR2.1:: Δ*potC*. In order to ligate this insert into pWM91, pCR2.1:: Δ*potC* was digested with *Apa*I and *Spe*I and ran on a 1% agarose gel. Lane 1 contains the NEB 2-log DNA ladder, lanes 3-6 contain the cut pCR2.1 plasmid containing the insert.

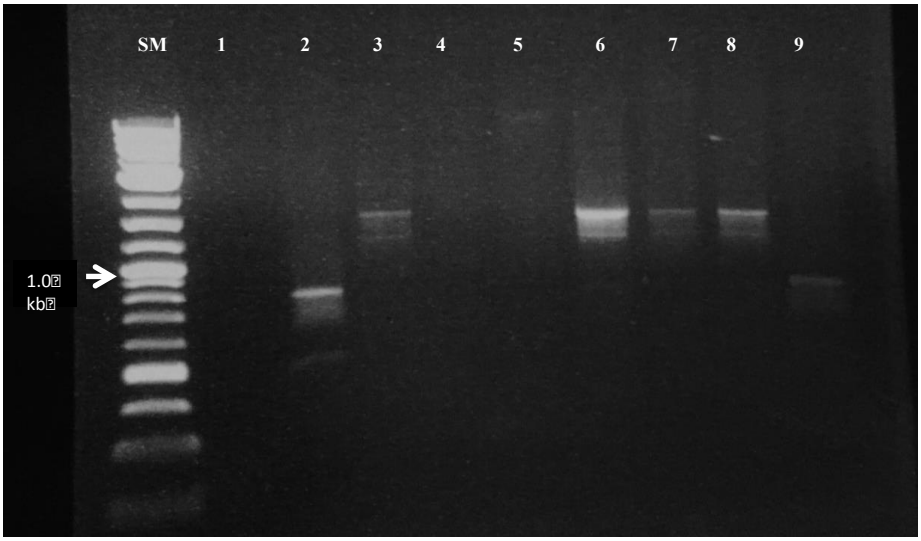


Fig. 18. Confirmation of presence of Δ*potC* insert in *Vibrio cholerae* PW357. Nine ampicillin sensitive and streptomycin resistant colonies were screened using colony PCR. Primers PA88 and 91 were used to verify presence of the Δ*potC* insert. SM (size marker): NEB 2-log ladder; lane 1: colony 1; lane 2: colony 2; lane 3: colony 3; lane 4: colony 4; lane 5: colony 5; lane 6: colony 6; lane 7: colony 7; lane 8: colony 8; lane 9: colony 9. Colonies 2 and 9 indicate successful homologous recombination due to presence of an 800 bp band indicative of the Δ*potC* insert.

Construction of nspC::kan ΔpotB and of nspC::kan ΔpotC double mutants

Because *V. cholerae* can synthesize norspermidine, a strain unable to synthesize or transport this molecule needed to be constructed in order to assess norspermidine import. A double mutant was generated by SacB counter selectable mutagenesis as previously described in materials and methods. *V. cholerae* PW357 *nspC::kan* mutant strain was used for the recipient, while SM10 λpir *E. coli* containing the plasmid pWM91::Δ*potB* or pWM91::Δ*potC* were used for the donor. The streptomycin, kanamycin resistant and ampicillin sensitive colonies were screened by colony PCR to confirm the presence of the Δ*potB* insert and Δ*potC* insert. Nine colonies were tested to detect the presence of the Δ*potB* insert in the *nspC::kan* background by using colony

PCR (Fig. 19). When ran on an agarose gel, two of the nine colonies screened contained the 800 bp insert (Fig. 19). Eight colonies were chosen and tested to detect the presence of the Δ*potC* insert in the *nspC::kan* background by using colony PCR (Fig. 20). When these reactions were ran on an agarose gel, three individual colonies were shown to contain the insert indicated by the band around 800 bp (Fig. 20).

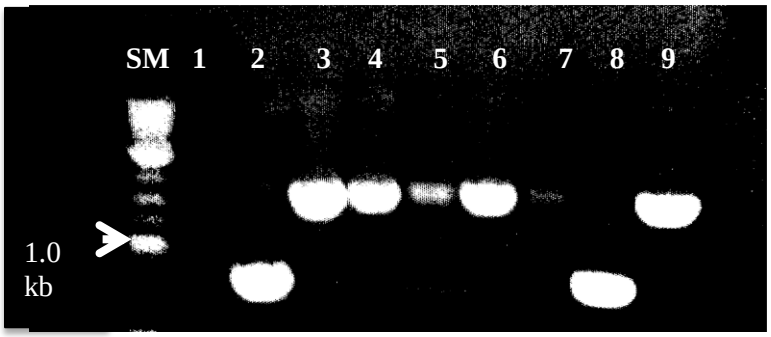


Fig. 19. Confirmation of presence of Δ*potB* insert in *V. cholerae nspC::kan* Δ*potB*. Nine ampicillin sensitive and streptomycin, kanamycin resistant colonies were screened using colony PCR. Primers PA84 and PA87 were used to verify presence of the Δ*potB* insert. SM (size marker): NEB 2-log ladder; lane 1: colony 1; lane 2: colony 2; lane 3: colony 3; lane 4: colony 4; lane 5: colony 5; lane 6: colony 6; lane 7: colony 7; lane 8: colony 8; lane 9: colony 9. Colonies 2 and 8 indicate successful homologous recombination due to presence of an 800 bp band indicative of the Δ*potB* insert.

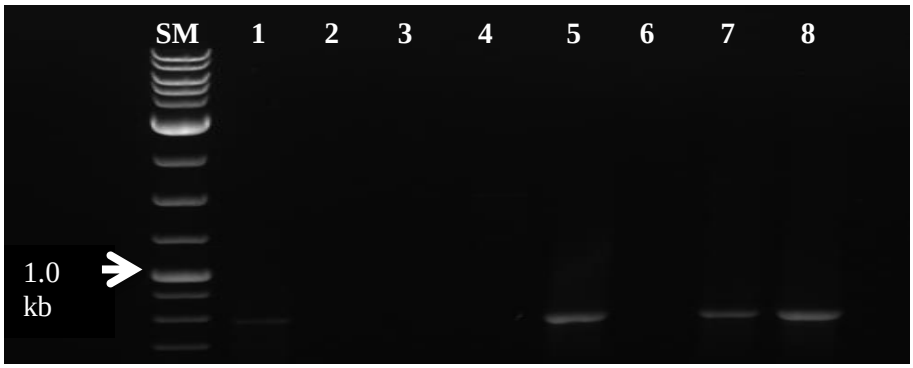


Fig. 20. Confirmation of presence of Δ*potC* insert in *V. cholerae nspC::kan* Δ*potC*. Eight ampicillin sensitive and streptomycin, kanamycin resistant colonies were screened using colony PCR. Primers PA88 and PA91 were used to verify presence of the Δ*potB* insert. SM (size marker): NEB 2-log ladder; lane 1: colony 1; lane 2: colony 2; lane 3: colony 3; lane 4: colony 4; lane 5: colony 5; lane 6: colony 6; lane 7: colony 7; lane 8: colony 8. Colonies 5, 7, and 8 indicate successful homologous recombination due to presence of an 800 bp band indicative of the Δ*potC* insert.

Construction of the complementation plasmid carrying the potB gene

The *pot* mutants were complemented with plasmids carrying each of the genes in order to further validate the role of the transmembrane permeases of the transport system. To complement the *potB* mutant, the entire *potB* gene was amplified using primers, PA247 and 248, to yield a 953 bp PCR product (Fig. 21). Gel electrophoresis was used to confirm the amplification of the *potB* gene. Presence of an approximately 1000-bp product confirmed that the *potB* gene was amplified. After purification of the PCR products, adenines were added to the 3' ends of the blunt ended fragments and the insert was cloned into a pCR2.1 TOPO plasmid, the construct was electroporated into *E. coli* DH5α, and nine colonies were tested to detect the presence of the insert by using colony PCR (Fig. 22). Seven colonies were found to contain the insert indicated by the band around 1000 bp (Fig. 22).

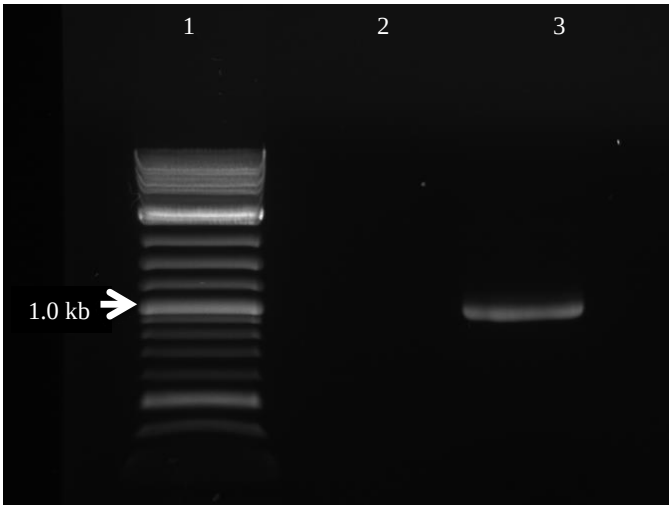


Fig. 21. Gel electrophoresis of *potB*-V5 PCR product. The entire *potB* gene was amplified using primers PA247 and 248. Lane 1 is the 2-log ladder. Lane 3 contains the 953 bp PCR product, which was used for TA cloning.

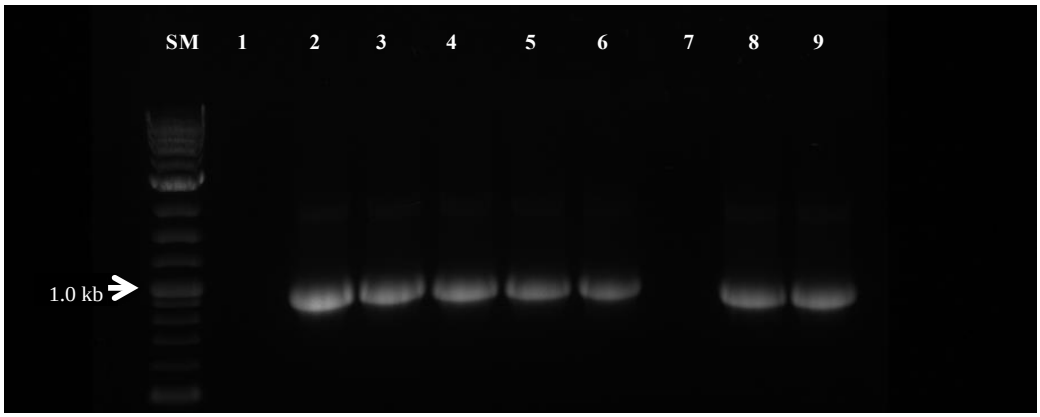


Fig. 22. Confirmation of the *potB*-V5 insert in pCR2.1. Nine isolated colonies were chosen and colony PCR was performed using primers PA247 and PA248 to verify presence of insert. SM (size marker): NEB 2-log ladder; lane 1: colony 1; lane 2: colony 2; lane 3: colony 3; lane 4: colony 4; lane 5: colony 5; lane 6: colony 6; lane 7: colony 7; lane 8: colony 8; lane 9: colony 9. Colonies 2, 3, 4, 5, 6, 8, and 9 indicate the correct sized product around 953 bp.

