A TALE OF TWO PATHWAYS:
SECRETIN ASSEMBLY IN *VIBRIO CHOLERAE*

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ABSTRACT

The Type 2 Secretion System (T2SS) is responsible for the transport of toxins and enzymes across the outer membrane of many Gram-negative bacteria. A crucial component of the T2SS is a large pore, composed of a multimer of EpsD, named the secretin. This pore inserts in the outer membrane with the assistance of a pilotin (EpsS) or assembly factors (EpsAB), both of which are present within the genome of *Vibrio cholerae*. The goal of this study was to determine whether or not both assembly mechanisms operate on the same secretin assembly in *V. cholerae*.

Protease deficient mutants generated from an insertion transposon library in *V. cholerae epsAB* were analyzed. The transposon was found to disrupt the operon encoding VC1702 and *epsS*.

Mutant strains of *V. cholerae* were constructed or obtained that are deficient in *epsA, epsB, epsC,* and *epsS.* Double mutants were constructed that were deficient in *epsA* and *epsS* or *epsB* and *epsS*. These mutants were tested for assembly of the secretin and secretion of lipase, protease, and cholera toxin. The *epsA* and *epsB* mutants have slightly reduced levels of secretion and secretin assembly, while the levels in the pilotin mutant are drastically reduced. The double mutants had little to no assembly, and secretion was reduced to the levels of the control mutant *epsC.*

In an attempt to restore function *epsAB* was over-expressed in all strains. It successfully complemented the *epsA* and *epsB* mutants, and restored levels of secretion to *epsS* levels in the double mutant, *epsAS.*

In a similar manner to *epsAB* complementation, *epsS* was over-expressed. It was found to require the preceding gene VC1702 to complement. The operon, encoding both *epsS* and VC1702, could complement both *epsA* and *epsS* mutations and over-expression increased secretin assembly and secretion to levels greater than wild-type levels.

Lastly, a phylogenomic analysis demonstrates that the EpsAB protein complex is found in most orders of the gamma proteobacteria and is ancestral. The pilotins appear to be a late acquisition as they are only found in the family Enterobacteriales.

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I am thankful for my committee members Dr. Goldie and Dr. Bull. I lacked knowledge about bacterial physiology when starting this degree and Dr. Goldie taught me the essentials. He also ensured my enzyme assays were done properly. Dr. Bull was a great help in that he knows an incredible amount of bacterial genetics and always asked the most pertinent questions. Outside of my committee Dr. Gribaldo proved to be as essential and I could not have performed the bioinformatic work without her. Dr. Ireland proved to support through his typical British comments about science.

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LIST OF ABBREVIATIONS

Abbreviation	Description
AA	Amino acid
ATP	Adenosine triphosphate
Amp	Ampicillin
BHI	Brain Heart Infusion
bp	Base pairs
ETEC	Enterotoxigenic Escherichia coli
PCR	Polymerase chain reaction
CT	Cholera Toxin
Cat	Chloramphenicol
D	Dalton
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
Eps	Extracellular protein secretion
Gsp	General secretion pathway
HMM	Hidden Markov Model
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase pairs
Kan	Kanamycin
LB	Luria-Bertani
LPS	Lipopolysaccharide
Lol	Localization of Lipoproteins
LT	Heat-labile enterotoxin
ORF	Open Reading Frame

OMV	Outer Membrane Vesicle
PCR	Polymerase chain reaction
pNP	p-nitrophenol
pNPL	L-Leucine-4-nitroanalide
pNPO	4-nitrophenyl octanoate
PVDF	Polyvinylidene fluoride
RBS	Ribosomal Binding Site
Rif	Rifampicin
RS	Restriction Site
SD	Shine-Dalgarno
Sm	Streptomyocin
SEM	Standard error of the mean
T2SS	Type 2 Secretion System
T3SS	Type 3 Secretion System
T4P	Type 4 pili
Tn	Transposon
UTI	Urinary Tract Infections
WHO	World Health Organization

TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

1.0 HYPOTHESIS

Assembly pathways involve complex interactions, redundancy, and essential components. In order to better understand outer-membrane assembly pathways one must therefore understand the non-canonical pathways. Studying the assembly pathways of proteins will give us a better understanding of how all proteins are inserted in the outer membrane, specifically that of the type 2 secretin. Assembly factors and pilotins assist in assembling the secretin. Determining if these are distinct or redundant pathways will enhance the current theory of secretin assembly.

2.0 OBJECTIVES

1) Construct or obtain mutant strains of *epsA, epsB, epsC, epsS,* and double mutants deficient in both *epsA* and *epsS* or *epsB* and *epsS*.

2) Test these strains for T2SS secretion and secretin assembly

3) Build constructs for expressing each of these genes

4) Test constructs in each strain for the complementation of secretion and secretin assembly

5) Test transposon library for insertion within *epsS*

6) Characterize the genes using bioinformatics

3.0 INTRODUCTION

3.1 Pathogenic Gamma proteobacteria

Disease has been a constant burden to mankind since before the beginning of history. It has killed countless, maimed many more, and slowed the progress of human development. Some of the best known human diseases, such as the bubonic plague, are caused by bacteria of the Gammaproteobacteria.

The Gammaproteobacteria is one of the richest clades of all bacterial phyla. It is one of the best studied clades as it includes members of the human microbiota, such as the archetypal *Escherichia coli*, and the pathogens *Yersinia, Vibrio, Pseudomonas,* and *Salmonella*. This clade is diverse in metabolism, temperature adaptation, and morphology. It is of great interest to human health (Garrity *et. al.,* 2005).

3.1.1 *Klebsiella oxytoca*

An opportunistic pathogen belonging to the Gammaproteobacteria is *Klebsiella oxytoca*. *K. oxytoca* is a diazotroph and has been found to associate with the root nodules during salinity stress (Wu *et. al.,* 2014). It is a pathogen responsible for many hospital acquired infections, including urinary tract infections (UTI), pneumonia, and septicemia (Janda *et. al.,* 2006).

3.1.2 Enterotoxigenic *Escherichia coli* (ETEC)

Since its discovery in 1885 by Theodor Escherich (Shulman *et. al.,* 2007) *Escherichia coli* has been a model organism. This bacteria is of keen interest to scientists as it is part of the human microbiota. The organism is a Gram-negative, facultatively anaerobic, rod shaped bacterium. Enterotoxigenic *E. coli* (ETEC), a pathogenic form of *E. coli*, is the leading cause of traveler's diarrhea, and is one of the most prevalent agents of diarrhea in the developing world. The organism's pathogenesis is similar to that of cholera as it produces enterotoxins and colonization factors (Garrity *et. al.,* 2005).

3.1.3 *Vibrio cholerae*

Cholera is one of the most feared diseases on the planet and has been responsible for hundreds of thousands of illnesses since the 1800's. The causative bacteria, *V. cholerae,* was spread worldwide by the bilge water of British ships and is now found on every continent (Rosenberg 1987). The disease has been on the rise since 2007 after a large outbreak in Haiti

began the dissemination of the bacteria to new populations. In 2012 over 245,393 cases were reported including 3034 deaths (WHO, 2013).

This organism circulates between two drastically different niches; reservoirs of water, and the alimentary tract of the human body. When the organism is present within its aquatic reservoir it associates with chitin particles, zooplankton, and Chironomidae egg masses (Cottingham *et. al.,* 2003). This is facilitated by several Type 2 Secretion System (T2SS) substrates, including the chitin binding protein, GbpA (Stauder *et. al.,* 2012). Infection occurs when the host drinks water contaminated with *V. cholera.* Following infection the bacterium colonizes the small intestine and starts to secrete pathogenicity factors.

V. cholerae has multiple pathogenicity factors that play a role in establishing an infection. Many of the factors aid in colonization and allow the bacteria to colonize and thrive in the intestinal epithelium. These factors include charged lipopolysaccarides, type 4 pili, and many others (Garrity *et. al.,* 2005). By far the most important factor for disease (and with relevance to this study) is the cholera toxin (CT). The cholera toxin (Figure 1) is an AB5 toxin which forms a five membered ring from the B subunits. It has a single A subunit which activates adenylate cyclase (Zhang *et. al.,* 1995). This activation results in watery diarrhea by inducing the secretion of water and ions into the intestinal lumen. This is similar to the action of the enterotoxin produced by ETEC, another AB5 toxin. Both cholera toxin and enterotoxin are secreted by the T2SS (Murthy *et. al.,* 2013).

Following infection, *V. cholerae* is shed back into the environment through watery stools, and continues its deadly cycle.