Plasmids were isolated from colonies 2, 3, and 4, and correct construction was verified by sequencing. Once the sequence of pCR2.1::*potB*-V5 was verified, the plasmid pCR2.1::*potB*-V5 was digested with *EcoR*I to release the insert, the reaction was ran on an agarose gel, and the insert was cut out and gel-purified. The insert was then ligated into the pACYC184 plasmid linearized with the same enzyme. The ligation was then transformed into *E. coli* DH5α and colonies containing the plasmids with the insert were verified using colony PCR (Fig. 23). Three colonies testing positive for insert were grown overnight and plasmids were isolated. The pACYC184::*potB*-V5 plasmid (pBS3) was transformed into both Δ*potB* and *nspC:kan* Δ*potB* mutant strains and verified using colony PCR (Fig. 24 and 25). Nine of ten colonies screened contained the 1000 bp *potB*-V5 insert in the *V. cholerae* Δ*potB* background (Fig. 24). All of the colonies screened contained the *potB*-V5 insert in the *V. cholerae nspC::kan* Δ*potB* background (Fig. 25).

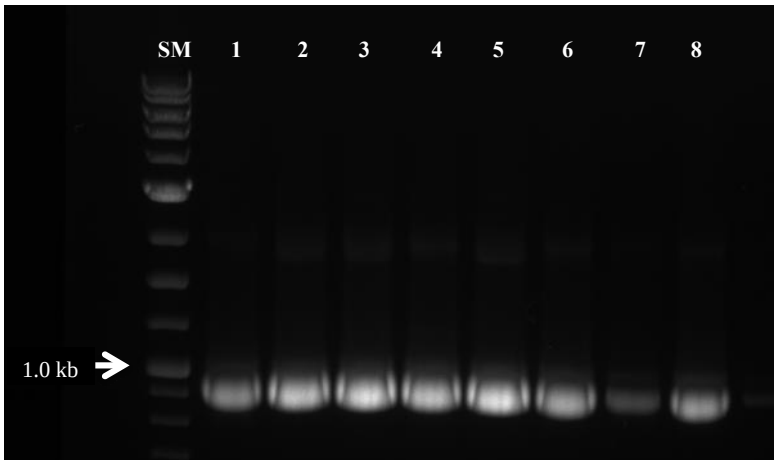


Fig. 23. Confirmation of the *potB*-V5 insert in pAYCY184. Eight isolated colonies were chosen and colony PCR was performed using primers PA247 and PA248 to verify presence of insert. SM (size marker): NEB 2-log ladder; lane 1: colony 1; lane 2: colony 2; lane 3: colony 3; lane 4: colony 4; lane 5: colony 5; lane 6: colony 6; lane 7: colony 7; lane 8: colony 8. All colonies indicate the correct sized product around 953 bp, colonies 2, 3, and 4 were used for further manipulation.

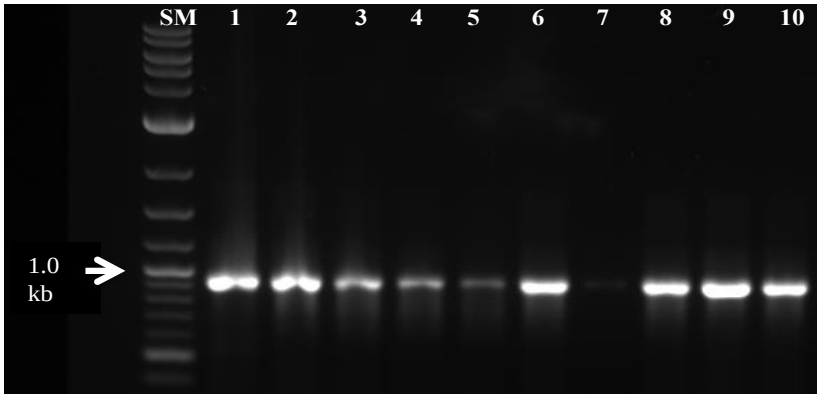


Fig. 24. Confirmation of the plasmid pACYC184::*potB*-V5 in *V. cholerae* Δ*potB*. Ten isolated colonies were chosen and colony PCR was performed using primers PA247 and PA248 to verify presence of insert. SM (size marker): NEB 2-log DNA ladder; lanes 1-10: colonies 1-10. Colonies 1, 2, 3, 4, 5, 6, 8, 9, and 10 indicate the correct sized product around 953 bp.

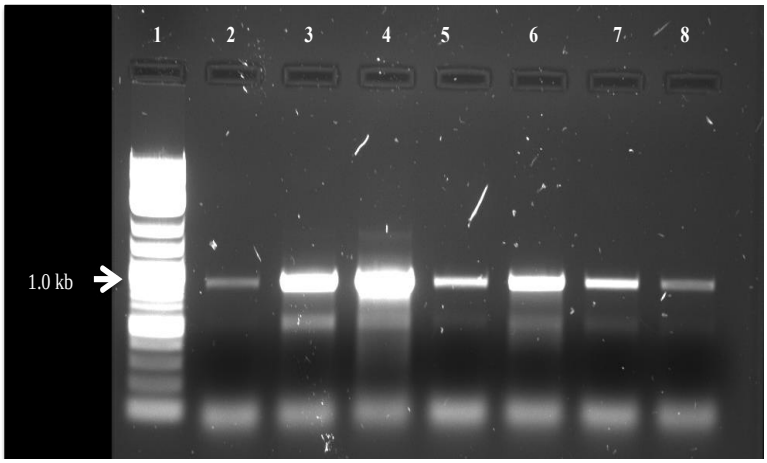


Fig. 25. Confirmation of the plasmid pACYC184::*potB*-V5 in *V. cholerae nspC::kan* Δ*potB*. Seven isolated colonies were chosen and colony PCR was performed using primers PA247 and PA248 to verify presence of insert. Lane 1: NEB 2-log ladder; lane 2: colony 1; lane 3: colony 2; lane 4: colony 3; lane 5: colony 4; lane 6: colony 5; lane 7: colony 6; lane 8: colony 7. All lanes indicate the correct sized product around 953 bp.

Construction of the complementation plasmid carrying the potC gene

To complement the *potC* mutant, the entire *potC* gene was amplified using primers PA249 and 250 to yield a 905 bp PCR product (Fig. 26). Gel electrophoresis was used to assess the amplification of the *potC* gene. When ran on the gel the PCR product was seen at around 1000 bp, confirming that the *potC* gene was amplified (Fig. 26). Adenines were added to the 3' ends of the blunt ended fragments and the insert was cloned into a pCR2.1 TOPO plasmid, the construct was electroporated into *E. coli* DH5α, and nine colonies were tested to detect the presence of the insert by using colony PCR. All nine colonies were found to contain the insert indicated by the band around 1000 bp (Fig. 27).

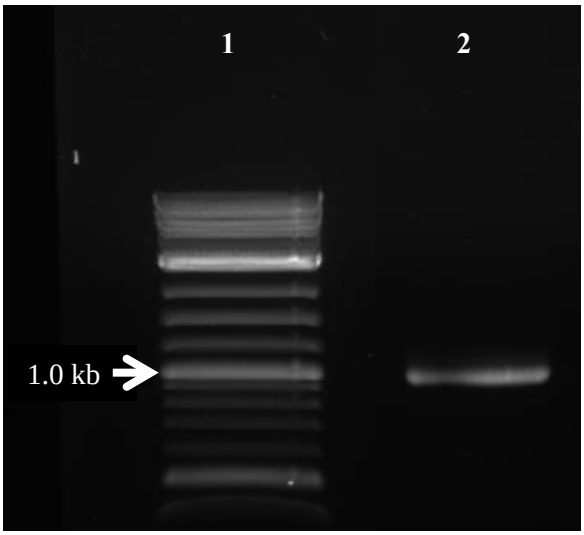


Fig. 26. Gel electrophoresis of *potC*-V5 PCR product. The entire *potC* gene was amplified using primers PA249 and 250. Lane 1 is the NEB 2-log DNA ladder. Lane 2 contains the 905 bp PCR product, which was used for TA cloning.

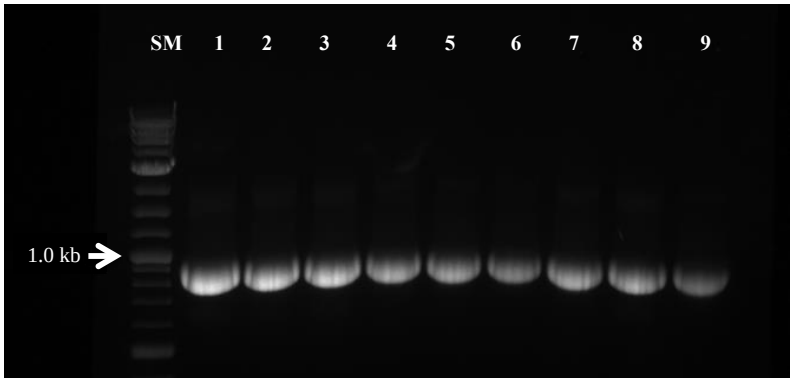


Fig. 27. Confirmation of the *potC*-V5 insert in pCR2.1 . Nine isolated colonies were chosen and colony PCR was performed using primers PA249 and PA250 to verify presence of insert. SM (size marker): NEB 2-log ladder; lane 1: colony 1; lane 2: colony 2; lane 3: colony 3; lane 4: colony 4; lane 5: colony 5; lane 6: colony 6; lane 7: colony 7; lane 8: colony 8; lane 9: colony 9. All lanes indicate the correct sized product around 953 bp.

Plasmids were isolated from colonies 1, 2, and 3, and the correct construction was verified by sequencing. Once the sequence was verified, the plasmid pCR2.1::*potC*-V5 was digested with *Nco*I to release the insert, the reaction was ran on an agarose gel, and the insert was cut out and gel-purified. The insert was then ligated into a linearized

pACYC184 plasmid with the same enzyme. This was then transformed into *E. coli* DH5α and colonies containing the insert were verified using colony PCR (Fig. 28). Colonies testing positive for insert were grown overnight and plasmids were isolated. The pACYC184::*potC*-V5 plasmid (pBS4) was transformed into both Δ*potC* and *nspC:kan* Δ*potC* mutant strains and verified using colony PCR (Fig. 29). All nine colonies screened contained the 1000 bp *potC*-V5 insert in the *V. cholerae* Δ*potC* background (Fig. 29). Four of the nine colonies screened contained the *potC*-V5 insert in the *V. cholerae nspC::kan* Δ*potC* background (Fig. 29).

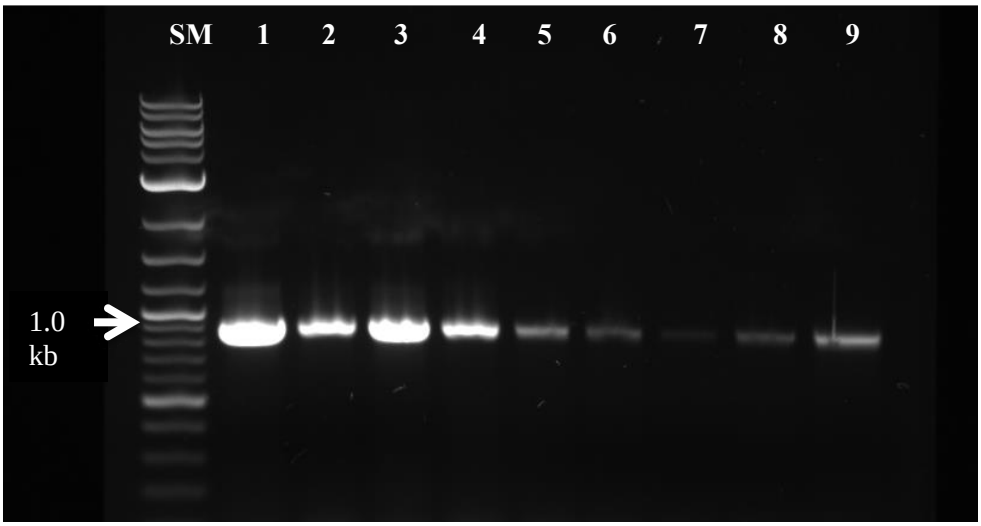


Fig. 28. Confirmation of the *potC*-V5 insert in pAYCY184. Nine isolated colonies were chosen and colony PCR was performed using primers PA249 and PA250 to verify presence of insert. SM (size marker): NEB 2-log DNA ladder; lanes 1-9: colonies 1-9. All colonies indicate the correct sized product around 905 bp, colonies 1, 2, and 3 were used for further manipulation.

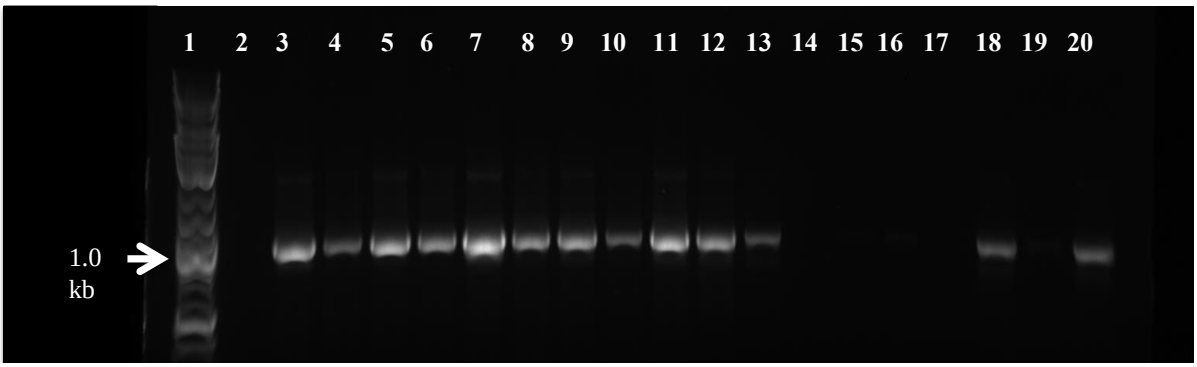


Fig. 29. Colony PCR confirming pACYC184::*potC*-V5 in *V. cholerae* Δ*potC* and *nspC:kan* Δ*potC* mutant strains. Lane 1 contains 2-log DNA ladder, while lanes 3-11 contain the correct sized band indicative of *potC*-V5 insert in PW357 Δ*potC* mutant strain. Lanes 12, 13, 18, and 20 represent single colonies that contain *potC*-V5 insert in *nspC:kan* Δ*potC* double mutant strain.

Confirmation of the presence of PotB and PotC proteins

To confirm the presence of the Pot proteins in each complemented mutant strain, a Western blot was performed as described in materials and methods. In a separate experiment, *nspC::kan* Δ*potD1* complemented with a plasmid containing the *potD1*-V5 construct was used as a positive control to detect the V5 epitope tag, while *nspC::kan* with an empty pACYC184 vector was used as a negative control. In analyzing the blot, the PotD1 protein can be visualized in lane 3 around 40 kDa (Fig. 30). A smaller band lower than 40 kDa was present in the Western blot. Lower molecular weight bands are usually indicative of degradation of target proteins. There was no band observed in the negative control as expected (Fig. 30). To confirm the presence of PotB and PotC in the complemented mutants that were constructed, a Western blot was performed and analyzed. Bands were detected around 31 kDa for PotB and 28 kDa for PotC (Fig 31). This verified that the complemented mutants with the plasmid carrying the entire gene express the V5 tagged PotB and PotC proteins.

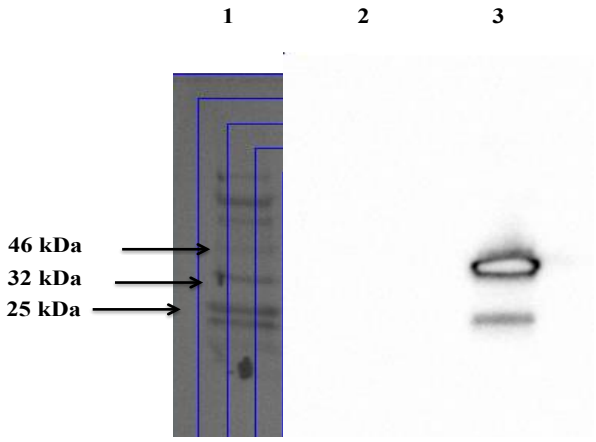


Fig. 30. Western Blot analysis showing expression of PotD1 complement mutants. Equal concentration of whole cell lysates were separated by SDS-PAGE, blotted, and reacted with V5-antibody. Mutants were detected using the V5 epitope tag. Lane 1: New England Biolabs Color Plus Protein Standard, Broad Range; Lane 2: *nspC::kan* with empty pACYC184 vector; Lane 3: *nspC::kan* Δ*potD1* with *potD1*-V5, size of PotD1 protein is 40 kDa.

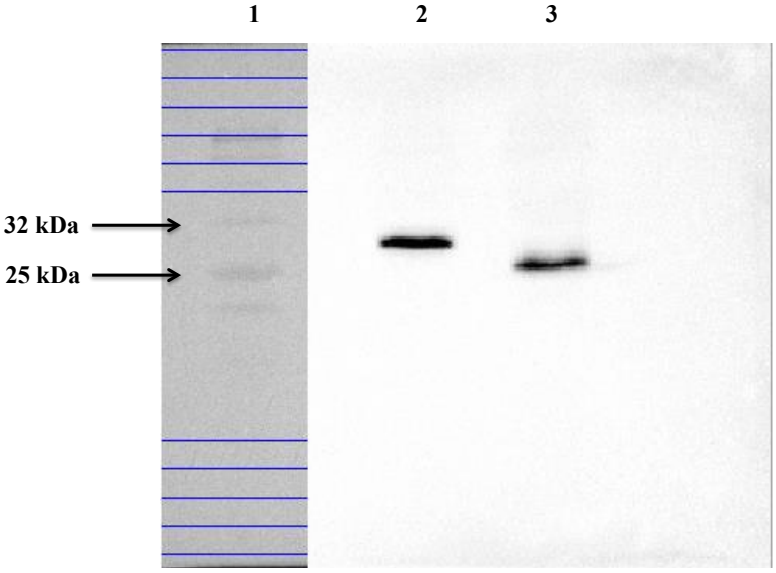


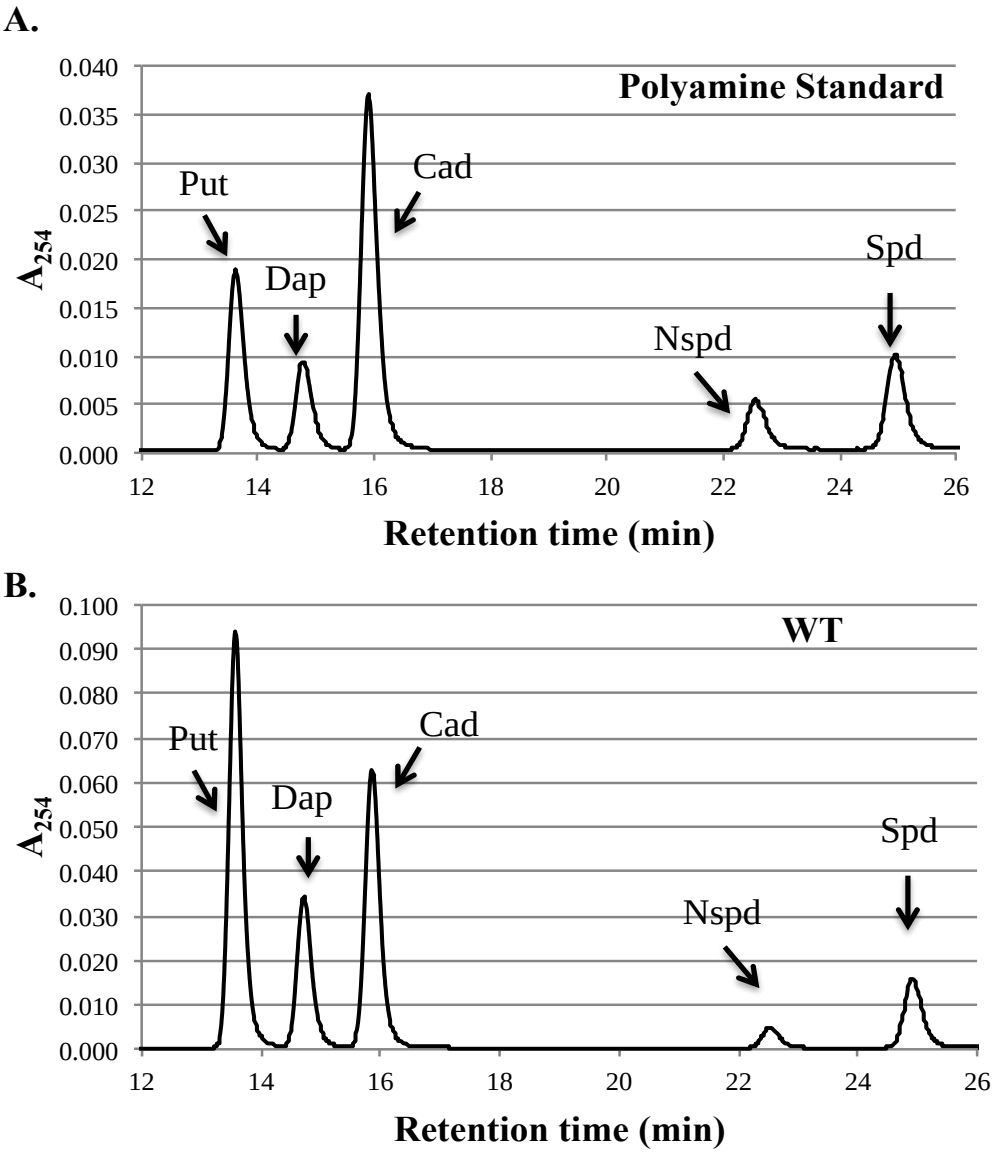
Fig. 31. Western Blot analysis showing expression of PotB and PotC complement mutants. Equal concentration of whole cell lysates were separated by SDS-PAGE, blotted, and reacted with V5-antibody. Mutants were detected using the V5 epitope tag. Lane 1: New England Biolabs Color Plus Protein Standard, Broad Range; Lane 2: *nspC::kan* Δ*potB* with *potB*-V5, size of PotB protein is 31 kDa; Lane 3: *nspC::kan* Δ*potC* with *potC*-V5, size of PotC protein is 28 kDa. ExPASy (http://www.expasy.org/) was used to compute molecular weight of PotB and PotC proteins.