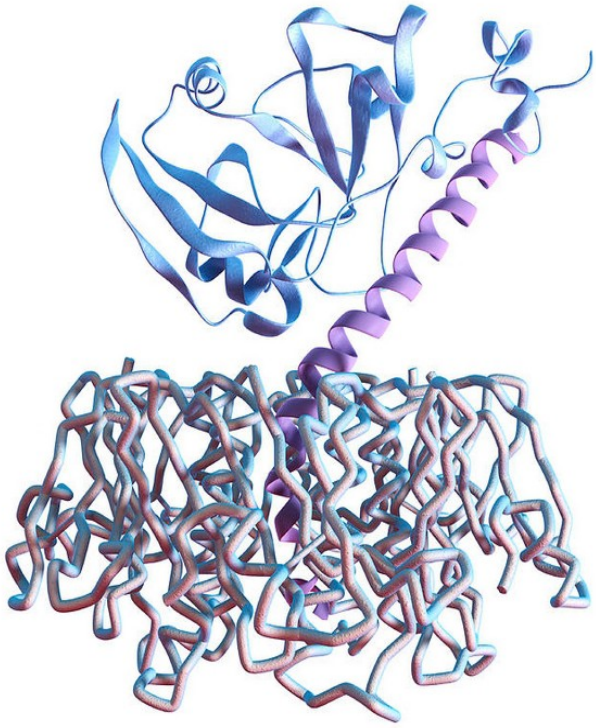

Figure 1. Cholerae Toxin. This figure shows the assembled toxin with the 28kDa A subunit (ribbon, top) and the five 11 kDa B subunits, (string, bottom) PDB ID:1XTC (Zhang *et. al.,* 1995)

3.2 Secretion Systems

Secretion systems are essential for all forms of life. These mechanisms are how organisms can interact with the environment or other cells. This effect can be for cell localization in multi-cellular organisms or the secretion of toxins as in bacteria. Gram-negative bacteria have a unique difficulty in that they must export proteins through two membranes as opposed to one. This difficulty is increased by the fact that the second membrane is almost devoid of energy, in the form of ATP, and lacks a proton gradient (Sutcliffe 2010).

3.2.1 Sec Secretion Pathway

The most well studied and ubiquitous secretion pathway is the Sec pathway. This system of exporting proteins through a single membrane is utilized by all forms of life and was likely present in the last common ancestor. In bacteria, it is responsible for the secretion of most extracellular proteins. These proteins have diverse functions in structure, communication, metabolism, and pathogenicity. For a recent review please read Denks *et. al.,* 2014.

Proteins are targeted to the Sec translocase by two different mechanisms: co-translational and post translational targeting. Co-translational targeting involves a signal recognition particle binding to the signal sequence, a hydrophobic region, and targets the entire ribosomal complex to the Sec-translocase. As the name suggests, in post translational targeting the protein is released from the ribosome and then targeted to the translocase by binding of SecB.

The translocase is composed of four components: a cytoplasmic ATPase SecA, a protein channel SecY and SecE, and a translocation stimulator SecG. Proteins are shuttled to the channel by either SecB or the signal recognition particle, at which point they are threaded through the channel utilizing ATP hydrolysis as an energy source. After the protein is exported through the channel the signal sequence is cleaved and the protein is released (Natale *et. al.,* 2008) .

3.2.2 TAT Secretion Pathway

The twin-arginine translocation (TAT) system is found in the cytoplasmic membranes of bacteria, archaea, and plant plastids. The TAT system is functionally different from the Sec system in that it utilizes proton motive force as energy and only translocates folded proteins. As it is capable of transporting folded proteins, this enables the cell to post-translationally modify the protein and add cofactors using cytoplasmic enzymes. (Natale *et. al.,* 2008)

Surprisingly the TAT translocase is much simpler than that of Sec and only contains two essential components: TatA and TatC which form the pore which the proteins are translocated

through. The Gammaproteobacteria have a third component TatB which is believed to aid in translocation. The signal sequence is similar to that of Sec except it usually contains two large charged amino acids (Twin Arginine) and is less hydrophobic than that of Sec.

3.2.3 Outer Membrane Secretion Systems

As stated earlier the Gram-negative bacteria have a difficult problem to surmount in secreting past two membranes. Life is capable of adapting to almost any problem or condition and so far seven distinct mechanisms have been discovered. These mechanisms are different and each serves a unique function.

3.2.3.1 Type I Secretion System

The Type I secretion system, commonly named ABC transporters are used to directly transport proteins to the extracellular space without the use of Sec or TAT. This system contains three major components. ATP-binding cassette, outer-membrane factors, and membrane fusion proteins. These three factors combine to form a transperiplasmic gated channel which forces peptides to the extracellular space through the hydrolysis of ATP. These systems have a specific signal sequence (GGXGXDXXX), located at the amino terminus and this is bound by HasA and targeted to the translocase complex. SecB has been shown to be involved in some cases of export.

The most studied type I secretion system is the TolC-HlyD-HlyB complex of E. coli. TolC is an integral membrane protein on the outer membrane while HlyD and HlyB (ABC) occupy the periplasmic space and inner membrane, respectively. The substrate in this system is a hemolytic toxin called HlyA (Delepelaire *et. al.,* 2004).

3.2.3.2 Type III Secretion System

The type III secretion system (T3SS) is a large multi-protein complex, composed of 30 different proteins, that is utilized to directly inject plant and animal hosts with effector cells. It has been classically studied in *Salmonella* as a pathogenicity factor, but is also used in symbiotic relationships. The machinery of the T3SS, also called the injectisome, contains a large complex which spans both membranes. Several components are wholly within the cytoplasm, including an ATPase which drives the system. A channel is formed at the inner-membrane and extends through the outer-membrane by another set of proteins. This channel does not lead into the extracellular space but to a thin hollow filament, termed the needle. The tip of this needle interacts with host cells to allow direct passage of the effector molecules. This system is of

interest to this study as the secretin is utilized as the pore for passage of the needle through the outer membrane. The T3SS has its own unique signal sequence, but unlike the other secretion systems, this signal is not cleaved (Coburn *et. al.,* 2007).

3.2.3.3 Type IV Secretion System

The previous secretion systems focused on the secretion of protein substrates, the type IV system, on the other hand, predominantly secretes DNA and RNA substrates. Unsurprisingly, this system is homologous to that of the conjugation system. This system is the least studied of all the secretion systems, with the possible exception of the type VII secretion system. This system is composed of 12 proteins and spans both membranes. Substrates do not requre translocation by Sec for this system, but the system does require Sec for assembly. The complex itself appears similar to the T3SS as it contains a cytoplasmic ATPase and has a filament which extends past the outer-membrane to directly interact with both eukaryotic and prokaryotic cells (Fronzes *et. al.,* 2013).

3.2.3.4 Type V Secretion System

The most simple transport systems are the autotransporters, also called the type V secretion system. This secretion system does not rely on many other proteins as the substrates form a pore to transport themselves through the outer membrane. Autotransporters have several different features depending on the type, but all are initially transported through the inner-membrane *via* the sec pathway. Once these proteins are through the inner-membrane they transport themselves through the outer membrane without the use of any secondary machinery. Then the proteins associate with the outer membrane and form a pore, utilizing the BAM complex, which the remainder of the protein is translocated through. Finally the translocated domain either remains attached to the outer membrane or is cleaved and truly secreted. (Leo *et. al.,* 2012)

3.2.3.5 Type VI Secretion System

The Type VI secretion system of Gram-negative bacteria is an organelle that is structurally similar to a phage tail. This structure is used for cell to cell combat and can inject both antibacterial and antieukaryotic effectors. These effectors are loaded into the sheath of the complex in the cytoplasm and passed into the spike. The complex then induces a rapid conformational change that propels a spike into the host cell, wherein a cap on the spike hinges open and effectors are released. (Ho *et. al.,* 2014)

3.2.3.6 Type VII Secretion System

The most recently discovered secretion system of Gram-negative bacteria is the Type VII secretion system. As this system has not had the intense study the other systems have received, little is known about it. What is known is that it is a necessary component of *Mycobacteria tuberculosis* for pathogenicity. Five of these secretion systems are found within this bacteria, but unfortunately only preliminary studies on both the mechanism and the structure have been performed. These results suggest that both the mechanism and the structure are similar to that of the type I secretion system (Houben *et. al.,* 2014).