PotB and PotC facilitate the import of spermidine

Previous research in our lab has reported that PotD1 in *V. cholerae*, the presumed substrate binding protein of the putative ABC-type transporter, PotABCD1 was responsible for both norspermidine and spermidine uptake (Cockerell *et al.*, 2014; Mcginnis *et al.*, 2009). In this work, we further characterize this putative transport system and analyze the role of the other components of this system, specifically the transmembrane permeases of this system, PotB and PotC. To determine if PotB and PotC are responsible for transporting spermidine into the cell, I constructed mutant strains lacking *potB* or *potC* and analyzed the intracellular polyamine content. Polyamines were extracted, derivatized by benzoylation, and analyzed by HPLC as described in materials and methods. A standard mix containing 0.1 mM of each polyamine was prepared each time in order to determine the identity of the polyamines. The standard mix included putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd) (Fig. 32A).

The *V. cholerae* wild-type cellular polyamine profile showed all five of the polyamines in the standard mix (Fig. 32B). The biosynthetic pathways for putrescine, cadaverine, diaminopropane, and norspermidine have been detected in *V. cholerae* (Lee *et al.*, 2009; Mcginnis *et al.*, 2009; Merrell & Camilli, 1999). However, *V. cholerae* lacks the genes encoding enzymes responsible for spermidine synthesis (Tabor *et al.*, 1986). Under the conditions of our experiment, *V. cholerae* is unable to synthesize spermidine, so any spermidine that is observed in the chromatograms is imported from the media. Polyamine levels in the tryptone medium used in our experiments have been determined. This media contains on average approximately 3.6 μM spermidine (data not

shown). The *ΔpotB* and Δ*potC* mutants contained all of the polyamines in the standard except for spermidine (Fig. 32C and 32D), indicated by the absence of the peak around 25 min. These results were similar to previous research in our lab on the substrate binding protein PotD1 (Mcginnis *et al.*, 2009). This data suggests that the transmembrane permeases, PotB and PotC, the transmembrane permeases, are required for spermidine transport into the cell.



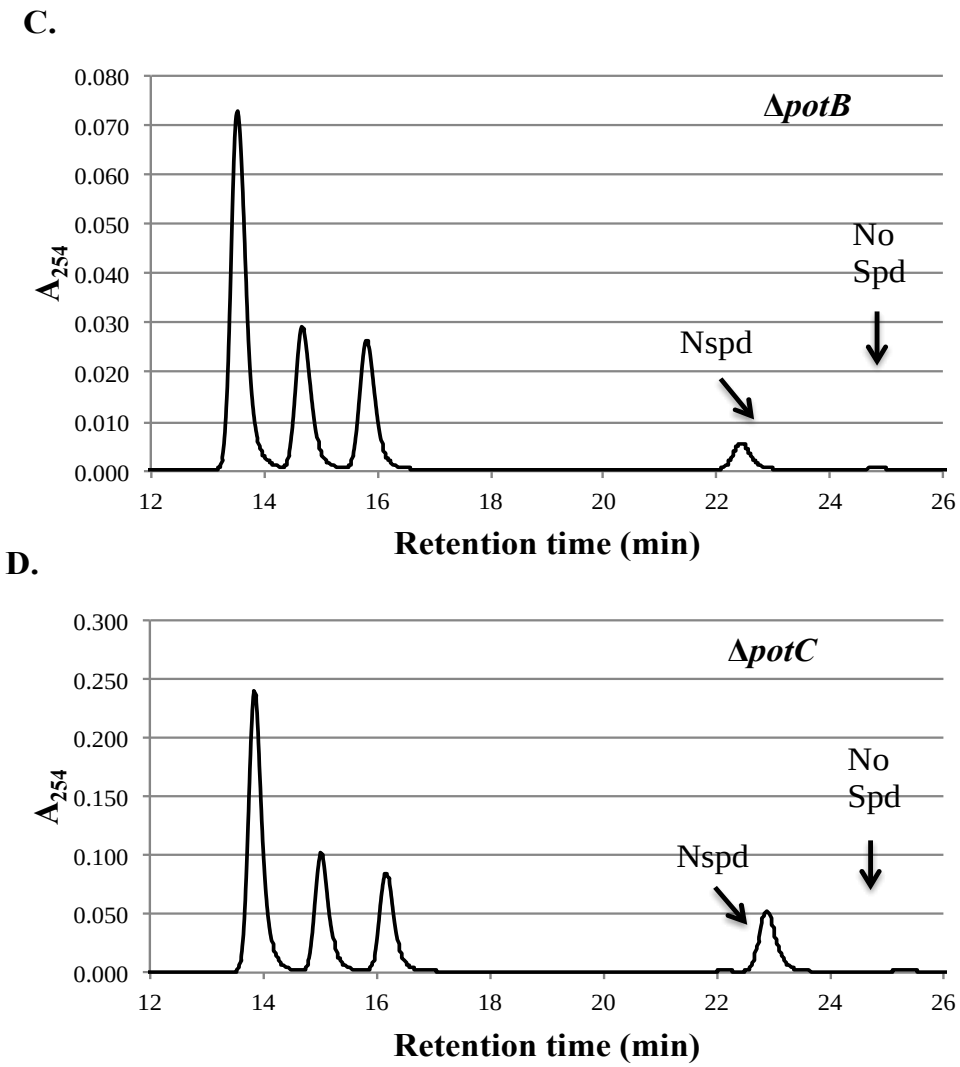
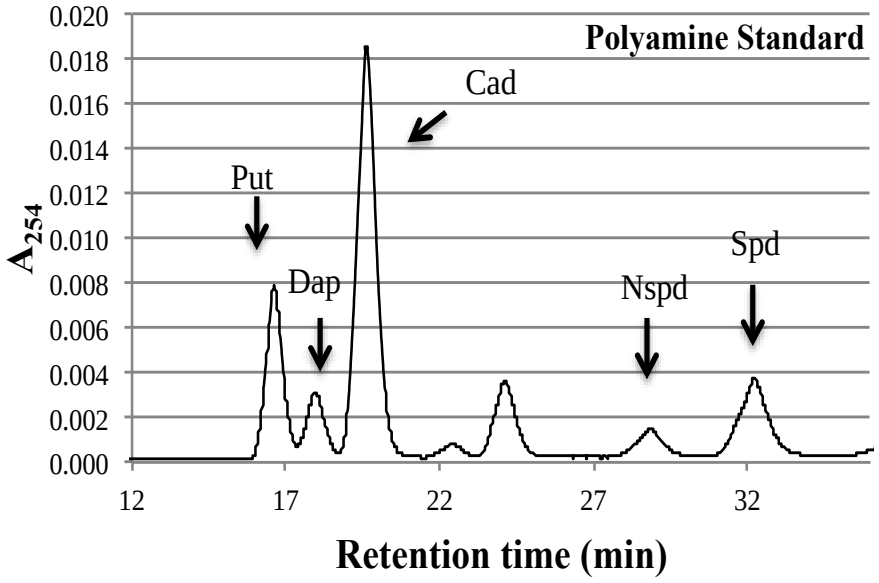


Fig. 32. Polyamine composition of the *pot* single deletion mutants. Cellular polyamines were extracted, derivatized by benzoylation, and analyzed by HPLC as previously described in Materials and methods. For clarity, only data obtained between 12 and 26 min of a 40 min run are plotted. **A.** HPLC chromatogram depicting the retention times of each polyamine; peaks labeled in the standard chromatogram correspond to putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd). **B.** HPLC chromatogram depicting the polyamine composition of wild-type *V. cholerae* **C.** Chromatogram confirming a lack of spermidine in the *V. cholerae* Δ*potB* mutant strain. The spermidine peak at 25 min of the run is absent. **D.** Chromatogram confirming a lack of spermidine in the *V. cholerae* Δ*potC* mutant strain. The spermidine peak at 25 min of the run is absent. A_{254}, absorbance at 254 nm.

To further validate and confirm the role of PotB and PotC, I complemented the mutant strains with a plasmid carrying the entire *potB* or *potC* genes. These mutants were grown in media with 100 μM exogenous spermidine, and intracellular polyamine content was analyzed as previously described. A standard mix containing 0.1 mM of each polyamine was prepared in order to determine the identity of the polyamines. The standard mix included putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd) (Fig. 33A). The Δ*potB* mutant contained all polyamines except spermidine, even with the addition of increased amounts of exogenous spermidine further confirming that PotB is required for spermidine transport (Fig. 33B). When I complemented this mutant strain with a plasmid carrying the *potB* gene, the spermidine peak was recovered (Fig. 33C). This confirms that PotB facilitates the import of spermidine into the cell.

A.



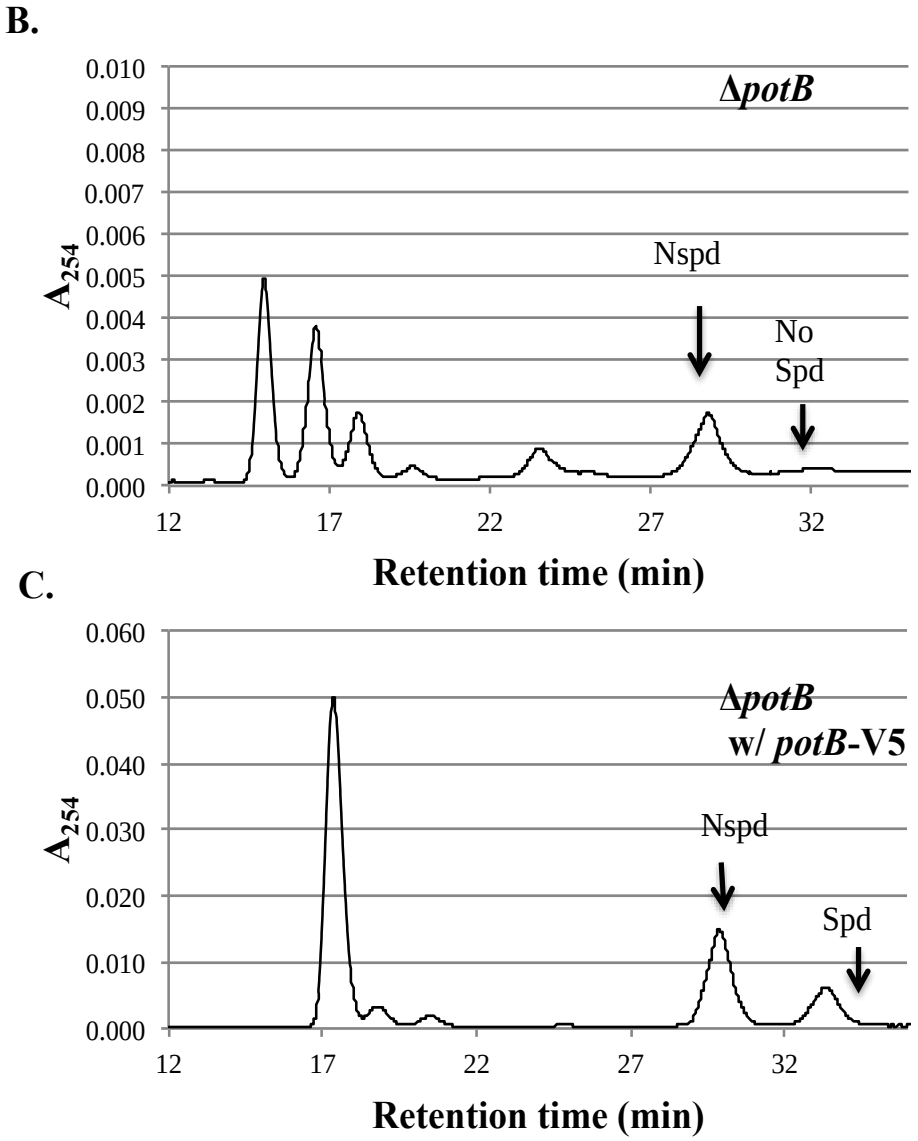
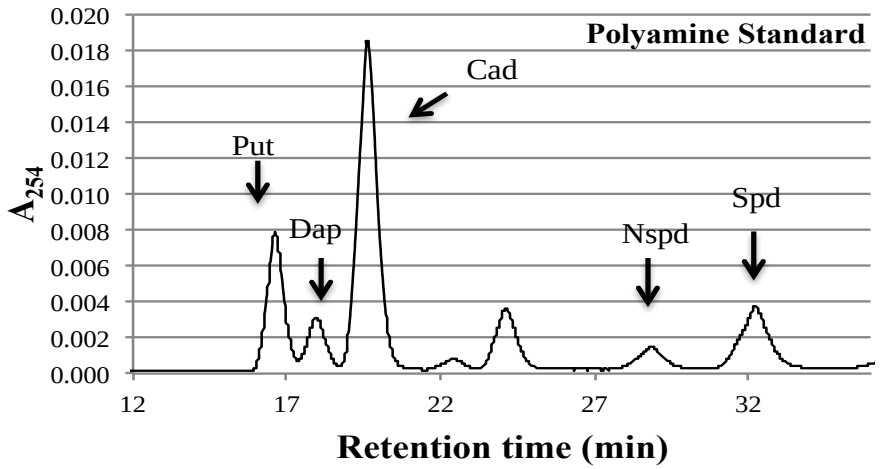


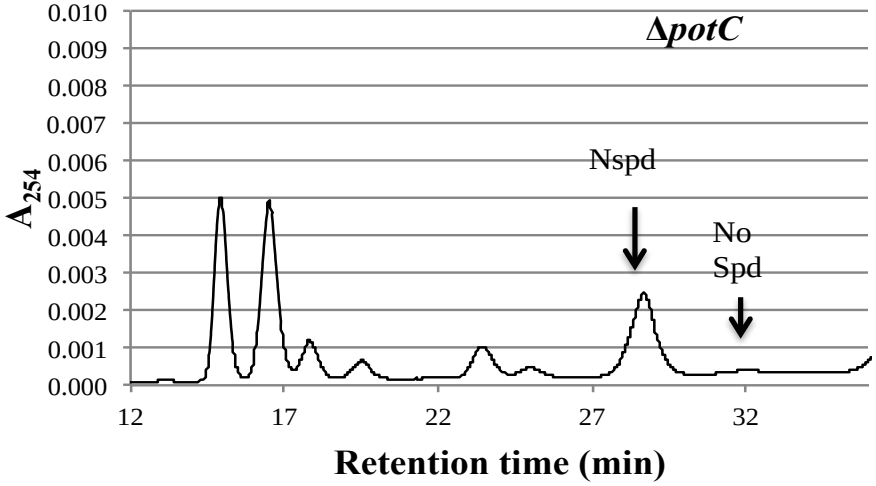
Fig. 33. Confirmation that PotB is responsible for spermidine import into the cell. Cellular polyamines were extracted from media with exogenous spermidine added, derivatized by benzoylation, and analyzed by HPLC. For clarity, only data obtained between 12 and 36 min of a 60 min run are plotted. **A.** HPLC chromatogram depicting the retention times of each polyamine; peaks labeled in the standard chromatogram correspond to putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd). **B.** Chromatogram confirming a lack of spermidine in the *V. cholerae* Δ*potB* mutant strain. **C.** HPLC chromatogram of the Δ*potB* mutant strain complemented with the plasmid carrying the entire *potB* gene. The spermidine peak is present around 32-33 min, indicating that the PotB is responsible for spermidine import.

Similar results were also observed with the polyamine profile of the *potC* mutant (Fig. 34B). A standard mix containing 0.1 mM of each polyamine was prepared in order to determine the identity of the polyamines. The standard mix included putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd) (Fig. 34A). The Δ*potC* mutant contained all polyamines except spermidine, even with the addition of increased amounts of exogenous spermidine further confirming that PotC is required for spermidine transport (Fig. 34B). When we complemented this mutant strain with a plasmid carrying the *potC* gene, the spermidine peak was recovered (Fig. 34C). This confirms that PotC facilitates the import of spermidine into the cell.

A.



B.



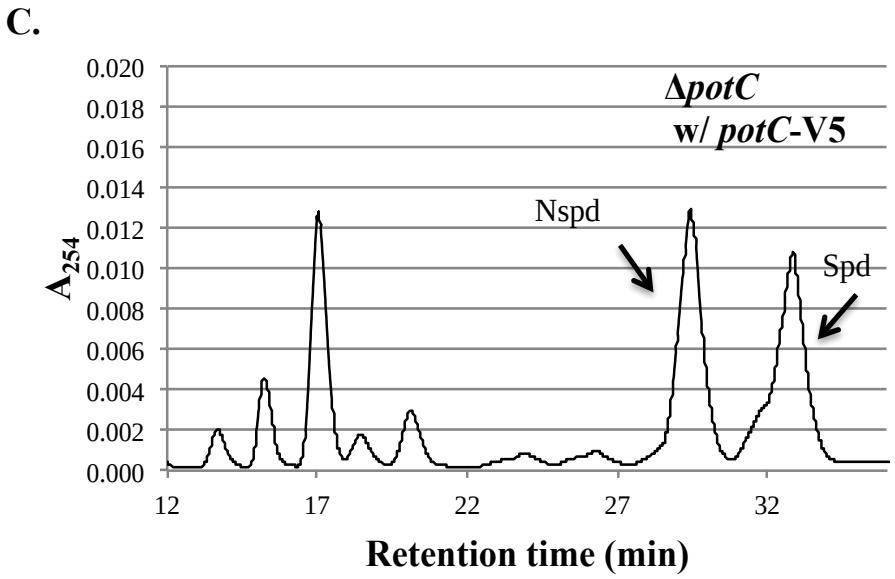


Fig. 34. Confirmation that PotC is responsible for spermidine import into the cell. Cellular polyamines were extracted from media with exogenous spermidine added, derivatized by benzoylation, and analyzed by HPLC. For clarity, only data obtained between 12 and 36 min of a 60 min run are plotted. **A.** HPLC chromatogram depicting the retention times of each polyamine; peaks labeled in the standard chromatogram correspond to putrescine (Put), diaminopropane (Dap), cadaverine (Cad), norspermidine (Nspd), and spermidine (Spd). **B.** Chromatogram confirming a lack of spermidine in the *V. cholerae nspC::kan* Δ*potC* mutant strain. **C.** HPLC chromatogram of the *nspC::kan* Δ*potC* mutant strain complemented with the plasmid carrying the entire *potC* gene. The spermidine peak is present around 32-33 min, indicating that the PotC is responsibe for spermidine import into the cell.

PotB and PotC facilitate the import of norspermidine

To determine the ability of PotB and PotC to facilitate norspermidine import, a double mutant was constructed. Homologous recombination was used to delete the *potB* and *potC* gene from the *V. cholerae nspC::kan* mutant. In order to confirm that these strains were deficient in uptake of norspermidine, exogenous norspermidine was added to the media at 100 μM concentration. Cellular polyamines were extracted, derivatized by benzoylation, and analyzed by HPLC as described in materials and methods.

The *nspC::kan* Δ*potB* double mutant contained putrescine, diaminopropane, and cadaverine due to the capability of this mutant to synthesize these three polyamines. The norspermidine peak was absent because the gene responsible for norspermidine synthesis has been disrupted. The lack of spermidine was due to the absence of an integral transport component as seen before with the single deletion mutants. Even with the addition of exogenous norspermidine to the media, *nspC::kan* Δ*potB* was unable to import this polyamine further suggesting that PotB is responsible for norspermidine transport (Fig. 35A). When I complemented this mutant strain with a plasmid carrying the *potB* gene, the norspermidine peak was recovered around 30-31 min, as seen in the chromatogram below (Fig. 35B).

Similar results were observed regarding PotC, the *nspC::kan* Δ*potC* double mutant contained the first three polyamines due to this mutant being able to synthesize these. The norspermidine peak was absent due to the lack of the *nspC* gene and the spermidine peak was missing because the gene encoding for one of the transmembrane permeases of the spermidine transport system has been deleted. Even with the addition of exogenous norspermidine to the media, this mutant could not import norspermidine suggesting that PotC is required for norspermidine transport (Fig. 36A). When the polyamine content of the complemented mutant strain was analyzed by HPLC, the norspermidine peak was recovered around 30-31 min (Fig. 36B).

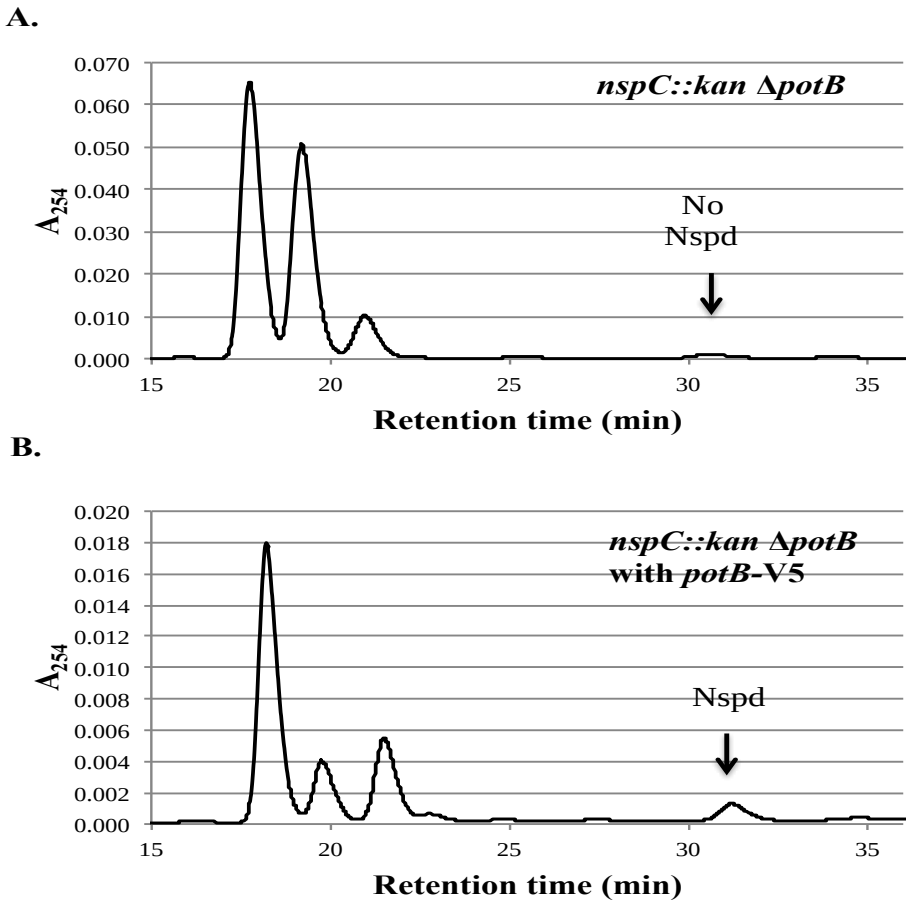


Fig. 35. Confirmation that PotB is responsible for norspermidine import into the cell. Cellular polyamines were extracted from media with exogenous norspermidine added, derivatized by benzoylation, and analyzed by HPLC. For clarity, only data obtained between 15 and 36 min of a 60 min run are plotted. **A.** Chromatogram confirming a lack of norspermidine in the *V. cholerae nspC::kan* Δ*potB* mutant strain. **B.** HPLC chromatogram of the *nspC::kan* Δ*potB* mutant strain complemented with the plasmid carrying the entire *potB* gene. The norspermidine peak is present around 30-31 min, indicating that the PotB functions as part of a spermidine importer.