3.3 Type II Secretion System

The T2SS (Figure 2) is one of the major pathways utilized by Gram-negative bacteria (including *V. cholera, A. hydrophila,* enterotoxigenic *E. coli*, and *K. oxytoca*) in transport of folded enzymes and toxins from the periplasm to the extracellular space. The T2SS complex is composed of 12-16 proteins which span both membranes. The proteins exported by this pathway are fully folded before leaving the cell and include toxins such as: cholera toxin and LT, and hydrolytic enzymes such as: lipases and proteases. For a recent review see Zuckert 2014. This system is homologous to the type 4 pili (T4P) of many diderm (double membraned) bacteria and contains many of the same components.

3.3.1 Genetic Organization

The T2SS functions as the terminal exporter of the General Secretory Pathway (Gsp). The system takes substrates which have been transported from the cytoplasm via the Sec or TAT pathways and exports them past the outer membrane. The genetic nomenclature of the system usually follows a convention of an alphabetical naming system (Figure 2) with a three letter species specific prefix; *e.g.* EpsC naming the C protein of *V. cholerae*. Non-specific nomenclature or reference to the *E. coli* systems use the prefix Gsp. The *Pseudomonas* systems follow a naming convention other than the alphabetical system.

The genetic organization of the T2SS (Figure 2) shows that the key components of the system, *gspC-N,* are usually expressed from a single operon. The assembly factors, *gspA* and *gspB*, and the pilotins *gspS*, are each expressed on distinct operons (Strozen *et. al.,* 2012).

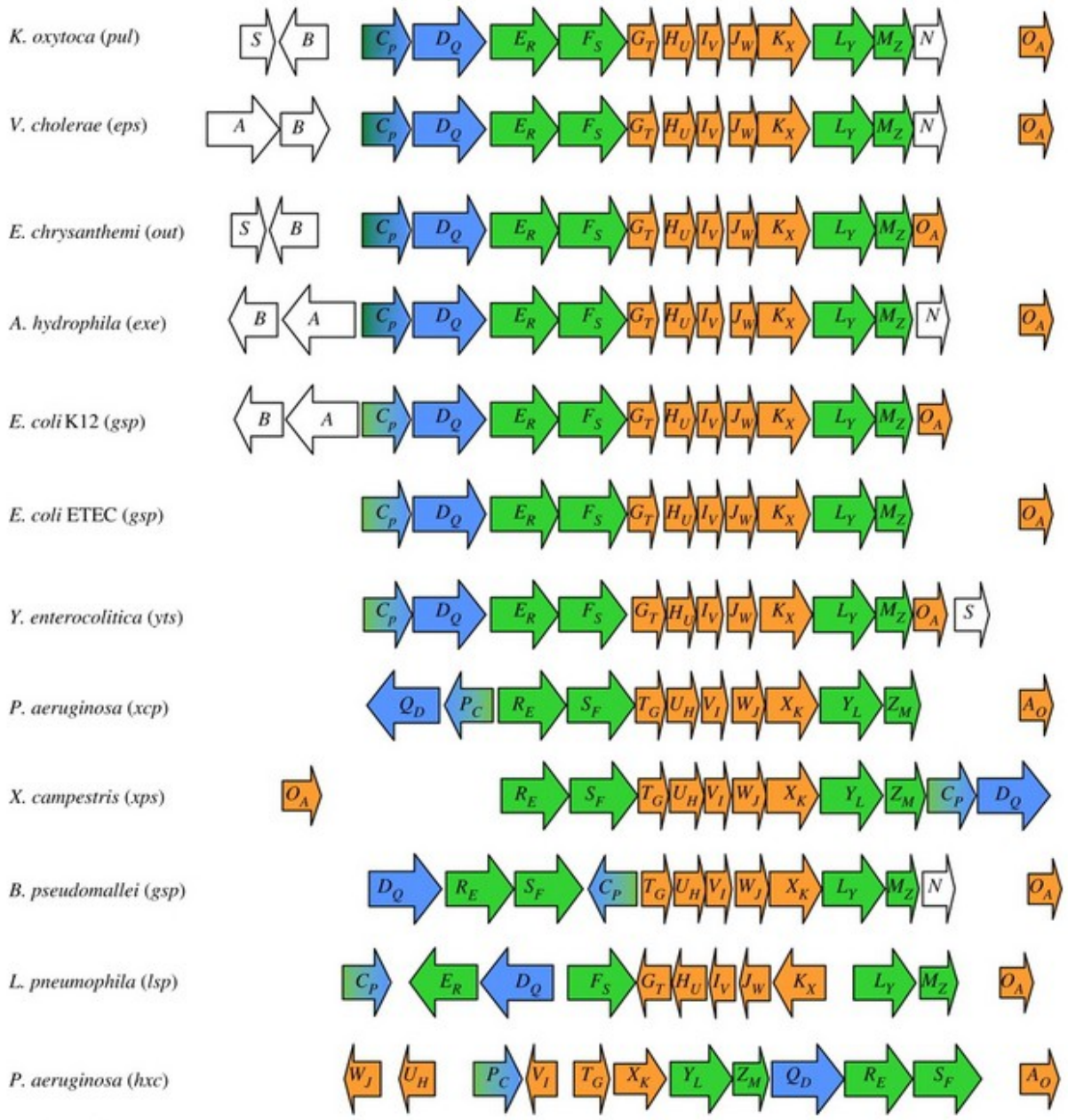

Figure 2. Genetic organization and nomenclature of the T2SS. Organisms are listed with the acronym for each T2SS. Shading represents homologous groups. (Douzi *et. al.,* 2012)

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3.3.2 Structure of the T2SS

The genes of the T2SS encode 12-16 proteins which form a multi-protein complex containing 40-70 proteins that span both membranes. This complex consists of four parts: an outer membrane pore (the secretin), an inner membrane platform, a cytoplasmic ATPase, and a pseudopilus. Transport by this complex is currently believed to be achieved by loading a pseudopilus with substrate, after which ATP driven extension of this pseudopilus pushes the protein through the secretin (Figure 3).

The pseudopilus is composed of major and minor subunits. The major pilin subunit is GspG, and it has been shown to be the predominant protein within the fibers of the pseudopilus (Sauvonnet *et. al.,* 2000). Most experiments have not detected the minor pilin subunits GspI, J, and K. Another minor pilin subunit, GspH, has been detected in the fibre of *Xanthamonas campestris* (Hu *et. al.,* 2002).

The complex also has an inner membrane platform, that serves as the base of the pseudopilus. The inner membrane platform is composed of the proteins GspC, F, L and M. These proteins form a platform on the inner-membrane, upon which is the base of the pseudopilus. An essential component of the system is GspC. Mutants of this protein are used in this study as controls. GspC has been shown to bind to the N0 domain of the secretin and may be the sole factor involved for linking the inner and outer membrane regions (Korotkov *et. al.*, 2011; Lee *et. al.,* 2004). Other studies looking at GspC suggest this protein may be involved in determining substrate specificity (Bouley *et. al*., 2001).

Although many of the proteins that are included within the inner membrane platform have cytoplasmic components, only one protein is entirely within the cytoplasm. This protein is the ATPase, GspE. GspE is a classical ATPase containing a Walker A and B motif (Patrick *et. al.,* 2011), and has been suggested to be the energy source for the entire system. The mechanism of this energy transfer has been suggested to be conformational changes that are first transferred through a conserved interface within GspL and transfered to GspG, the major pilin subunit (Sandkvist *et. al.,* 1995; Patrick *et. al.,* 2011). This is not the only source of energy as it has been shown that some species require the proton motive force (Letellier *et. al.,* 1997).

The secretin will be discussed in depth in section 3.4.

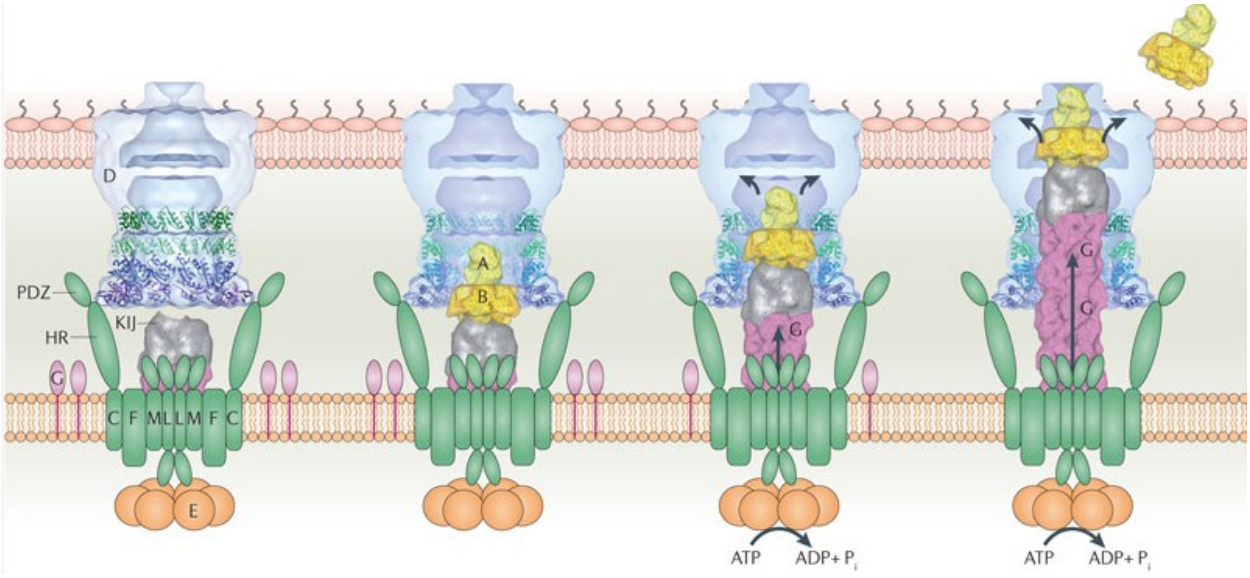

Figure 3. Type 2 Secretion System. This figure shows the key components of the system and demonstrates the hypothesized mechanism of secretion Each component of the system is labeled with its single letter code (GspE, located at the bottom is E) PDZ and HR are domains of GspC. The outer-membrane is positioned at the top of the figure (light blued), the inner-membrane is located at the bottom(Green), the Atpase GspE is coloured orange, and the pseudopilus is both purple and grey.(Kortokov *et. al.,* 2012) Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology (10, 336-351), copyright 2012

3.4 T2SS of *Vibrio cholerae*

The genetic structure of the T2SS of *V. cholerae* is not contained on a single operon; it has a functional operon containing *epsC-N*, a second operon containing epsAB, and a third operon containing *epsS* and VC1702, an uncharacterized protein. To test if this is a unique feature a Hidden Markinov Model (HMM) search was conducted. Similar to Blast this technique looks for homologs of a protein within a database. No genomes were found outside of the *Vibrio's* which contained both *epsAB* and *epsS* (Data not shown).

Extensive studies have been conducted to better understand the *Vibrio* species T2SS components. These studies have included electron microscopy of the secretin (Reichow *et. al.,* 2011), characterization of the interactions of EpsC (Hwang *et. al.,* 2011), EpsL (Abendroth *et. al.,* 2009), EpsF (Abendroth *et. al.,* 2004), EpsI and J (Yanez *et. al.,* 2008), and EpsH (Yanez *et. al.,* 2007). Aside from the structural studies, a key study was conducted which determined the secretome, all proteins exported by the T2SS, for *V. cholerae* (Sikora *et. al.,* 2011). This study found that the secretome contained cholera toxin, hemagglutinin protease, chitinase, GbpA, lipase, serine protease, chitin binding proteins, cytolysin, and several uncharacterized proteins. Unsurprisingly *V. cholerae* is also capable of secreting the ETEC heat-labile enterotoxin B-subunit (Sandkvist 1997), which is closely related to the B-subunit of cholera toxin.

3.5 Secretin (GspD)

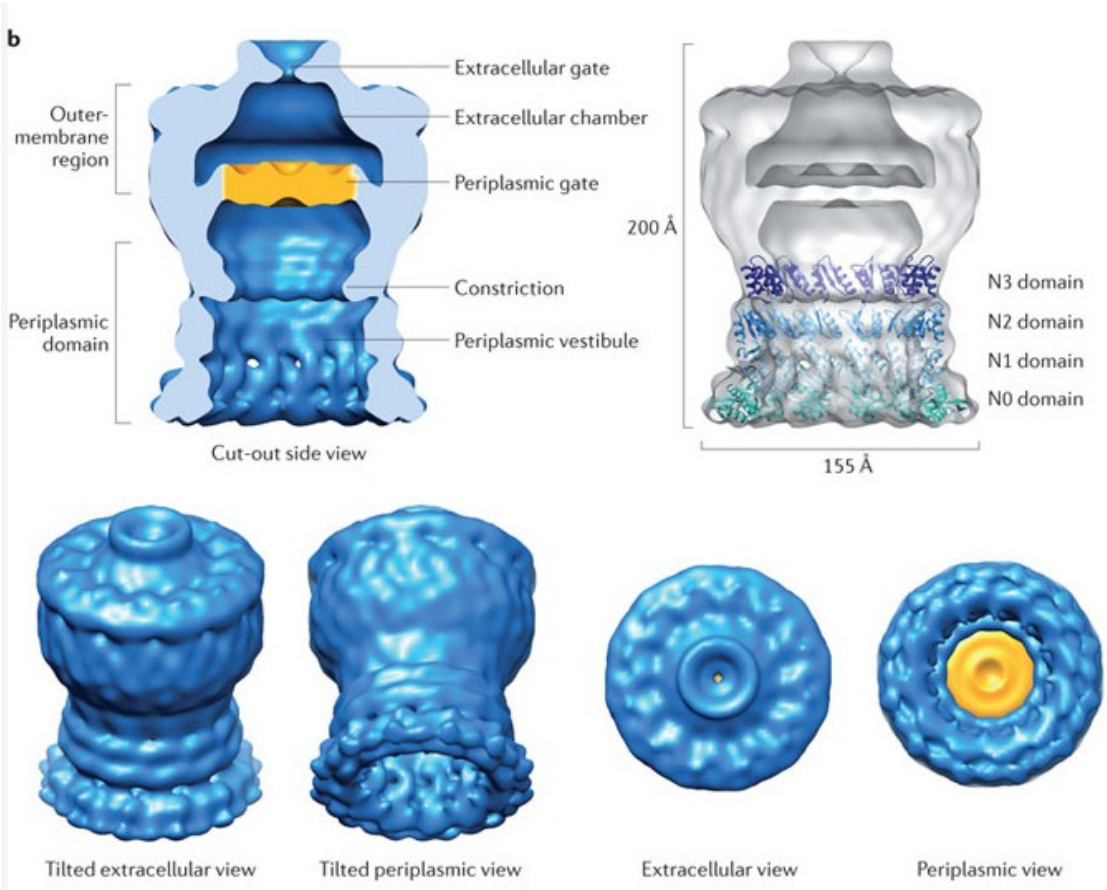

Figure 4. Structure of GspD (Kortokov *et. al.,* 2012)**.**
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To keep equilibrium within a cell, transport must be precisely controlled. Most channels in bacterial outer-membranes are small trimeric β barrels inserted via the BAM complex (Selkrig *et. al.,* 2014), however these are not used for protein export. There are limitations to the size of substrates which can be exported through these small complexes and as such bacteria have developed a larger pore, the secretin, which allows the passage of large substrates (Collin *et. al.,* 2007).

The secretin is found in all diderm (double membraned) bacteria and used to facilitate trafficking of large proteins. This pore is common to several systems including

the T2SS, Type Three Secretion Systems (T3SS), Type 4 pili (T4P), the DNA uptake systems, and the filamentous bacteriophage extrusion system (Burkhardt *et. al.*, 2011; Collins *et. al.*, 2003; Jain *et. al.,* 2011; Korotkov *et. al.,* 2011; Opalka *et. al.,* 2003; Schraidt and Marlovitz, 2011). In T3SS the pore facilitates the passage of the needle complex through the outer membrane to allow transfer of effector molecules to a target cell. The T4P are homologous to the T2SS in general and share a similar structure. The difference is that the pilus in the T4P extends through the outer membrane to interact with surfaces. The pore the pilus extends through in T4P is homologous to the T2SS secretin.

The secretin monomer, GspD, consists of three domains: an N-domain that interacts with the inner membrane proteins in the T2SS, the C-domain which is responsible for multimerization of the D protein, and the S domain at the C-terminus responsible for interactions with pilotins, which will be discussed in section 3.6.6. Some components have been crystallized individually, however the nature of the transmembrane region has yet to be determined.

The N-domain can be further divided into four domains: N0-N3. These regions are not present within all types of secretin, for example the T3SS secretin lacks the N1 domain, while most T4P lack both the N1 and N2 domains (Figure 5) (Kortokov *et. al.,* 2011). The N0 domain has been shown to be essential for secretion in ETEC and binds to GspC *in vitro* (Kortokov *et. al.,* 2011). Little is known about the function of the other domains. It has been hypothesized that they are responsible for transferring conformational changes (Kortokov *et. al.,* 2011). Recently the N0-N1 domain has been demonstrated to bind ExeB in *A. hydrophila* (Vanederlinde *et al.,* 2014) which is discussed further in the section 3.6.6. The N-domain was found, by electron microscopy, to bind cholera toxin near the N-terminus when the secretin was in its closed state (Riechow *et. al.,* 2011).