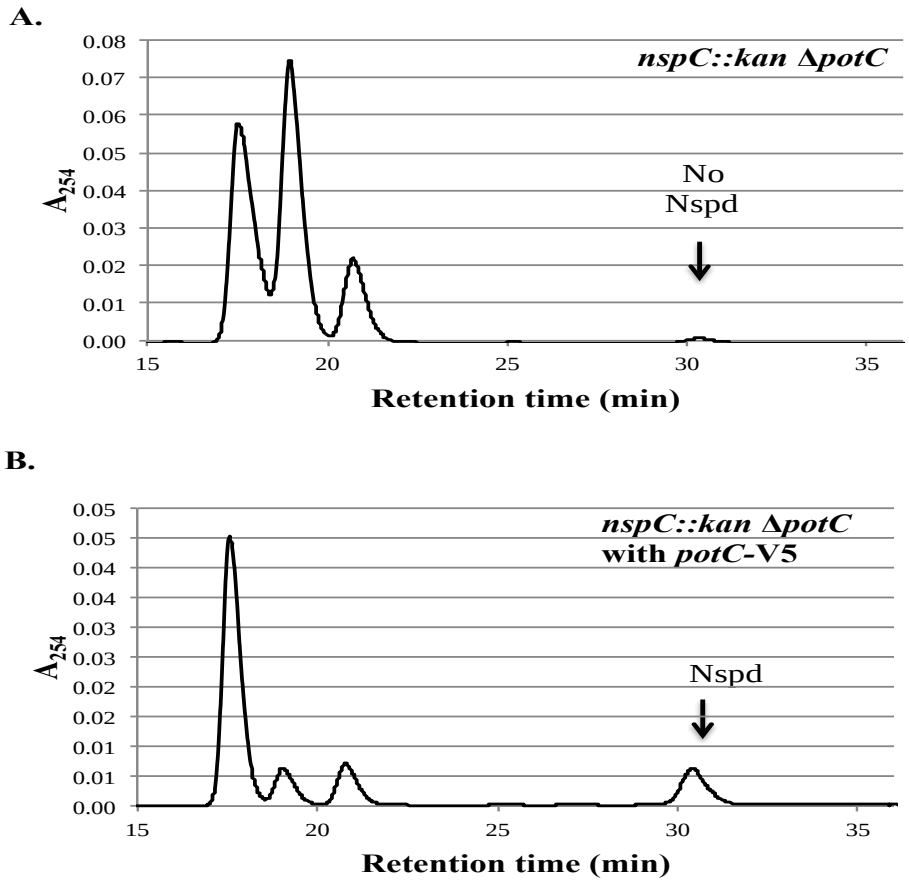


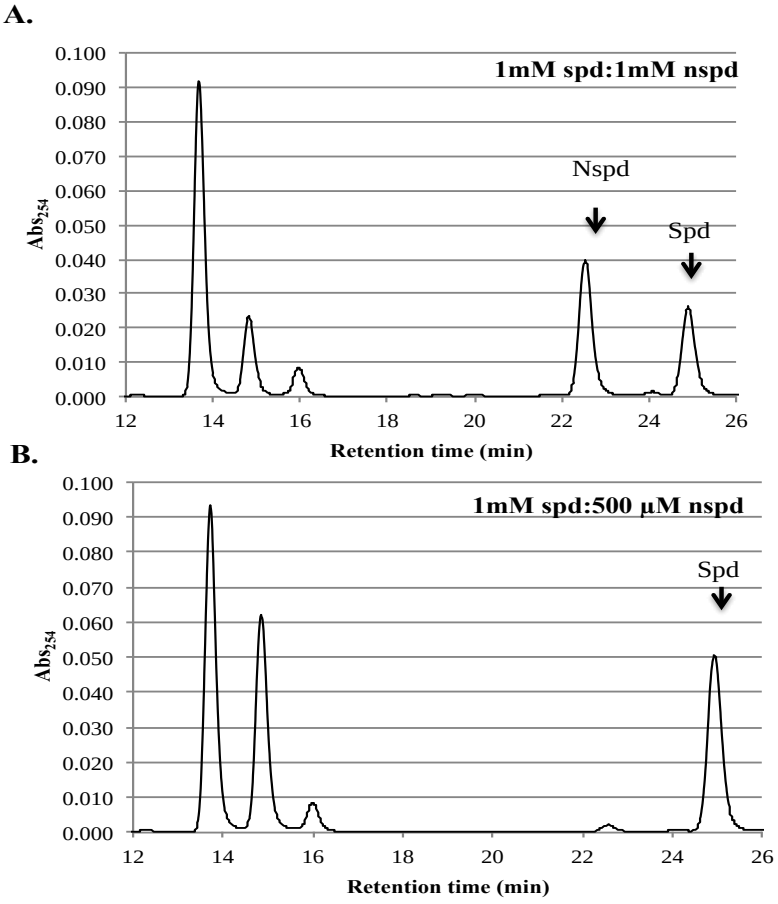
Fig. 36. Confirmation that PotC is responsible for norspermidine import into the cell. Cellular polyamines were extracted from media with exogenous norspermidine added, derivatized by benzoylation, and analyzed by HPLC. For clarity, only data obtained between 15 and 36 min of a 60 min run are plotted. **A.** Chromatogram confirming a lack of norspermidine in the *V. cholerae nspC::kan* Δ*potC* mutant strain. **B.** HPLC chromatogram of the *nspC::kan* Δ*potC* mutant strain complemented with the plasmid carrying the entire *potC* gene. The norspermidine peak is present around 30-31 min, indicating that the PotC functions as part of a spermidine importer.

Ligand preference of the substrate binding protein, PotD1

Due to the capability of PotD1 to facilitate import of both norspermidine and spermidine, the preference of this protein for one ligand over the other was studied. *V. cholerae nspC::kan* cultures were grown with varying amounts of norspermidine and spermidine. Polyamines were extracted, derivatized by benzoylation, and analyzed by

HPLC as described in materials and methods. The addition of 250 μM, 500 μM norspermidine to a *nspC::kan* culture with 1 mM spermidine resulted in a small amount of norspermidine import (Fig. 37B and 37C) indicated by the small peak around 22-23 minutes. The addition of 100 μM did not result in any norspermidine uptake (Fig. 37D). A large amount of norspermidine was imported only upon the addition of equal amounts of spermidine and norspermidine (Fig. 37A).

This trend was not seen with the addition of 100 μM, 250 μM, 500 μM spermidine to a *nspC::kan* culture with 1 mM norspermidine. There was no spermidine peak present upon addition of reduced concentrations of spermidine to 1 mM norspermidine (Fig. 38). Spermidine uptake only occurred upon the addition of equal amounts of spermidine and norspermidine (Fig. 38A).



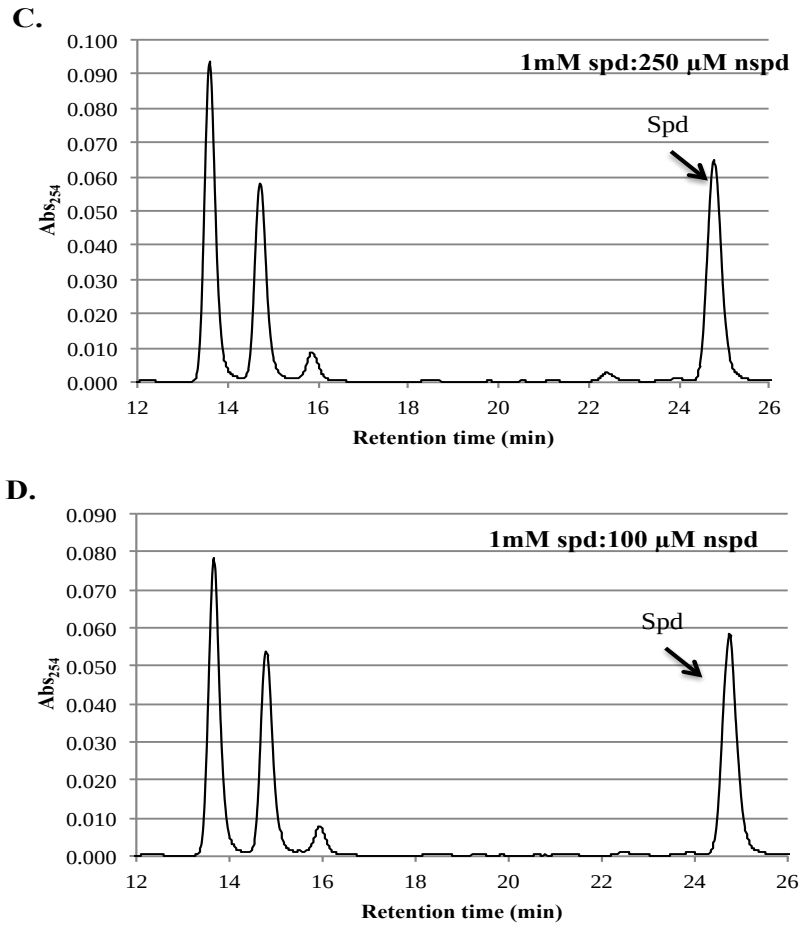
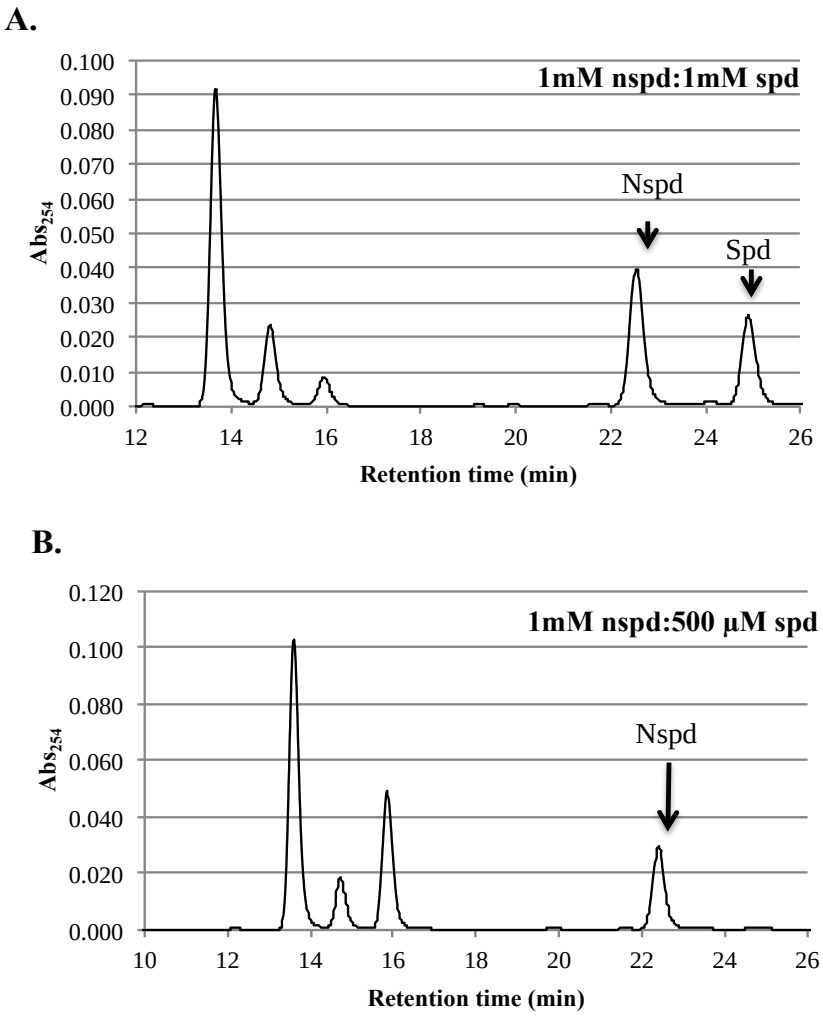