Most proteins destined for insertion in the outer membrane are first transported past the inner membrane via the sec pathway, and then inserted into the outer membrane by action of the β-barrel assembly machinery (BAM) complex (Figure 6). The secretin has been shown to be inserted independently of the BAM complex in *K. oxytoca* (Collin *et. al.,* 2007). In each of the systems, T2SS, T3SS and T4P, another factor is involved in assembly.

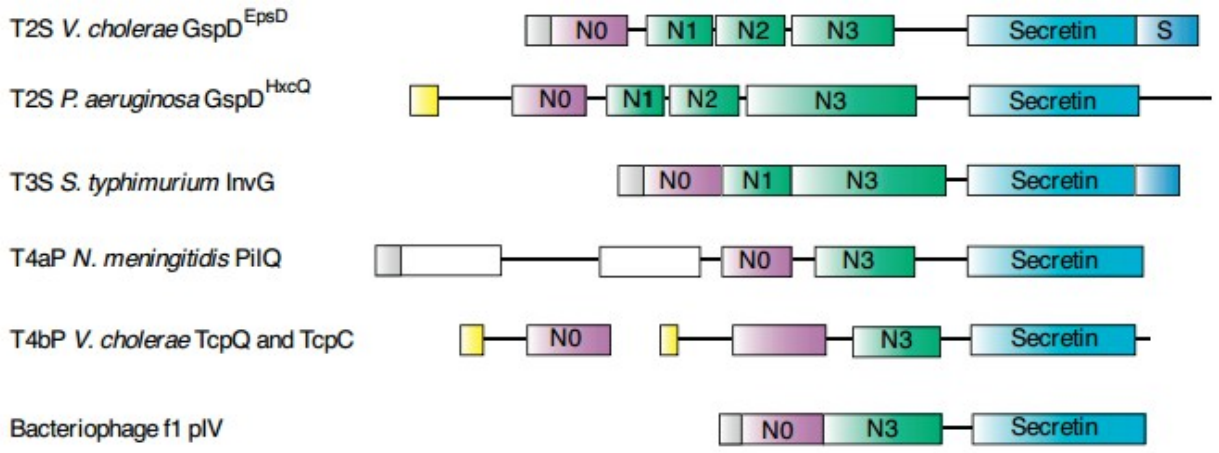
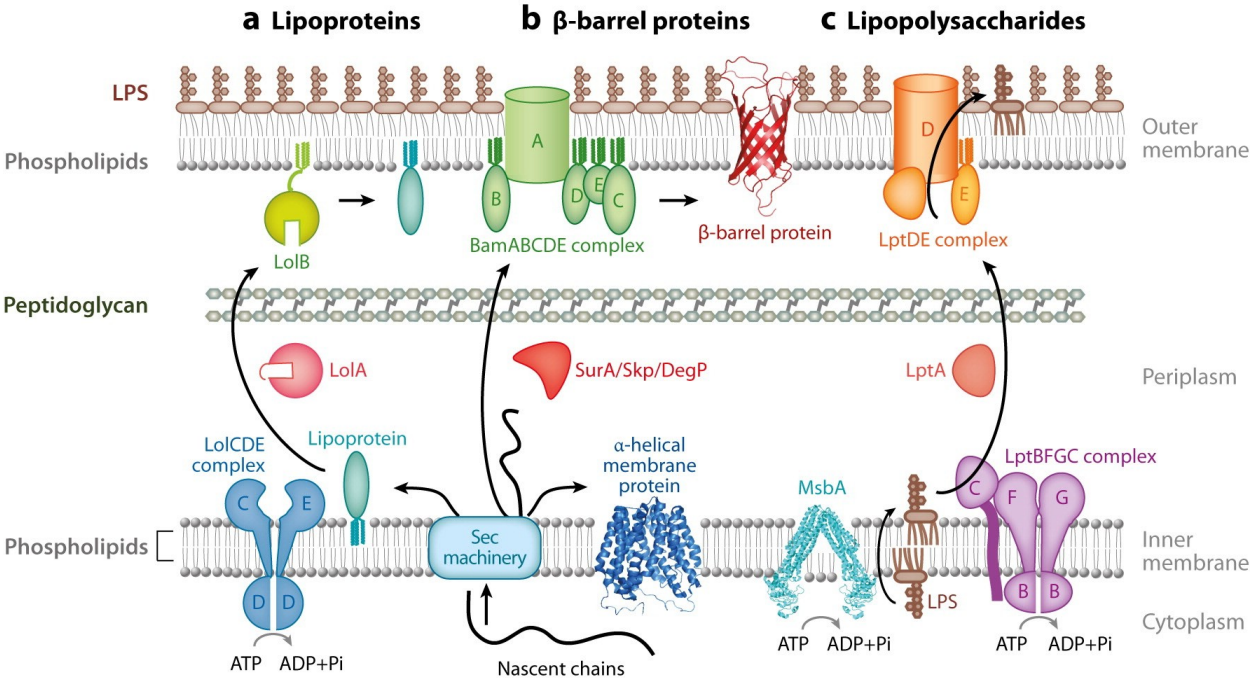


Figure 5. Presence and absence of domains within the secretin. The N0 domain represented in purple binds to GspC, N1-N3 (Green) are important in secretin multimerization, the secretin domain (blue) is the region embedded in the outer membrane, and the S domain (blue) is involved in pilotin binding. Yellow represents a LOL signal sequence (Kortokov *et. al.,* 2011). Reprinted from Trends in Biochemical Sciences, Volume 36 Isssue 8, Konstantin V. Korotkov, Tamir Gonen, Wim G.J. Hol, Secretins: dynamic channels for protein transport across membranes, Page 435, Copyright (2011), with permission from Elsevier .

3.6 Lol System

Background on the Lol (Localization of lipoproteins) is necessary to further enable discussion of how the secretin is formed in the outer membrane.



Okuda S, Tokuda H. 2011.
Annu. Rev. Microbiol. 65:239–59

Figure 6. Mechanisms of assembly in the outer membrane within Gram-negative bacteria. The LOL pathway is represented with (a), while the Bam pathway is shown under (b), while not discussed here, the lipopolysaccharide pathway is shown with (c). (Okuda & Tokuda 2011)

The Lol system is the primary pathway diderm bacteria use to traffic and lipid modify proteins for attachment to the outer membrane (Figure 6). The system itself contains three main components, an outer-membrane receptor, a periplasmic carrier protein and an ABC transporter complex. The proteins are synthesized in the cytoplasm and transported past the inner membrane *via* the sec pathway. Following transport they are lipidated if they posses the lipobox motif. These proteins will stay on this leaflet unless they posses the proper signal sequence within the first few amino acids. The lipoproteins bind the LolCDE complex which transfers the lipoprotein to LolB through ATP hydrolysis. Following this LolB carries the protein to the outer

membrane and finally releases the protein to the inner leaflet of the outer membrane (Okuda & Tokuda 2011).

3.7 Secretin targeting and assembly

Many hurdles must be overcome to assemble a secretin in diderm bacteria. First the secretin must not form in the inner-membrane as this would result in the loss of the proton gradient. Second, the subunits must be brought to the outer membrane without degradation. Third, subunits of GspD must pass through the peptidoglycan mesh that exists within the periplasm. Finally the complex must be properly assembled as it is incredibly stable once formed. It can survive boiling in the presence of SDS for periods of up to an hour (data not shown).

These hurdles aside, each system has independently evolved both similar and distinct mechanisms of targeting the secretin to the outer membrane. For a recent review on the subject please read Koo *et. al.,* 2011. These mechanisms are centered around two different pathways: pilotins, which are lipoproteins that target the secretin monomers to the outer-membrane with the Lol pathway, and assembly factors which interact with the peptidoglycan to assemble the complex. Previously the assembly factors have been named accessory factors, however this is incorrect as they are not an accessory to anything in *Aeromonas hydrophila* (Ast *et. al.,* 2002). As such they will be called “assembly factors”.

Pilotins have defining features that help keep them distinct from assembly factors. They are lipoproteins bound for the inner leaflet of the outer membrane, they bind to the C termini of the secretin monomers, and they promote assembly and prevent degradation of the secretin.

Until recently, pilotins were categorized by fold and fell into 3 classes. Class 1 pilotins consist of α-helical tetratricopeptide repeats and are double the size of all other pilotins. Class 2 pilotins are made entirely of β-strands while class 3 pilotins are predominantly non-repeating alpha helices (Dunstan *et. al* 2013). In recent studies of the two T2SS systems present in enterotoxigenic *E. coli*, we discovered the presence of a previously unrecognized novel GspS involved in secretin assembly, and bioinformatic analysis indicates that a homolog of this pilotin, termed $GspS_{\beta}$, is present in the *V. cholera* genome (Strozen 2012). Recently this pilotin was crystallized and it demonstrates a unique fold consisting of alpha helices and β-strands representing a fourth class of pilotin (Dunstan *et. al* 2013). Class 1 pilotins are found in T4P, Class 2 in T3SS, and Classes 3 and 4 are utilized by the T2SS.

3.7.1 T3SS Secretin Assembly Mechanism

The T3SS secretin is structurally different from the T4P and the T2SS secretins, as it lacks an N2 domain. The secretin functions as the pore through which the needle complex passes to reach the effector cell.

This secretin is assembled through the use of a pilotin MxiM (Figure 7). MxiM is understudied as a pilotin in comparison to T4P and T2SS. This protein was found necessary for T3SS function and assembly of the secretin within *Shigella* (Schuch & Maurelli 1999). MxiM shows all the characteristics of a pilotin; it is a small lipoprotien (which is associated with the inner leaflet of the outer-membrane) and binds near the C-terminus of MxiD, the secretin (Okon *et. al.,* 2008). This pilotin prevents degradation of MxiD monomers.

Aside from MxiM another factor effects stability of the secretin in the *Shigella* T3SS, MxiJ. This protein does not directly interact with the secretin yet it can stabilize monomers within the periplasm. It can induce multimer formation, however these multimers were not heat resistant, unlike multimers formed with assistance by MxiM, suggesting these are not the native conformations (Schuch & Maurelli 2001).

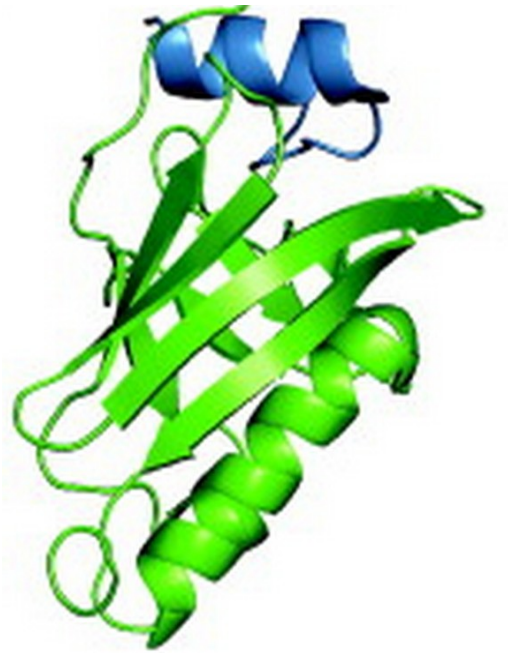


Figure 7. The T3SS pilotin MxiM (green) bound to the S domain of the secretin (blue). PDB ID: 2JW1 (Okon *et. al.,* 2008)

3.7.2 Type 4 pili Secretin Assembly Mechanism

Similar to the T3SS, the T4P have a pilotin, PilW (Figure 8) or PilF, that is necessary for the targeting of its secretin, PilQ, to the outer membrane. Homologs of this pilotin have been tested for secretin assembly in three different species; *Neisseria meningitidis* (Carbonelle *et. al.,* 2005), *Myxococcus xanthus* (Rodriguez-Soto & Kaiser 1997), and *Pseudomonas aeruginosa* (Watson *et. al.,* 1995). It has been found to be essential for T4P function in each. This pilotin is conserved among the Gammaproteobacteria which have a Type 4 pilus. Similar to other pilotins, this pilotin is lipidated and prevents proteolytic degradation of the secretin monomers (Koo *et. al.,* 2008).

Previously another lipoprotein, PilP, involved in the T4P was thought to be an assembly factor, but it was found to only influence expression and had no influence on secretin monomer stability or assembly (Balasingham *et. al,* 2007).

Figure 8: PilW the pilotin for the secretin of T4P in *Neisseria meningitidis* PDB ID: 2VQ2 (Trinidade *et. al.,* 2008)

3.7.3 The Self targeting Secretin of *Pseudomonas*

Secretins require a second system specific factor to assemble in the outer-membrane with the exception of HxcQ from *Pseudomonas*. This lipidated secretin was resistant to degradation and contained two inserts within the secretin: L1 and L2. L1 is located at the N terminus of the monomer and consists of a signal sequence with a linker region, while L2 is an addition between N3 and the secretin domain. This secretin was found to self target when expressed in *E. coli,* yet would localize to the inner-membrane if lipidation was prevented. It is worth noting that *Pseudomonas* contains a second T2SS that is not self targeting (Viarre *et. al.,* 2009).

3.7.4 The Class 3 Pilotins: PulS

The targeting mechanism of the secretin by a pilotin was originally discovered in the T2SS of *Klebsiella oxytoca* within the laboratory of Anthony Pugsley. Most work on pilotins continues to be from this laboratory (d'Enfert and Pugsley, 1989; d'Enfert *et al.,* 1987).

Initially the T2SS of *Klebsiella* was cloned into *E. coli* K12 and the pilotin, PulS, was found to protect the secretin monomers, PulD, from degradation and localize the secretin to the outer-membrane (Collin et al., 2011; Hardie *et al*., 1996a and Hardie *et al.,* 1996b; Nickerson *et al.*, 2011). Like all other pilotins PulS promoted the assembly of the secretin into dodecameric multimers in the outer membrane (Nouwen *et al.*, 1999).

Unlike many of the secretins, PulD continues to form multimers without its pilotin. The secretin would form in the inner-membrane as opposed to the outer-membrane (Hardie et al., 1996b). These pores induce the phage shock response due to membrane depolarization (Hardie et al., 1996b).

A variety of cross species studies were performed to test the pilotin's functions. A homolog of PulS in *Erwinia* is OutS. It was found to be functionally identical to PulS (Shevchik and Condemine, 1998) and could substitute for PulS in *Klebsiella oxytoca* (Hardie *et al.,* 1996b).

Like all other pilotins, PulS binds to the C-terminus of PulD in the S-domain (Nickerson *et al.,* 2011). This domain was swapped with the S domain of the filamentous bacteriophage f1 secretin and the bacteriophage's secretin monomers were then susceptible to degradation. This susceptibility is alleviated with the presence of PulS (Daefler *et al.,* 1997). The same effect is seen when MalE, a periplasmic protein, has the S domain attached to its C terminus (Hardie *et al.,* 1996b). These experiments demonstrated that the proteolytic degradation of PulD is an intrinsic property of the S-domain. The degradation is prevented by pilotin binding.

The pilotin is a stable protein while the S domain of PulD has been shown to be intrinsically disordered (Nickerson *et al*., 2011). Structure is induced by PulS binding to the S domain and is independent of PulS lipidation. If the lipidated cysteine residue is mutated on the pilotin, the pilotin will still protect PulD, however it will not target it to the outer membrane (Gu *et al*., 2012; Hardie *et al.*, 1996b).

This shuttling to the outer membrane done through action of the carrier protein LolA, and as such a complex of LolA and PulS can be isolated. No complex could be isolated that also included PulD (Collin et al., 2011).

A 1:1 complex is formed between PulS and PulD 12 copies of PulS have been found bound to a single secretin (Nouwen *et al.,* 1999).

Figure 9. The *Klebsiella* pilotin PulS PDB ID: 4A56 (Tosi *et. al.,* 2011)

3.7.5 The Class 4 pilotins: EpsS

Recently a second type of T2SS pilotin was discovered, $GspS_{\beta}$. This pilotin was discovered in ETEC and is necessary for LT (Heat-Liabile Toxin) secretion in that strain. In the discovery paper the protein was compared to the known pilotin PulS, and no sequence homology was found. This pilotin is a lipoprotein which targets GspD to the inner leaflet of the outer-membrane. Unlike PulS, if this pilotin is mutated only monomers will form in the inner membrane, not assembled secretin. (Strozen *et al.,* 2012).