Fig. 37. HPLC *V. cholerae nspC::kan* mutant with varying amounts of norspermidine and 1mM spermidine added to media. A. HPLC chromatogram representative of the *V. cholerae nspC::kan* mutant with 1 mM exogenous spermidine and 1 mM exogenous norspermidine. **B.** HPLC chromatogram representative of the *V. cholerae nspC::kan* mutant with 1 mM exogenous spermidine and 500 µM exogenous norspermidine. **C.** HPLC chromatogram representative of the *V. cholerae nspC::kan* mutant with 1mM exogenous spermidine and 250 µM exogenous norspermidine. **D.** HPLC chromatogram representative of the *V. cholerae nspC::kan* mutant with 1mM exogenous spermidine and 100 µM exogenous norspermidine.

Quantification of cellular polyamines showed that norspermidine and spermidine present at lower amounts was not imported (Fig. 39). However, with the addition of 250 μM and 500 μM there were slight peaks in the chromatograms indicating small amounts of imported norspermidine. When both polyamines are present at the same amount, 1mM concentration, more norspermidine is imported than spermidine (Fig. 39). The results of this competition assay suggest that PotD1 does appear to have a slight preference for norspermidine, under the condition tested.



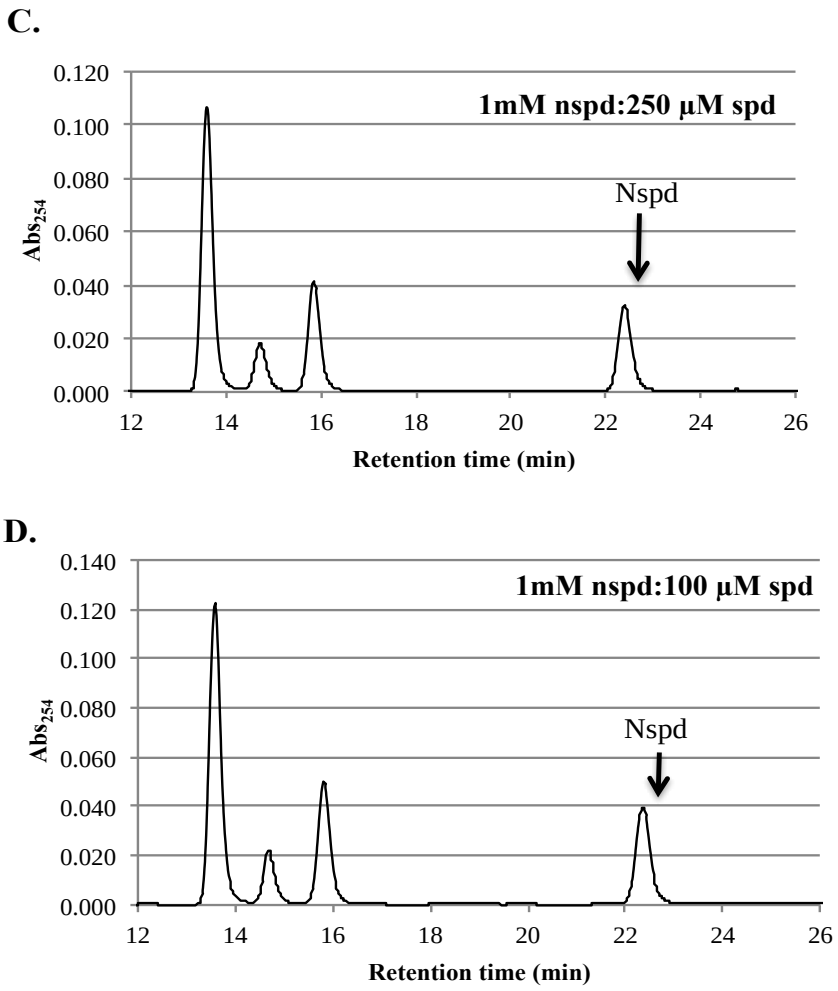


Fig. 38. HPLC *V. cholerae nspC::kan* mutant with varying amounts of spermidine and 1mM norspermidine added to media. A. HPLC chromatogram representative of the *V. cholerae nspC::kan* mutant with 1 mM exogenous spermidine and 1 mM exogenous norspermidine. **B.** HPLC chromatogram representative of the *V. cholerae nspC::kan* mutant with 1 mM exogenous norspermidine and 500 µM exogenous spermidine. **C.** HPLC chromatogram representative of the *V. cholerae nspC::kan* mutant with 1mM exogenous norspermidine and 250 µM exogenous spermidine. **D.** HPLC chromatogram representative of the *V. cholerae nspC::kan* mutant with 1mM exogenous norspermidine and 100 µM exogenous spermidine.

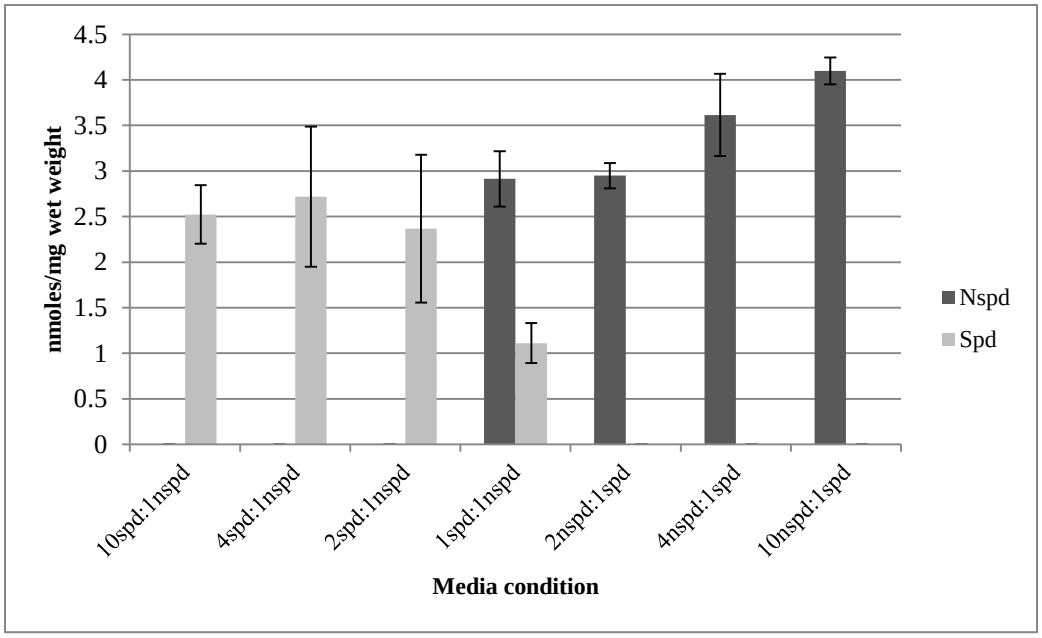


Fig. 39. Analysis of polyamines extracted from *nspC::kan* cells grown in presence of varying concentrations of spermidine (spd) and norspermidine (nspd). *V. cholerae nspC::kan* mutant was grown with varying concentrations of spermidine and norspermidine. The amount of polyamines, in nmoles/mg, was calculated by taking the area of the curve and comparing the results to the polyamine standard. Error bars indicate standard deviation of three biological replicates.

Spermidine import affects biofilm formation

Previous research in our lab has shown that when *potD1*, the gene encoding the substrate binding protein of the ABC transporter, is deleted there is an increase in biofilm formation (Mcginnis *et al.*, 2009). These results suggested that either spermidine imported into the cell decreased biofilm formation or PotD1 had a direct effect on this phenotype. The PotD1 orthologue PotD in *E. coli* has been previously shown to be involved in transcriptional regulation of the operon encoding the spermidine uptake system (Antognoni *et al.*, 1999). Thus, it was not clear from previous experiments in our lab whether PotD1 or the lack of spermidine in the cell was the determinative factor in regulating biofilms in *V. cholerae*. To differentiate between these two possibilities,

biofilm assays were performed as previously described and planktonic and biofilm cell density was measured in the *pot* mutants. Biofilm formation by all the *pot* mutants increased compared to *V. cholerae* wild-type strain (Fig. 36). These results suggest that intracellular spermidine affects biofilm formation. A decrease in the amount of planktonic cells was seen in all the *pot* mutants when compared to the wild-type strain as is typical with strains forming high amounts of biofilm (Fig. 36). This further shows the impact spermidine has on this dynamic change from a planktonic, motile lifestyle to a static or biofilm-associated state.

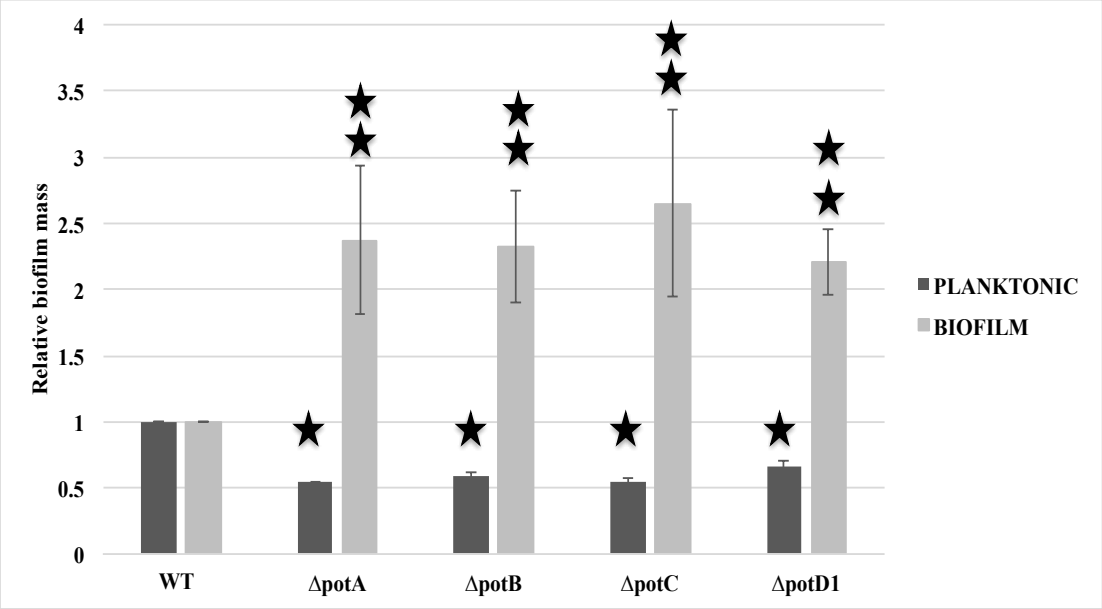


Fig. 40. Biofilm formation of *pot* mutants compared to wild type *V. cholerae*. Biofilm formation of PotA, PotB, PotC, and PotD1 mutants is compared to wild type strain. The biofilms were grown for 24 hours and this data is representative of three biological replicates. Relative biofilm mass was calculated by normalizing optical cell density readings of *pot* mutants to wild type. Error bars indicate standard deviation. A two-tailed t-test was used to determine if the differences seen between planktonic cells of *pot* mutants and wild type were statistically significant, shown with a black star. A two-tailed t-test was used to determine if the differences seen between biofilm associated cells of *pot* mutants and wild type were statistically significant, shown with two black stars.

Discussion

The purpose of this study was to further characterize the role of the components of the ABC-type transporter, PotABCD1, in the uptake of spermidine and norspermidine, while elucidating its effect on biofilm formation in the aquatic bacterium *V. cholerae*. This work establishes PotABCD1 as the first norspermidine transporter reported in any species. Norspermidine and spermidine are produced by both prokaryotes and eukaryotes, with norspermidine being one of the major polyamines in *Vibrio* as well as being found in the aquatic environment and organisms, such as sea urchins, sea cucumbers, aquatic plants, and algae (Hamana *et al.*, 1998; Hamana *et al.*, 1991). Norspermidine is suggested to be important to *V. cholerae* and its physiology due to the ability to synthesize this polyamine *de novo* as well as import it from the environment. Additionally, deletion of the *nspC* gene reduces the growth rate suggesting that norspermidine synthesis is essential for normal growth (Lee *et al.*, 2009). Furthermore, this polyamine makes up the backbone of the self-produced siderophore, vibriobactin, essential for scavenging iron for cellular processes and functions (Keating *et al.*, 2000).

Previous research in the Karatan lab on the periplasmic substrate binding proteins of this system, PotD1 and PotD2, warranted further characterization on the other components of this system. This work demonstrates that the other components, specifically the transmembrane permeases, PotB and PotC, also facilitate the import of spermidine and norspermidine. Following deletion of the *potB* and *potC* genes,

intracellular polyamines were quantified by HPLC. I observed the lack of spermidine uptake due to the absence of the spermidine peak. These results suggest that these proteins function in uptake of this polyamine. The role of these proteins in spermidine import into the cell was further confirmed by complementing the mutant strains with a plasmid carrying the *potB* or *potC* gene. When intracellular polyamines were quantified by HPLC, these complemented strains showed a spermidine peak indicating that spermidine uptake was recovered, further validating the role of PotB and PotC. To assess the import of norspermidine, double mutants were constructed by disrupting the synthesis of norspermidine and functional components of the transport system. When exogenous norspermidine was added to the culture media, the peaks for both norspermidine and spermidine were absent in the polyamine profile of both *nspC::kan* Δ*potB* and *nspC::kan* Δ*potC*. This indicated that PotB and PotC are responsible for the import of norspermidine as well. In order to validate and confirm the uptake of norspermidine, these double mutant strains were complemented with a plasmid carrying the entire *potB* or *potC* gene and cellular polyamines were quantified by HPLC. When exogenous norspermidine was added to the media, a recovery of norspermidine import was observed; however, this complementation did not restore uptake to levels seen in the wild-type strain. Despite this, recovery of the norspermidine peak does suggest that these proteins are involved in norspermidine transport.

An interesting aspect of this transport system is the fact that PotD1 can facilitate uptake of both spermidine and norspermidine. Due to the ability of this substrate binding protein to bind both ligands, the affinity or preference to bind to one over another was elucidated in this work. Using the *nspC::kan* mutant, exogenous spermidine and

norspermidine were added to the media in varying ratios. In analyzing HPLC data, a small amount of imported norspermidine was observed with the addition of 250 μM and 500 μM exogenous norspermidine to the media with 1mM spermidine, suggesting a slight preference for norspermidine even with a larger amount of spermidine present. Furthermore, when cellular polyamines were quantified, a larger amount of norspermidine was imported compared to spermidine when both polyamines were present at equal amounts. Under the conditions tested, PotD1 does not appear to have a large preference for one polyamine over the other. To further understand binding preference of PotD1 and quantify affinity, isothermal titration calorimetry will be utilized to obtain a binding constant for PotD1 to both spermidine and norspermidine.

This work further provides evidence in support of the role of polyamine transport and biofilm formation in *V. cholerae*. Previous work has shown that norspermidine and spermidine are exogenous signals that are suggested to influence the levels of cellular c-di-GMP levels through interaction with the NspS and MbaA signaling pathway (Cockerell *et al.*, 2014; Karatan *et al.*, 2005; Mcginnis *et al.*, 2009). Norspermidine and spermidine are capable of binding to NspS, which then influences MbaA and its enzymatic activity altering c-di-GMP levels in the cell. Norspermidine increases NspS influence on MbaA, increasing c-di-GMP, which increases biofilm formation. Whereas, spermidine acts inversely, thus hindering the inhibitory role of NspS on MbaA, which results in c-di-GMP hydrolysis, and subsequently a decrease in biofilm formation. The effect of extracellular polyamines on biofilm formation has been studied, but the mechanisms by which intracellular polyamines influence biofilm formation in *V. cholerae* is still unknown.

Previous work with the substrate binding protein of the PotABCD transporter in *E. coli*, found that PotD could act as a transcriptional regulator. So it was not clear in previous research in our lab if PotD1 was responsible for this biofilm phenotype or internal spermidine was influencing biofilm. One of the goals of this research was to establish that effects on biofilm formation are due to transport of extracellular spermidine into the cell rather than in response to direct regulation by transport system components. This study of the other components of the transport system confirms that spermidine inhibits biofilm formation in *V. cholerae* not only as an extracellular effector, but also intracellularly through an unknown mechanism following import by the ABC-type transporter, PotABCD1.

Due to their cationic nature, polyamines have been implicated in a number of cellular processes. Polyamines within the cell are predominately found in complexes with RNA and work together with cations like Mg^{2+} to stabilize higher orders of structure (Shah & Swiatlo, 2008). Because of these interactions, polyamines can influence and affect protein production on a number of levels. In *Pseudomonas aeruginosa*, a Gram-negative, opportunistic human pathogen, exogenous polyamines, specifically putrescine, have been shown to increase transcription of several loci including the *spuABCDEFGH* operon involved in polyamine uptake and utilization (Lu *et al.*, 2002). Additionally, putrescine was shown to stimulate synthesis of key response regulators in *E. coli* that mediate biofilm formation as well as affect enzymes involved in production of the biofilm polysaccharide of *Y. pestis*. Based on this knowledge, spermidine imported into the cell could possibly be stimulating production of key proteins that repress biofilm formation by numerous mechanisms. To further study and elucidate this mechanism of

intracellular spermidine, transcriptomic approaches such as RNA Sequencing could be used to identify the genes that are differentially expressed when spermidine is imported, as well as when transport is abolished. By comparing these two different conditions, possible factors that are influenced by imported spermidine may be identified. We could then identify a link between those factors and biofilm formation. If spermidine is not affecting transcription, then proteomics could be utilized in order to observe and identify the major protein products that are differentially regulated under the same conditions. This could allow us to detect and study the networks and pathways within the cell that are possibly regulating biofilm formation when spermidine is imported.

The role of the ATPase component of this system, as well as confirmation of co-transcription of the putative *pot* operon is still ongoing. In most bacteria, genes found in an operon usually function in the same pathway. In characterizing this putative ABC transporter, confirming that these genes are in an operon will further validate the role these proteins play in the transport of norspermidine and spermidine. Genes in an operon are co-transcribed in a polycistronic RNA. In order to detect that the *pot* genes are in an operon, RNA will be extracted from *V. cholerae*, cDNA will be made, and then used in a PCR with gene-specific primers to amplify the intergenic space between each gene. Successful amplification of the intergenic region will confirm that a polycistronic RNA is being produced.

ABC transporters have a profound impact on bacterial physiology; therefore a better characterization of these systems is crucial. This research further provides evidence to support the relevance of this novel norspermidine and spermidine transporter to *V. cholerae* physiology, especially during two critical stages of its life cycle that are

influenced by biofilm formation; these stages being colonizing the human host and dissemination and survival in the aquatic environment. It is suggested that the biofilm aids in passage through the gastric acid barrier of the stomach, however once reaching the small intestines *V. cholerae* must disperse from the biofilm to be able to colonize the small intestine and secrete cholera toxin causing disease. It has been reported that the typical human diet contributes large amounts of polyamines per day to the lumen (Bardocz, 1993); despite these large amounts of natural polyamines like spermidine, putrescine, and spermine arriving to the gut, research has shown that these polyamines are present at micromolar concentrations (Seidel & Scemama, 1997). This disappearance of polyamines from the intestinal lumen may likely be due either to rapid absorption, utilization *in situ*, or rapid degradation in the gut (Milovic, 2001). Based on this knowledge, the PotABCD1 transporter may possibly play a role in uptake of polyamines in the environment regulating the switch between planktonic and biofilm-associated states. This work further provides evidence that spermidine is a deterrent of biofilm formation, so once *V. cholerae* passes through the gastric acid barrier of the stomach in a biofilm and reaches the small intestines, where spermidine is found at micromolar concentrations, the transporter most likely initiates import of this polyamine to inhibit biofilm formation. Subsequently, *V. cholerae* will disperse from the biofilm and colonize the small intestine.

The other stage of life in which the ABC-type transporter may play a significant role is the dissemination of *V. cholerae* back into its natural aquatic environment after infection. After colonization of the small intestine and secretion of cholera toxin, *V. cholerae* cells are shed back into the environment by the host in secretory diarrhea. *V.*

cholerae will most likely need to scavenge nutrients and micronutrients from its natural aquatic environment (Kamp *et al.*, 2013). Research has determined through transposon sequencing the influence of non-essential genes on fitness of *V. cholerae* during survival in the aquatic environment. The genes encoding the ABC-type transporter were among the genes identified through this screen. Furthermore, disruption of *potA*, *potC*, and *potD* resulted in fitness defects in an aquatic environment (Kamp *et al.*, 2013). Based on this knowledge, as well as this characterization of the novel ABC-type transporter, PotABCD1, polyamine uptake is crucial for survival in the aquatic environment, where norspermidine is found generally. Due to the ability to uptake both norspermidine and spermidine, this transporter is capable of mediating biofilm formation not only in the natural environment, but quite possibly in the host environment, further establishing novel ABC-type transporters, like PotABCD1 as essential components to bacterial physiology.

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