A second paper (Dunsten *et. al.,* 2013) was published on this pilotin which focused on the evolution of this pilotin with physiological studies performed in *V. cholerae.* This study found that the Class 3 and Class 4 pilotins form two distinct evolutionary groups. Homology between them could not be proven. They repeated many of the experiments performed in ETEC and found that the Vibrio type pilotin has the same characteristics as the ETEC pilotin, notably it is necessary for full secretin assembly. This secretin would form in the inner-membrane without its pilotin, but only in small amounts. This was a much smaller percentage than in *Klebsiella*. The authors elucidated the structure of the protein through X-ray crystallography (Dunsten *et. al.,* 2013) (Figure 10).

In a third study, an undergraduate researcher in our laboratory did two key experiments that are worth mention. She expressed the *Vibrio* pilotin, EpsS, and found that it could restore LT secretion in a $GspS_{\beta}$ mutant, similar to how *Erwinia* OutS can complement a *Klebsiella* with a *pulS* mutation. The open reading frame (ORF) listed in genbank for VC1703 (*epsS*) is longer than $GspS_{\beta}$ ORF and contains 4 possible start codons. She established that the final start codon within the ORF is the native start codon by immunoblot (Rininsland *et. al.,* 2012).

A problem exists with the naming of this pilotin. In the initial study from our laboratory it was named $GspS_{\beta}$ (Strozen *et al.,* 2012) due to the fact it was discovered in the β T2SS of ETEC. A second study used a different naming system entirely and called it AspS (Dunsten *et. al.,* 2013). Even more confusing is a recent review, which has suggested that it be named GspP to continue with traditional nomenclature (Nivaskumar & Francetic 2014**)**. For this study the above forms will be rejected and its simple form, EpsS, will be used. I present this name as this pilotin is only present in systems that do not have the *Klebsiella* type pilotin (by HMM search of genbank) and the function is very similar. This will reduce the complication of the added β we previously proposed while remaining easily recognizable as a pilotin. It will be distinguished from class 3 pilotins by its prefix Eps or Gsp, as the other pilotins have the prefix Pul or Out.

Figure 10:The *Vibrio* pilotin EpsS PDB ID: 4FTF (Dunsten *et. al.,* 2013)

3.7.6 GspAB Complex

In *Aeromonas hydrophila* two proteins are necessary for functional assembly of the secretin in the outer membrane: ExeA and ExeB (Ast *et. al.,* 2002). When these genes are mutated the secretin is not formed and monomers are accumulated. The genes responsible for these proteins are located on a single operon and are likely co-expressed. It has been demonstrated that ExeA and ExeB form a heterodimer (ExeAB) and that multimerization is essential for function of these proteins (Jahagirdar and Howard 1994). It is worth noting that in *Vibrio vulnificus* the proteins are fused (Strozen *et. al.*, 2012) (Figure 11). ExeA spans the inner membrane with a cytoplasmic AAA ATPase domain (Schoenhofen *et. al*., 2005) and a periplasmic domain that binds peptidoglycan is located near the C-terminus (Li and Howard, 2010). ExeB has been shown to interact with the N0 and N1 domains of ExeD (Vanderlinde *et.*

al., 2014). It has been hypothesized that these proteins are responsible for remodeling the peptidoglycan and insertion of the secretin.

Our laboratory recently tested the homologs of ExeAB from *A. salmonicida, V. cholerae, V. vulnificus,* and *V. parahaemolyticus* and compared them to those from *A. hydrophilla* (Strozen *et. al.*, 2012). The Aeromonadales had the same phenotype, where mutation of *exeAB* prevented secretin assembly and prevented T2SS secretion, while all the Vibrionales had only partial reduction of secretin assembly and associated T2SS secretion when the *exeAB* homolog was deleted. When EpsAB from *V. cholerae* is expressed in an *exeAB* deficient strain of *A. hydrophila* secretin assembly is only partially restored.

Klebsiella's T2SS contains only one component of this system, PulB, yet it is not essential for secretion or secretin assembly (Pugsley *et. al.,* 1990). In *Erwinia* it was found that mutation of OutB reduces secretin assembly, but this defect could be complemented by over-expression of OutD (Condemine and Shevchik, 2000). A similar result is obtained with Aeromonadales, however the secretin would preferentially form in the inner-membrane (Ast *et. al.,* 2002). *Klebsiella* may have a phenotype with a *pulB* mutation, yet it has only been tested in *E. coli*, which can result in expression irregularities.

Figure 11. Structure of the Periplasmic domain of the EpsAB fusion from *V. vulnificus.*
PDB ID: 4G54 (Martynowski *et, al.,* 2013)

3.7.7 Global hypothesis of Secretin assembly

In a recently published article (Huysmans *et. al.,* 2014), a hypothesis of how the secretin forms in the outer membrane was proposed. They demonstrated that it was a multiple step process beginning with the formation of a pre-pore. The mechanism of formation is suggested by two alternate hypotheses; in the piloting theorem, the pilotin "drags" the secretin monomers across the periplasm via LolA and then the secretin monomers are brought together by the pilotins within a fluid membrane. Conversely, in the docking theory, the pilotin is transferred to the outer membrane alone, where it is bound by secretin monomers.

After the secretin monomers are in the outer membrane the transmembrane region of the monomers join and form the pre-pore. The protein then folds down the length of its periplasmic region until the protein is fully folded. This is a theory. Strong evidence exists for the formation of a pre-pore, however the remainder of the folding patterns have not been elucidated. This theory does not account for how ExeAB functions in *Aeromonas*.

3.8 YcII/Vc1702

A gene that is important in this thesis is the gene immediately upstream of *epsS.* This gene is separated from *epsS* by a single nucleotide. The two genes are likely on the same operon, as *epsS* does not have a Shine-Dalgarno (SD) sequence preceding it, yet VC1702 does. The protein product of this gene has been involved in two studies: a pathogenicity assay (John *et. al.,* 2005) and a crystallization study (Figure 12)(Willis *et. al.,* 2005). In the pathogenicity assay the authors found the gene was only one of many with unknown functions, however the gene associated strongly with pathogenicity, and was found to be localized in the inner membrane. Very little else is known about this gene or its protein product.

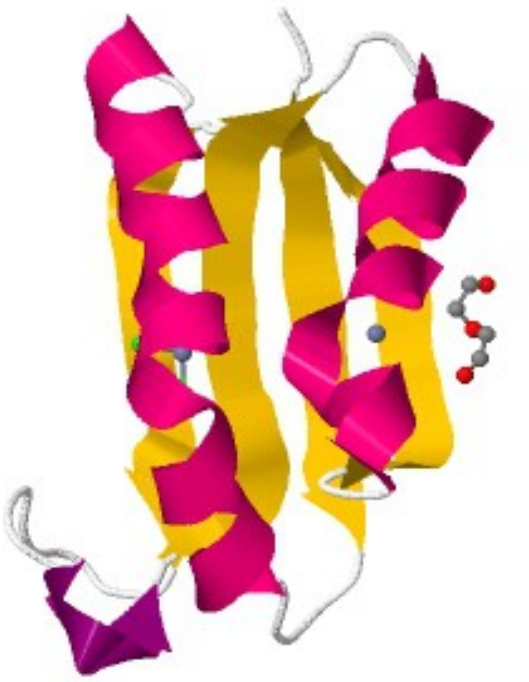


Figure 12. Crystal Structure of YciI from *Haemophilus influenzae* PDB ID: 1MWQ (Willis *et. al.,* 2005)

4.0 MATERIALS AND METHODS

4.1 Bacterial strains and culture conditions

A full list of strains used in this study can be found in Appendix C. All strains were maintained on plates of Luria-Bertani (LB) medium with 1.5% agar. Strains were incubated at 37°C until mid to late logarithmic phase of growth. Antibiotics were used at the following concentrations, unless specified otherwise: rifampicin (rif), 50 μg/ml; kanamycin (kan), 50μg/ml; chloramphenicol (cat), 1.25 μg/ml; ampicillin (amp), 200 μg/ml; and streptomyocin (sm), 50μg/ml.

In physiological growth experiments cultures were grown overnight in selective LB medium inoculated from freshly streaked selective plates. These cultures were diluted 1:100 and grown at 37˚C with moderate shaking until an OD_{600} of 2.0 was reached.

4.2 Purification of Genomic and Plasmid DNA and Polymerase Chain Reaction

Genomic DNA was isolated using DNeasy kit (Qiagen). Minipreparation of plasmid DNA was done utilizing the alkiline lysis method (Sambrook *et al.,* 1989). Large preparations of plasmid DNA were done using Plasmid Midi Kits (Qiagen). All DNA that was further manipulated in PCR reactions and restriction enzyme digestions was purified with NucleoSpin columns (Macherey-Nagel). Polymerase chain reactions (PCR) were performed using either Q5 or Phusion DNA polymerase (Bio-Rad) for cloning, while colony PCR was performed as mentioned in Molecular Cloning (Sambrook *et al.,* 1989) using Taq DNA polymerase (Bio-Rad).

4.3 Plasmid Construction and Conjugation

A list of plasmids is provided in Appendix B and a list of primers is provided in Appendix A and both are visually represented in Figure 13. The *epsS* open reading frame (ORF) was amplified using primers designed for cloning in the vector pMMB67HE. Two clones were constructed to determine the effect of ORF length. Upon failure of these clones to complement, a third clone was constructed to encompass the whole operon. To construct a vector which expressed a Myc tagged EpsS the reverse primer of each of these clones was replaced with a primer containing a Myc tag but all other procedures remained the same. The same procedure was used for cloning the *epsAB* clone.

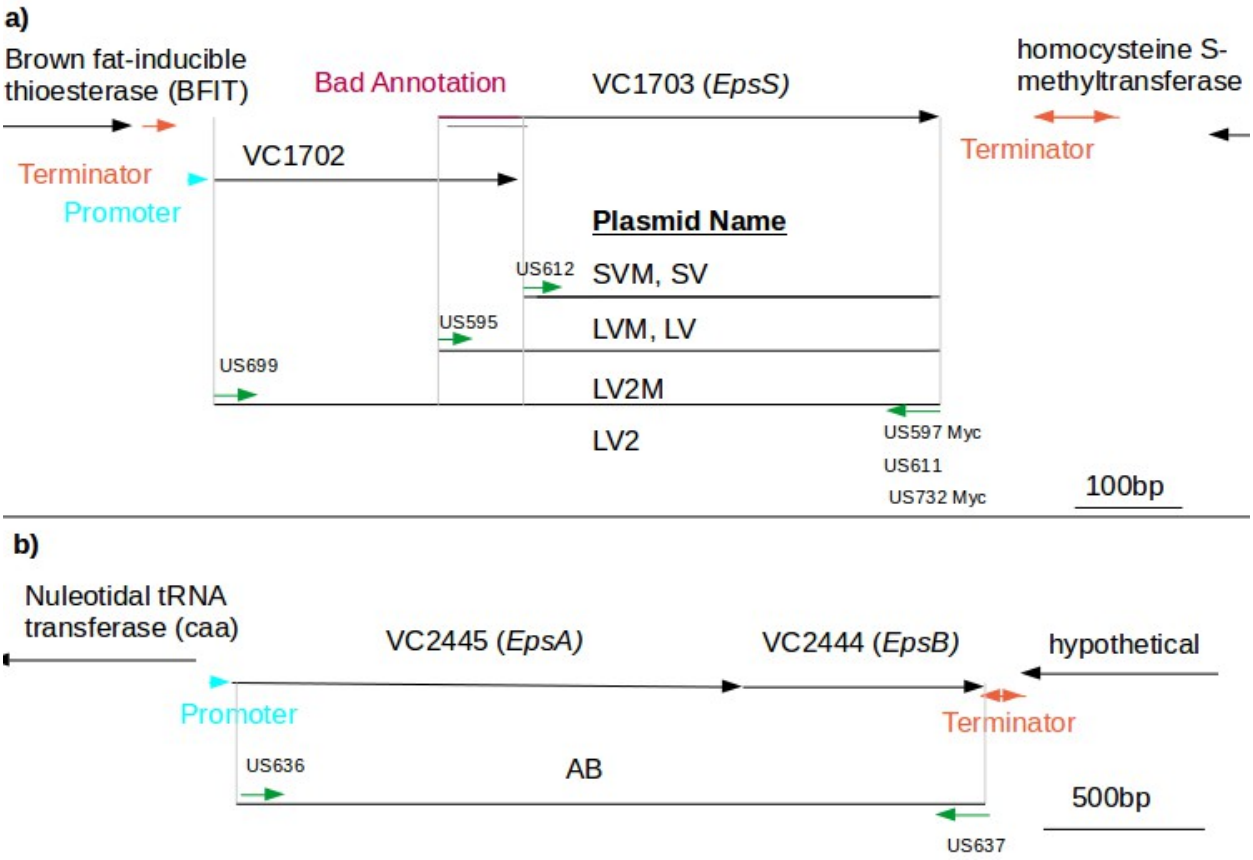


Figure 13. Genomic syntany of *epsS* (a) and *epsA* (b) illustrating the sections of DNA cloned and the primers used. For details of each primer please see appendix A. Primers, terminators, and promoters are not to scale.

Plasmids were digested with the same enzymes listed for each primer in Appendix A and digested DNA was purified and ligated using T4 DNA ligase (New England Biolabs). 1 µl of ligation mixture was electroporated into 50 µl of DH5α cells and recovered in 450 µl of SOC (2% Tryptone, 0.5% Yeast extract, 10mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20mM Glucose) for 2 hours and plated on selective LB plates. Minipreps were made from isolated colonies, and these colonies were tested by colony PCR and digestion for presence of the insert. Plasmids were then sequenced (NRC Plant Biotechnology Institute) for confirmation of proper clone construction.

To conjugate the plasmid into the *Vibrio* strains, it was electroporated into *E. coli* SM10 by selecting for ampicillin resistance. The donor strain and recipient strains were grown overnight with antibiotic selection, subcultured 1:100 in LB broth without antibiotics and incubated at 37˚C for 4 hour prior to conjugation. 900 µl of recipient were combined with 100 µl of donor, mixed and collected by centrifugation. The pellet was resuspended in 100 µl LB and

applied to BHI plates and incubated at 37˚C overnight. Copious amounts of culture was scraped and spread on LB plates containing streptomycin (500μg/ml) and ampicillin and incubated at 37˚C to screen for strains containing the plasmid. Confirmation of plasmid exchange was verified by colony PCR and minipreps.

4.4 Creation of Mutant strains of *Vibrio cholerae*

Most mutant strains were obtained through previous work in our laboratory or from the Cameron laboratory (Cameron *et al.*, 2008), for a complete list see appendix C. The Bah-2 *epsS* mutants and the C6706 double mutants, *epsAS* and *epsBS*, were constructed by marker exchange mutagenesis using the suicide vector pMRS101 (Sarker and Cornelis, 1997). The vector pMRS101/*epsS*::cat had previously been developed in our lab to generate the Bah-2 *epsS* strain. This plasmid was electroporated into *E. coli* S17 by selecting for kanamycin and chloramphenicol resistance. The donor strain (S17 containing pMRS 101/*epsS*::cat) and recipient strains (Bah-2 *epsA,* C6706 *epsA,* C6706 *epsB*) were grown overnight with antibiotic selection, subcultured 1:10 in brain heart infusion (BHI) broth without antibiotics and incubated at 37˚C for 1 hour prior to conjugation. 900 μl of recipient were combined with 100 μl of donor, mixed and collected by centrifugation. The pellet was resuspended in 100 μl BHI and applied to BHI plates and incubated at 37˚C overnight. Copious amounts of culture were scraped and spread on LB plates containing streptomycin (500 μg/ml), chloramphenicol, and sucrose (10%) and incubated at 37˚C to screen for recombinant colonies. Confirmation of allele exchange was verified by colony PCR.

4.5 Gel Electrophoresis and Immunodetection

Samples of whole cells grown in broth culture were mixed with an equal volume of 2X sample buffer (125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.01% bromophenol blue, 10% β-mercaptoethanol) and heated for 3 minutes at 95˚C.

Samples with proteins larger than 20kDa were separated on 10-12% SDS-PAGE gels (Sambrook *et. al.,* 1989), while smaller proteins were separated on 16% Tris/Tricine SDS-PAGE (Schaegger and von Jagow, 1987). Secretin assembly was determined by separating samples on 3-8% Criterion gradient gels (Bio-Rad) or 4-15% gradient Ready Gel (Bio-Rad). Secretin assembly gels were separated at 100 V until an 84 kDa protein standard was approximately 1.5 cm from the bottom of the gel. All other gels were run at 100 V until the bromophenol blue reached the bottom of the gel.

All gels were transferred to PVDF membranes using a Trans-Blot semi-dry apparatus and prepackaged Trans-Blot transfer kits (Bio-Rad). Samples run on Tris/Tricine gels were transferred using the Low MW program (2.5 A midi, 1.3 A mini; up to 25 V, 5 min) , while standard gels were transferred using the Standard SD program (up to 1.0 A; 25 V, 30 min). Secretin assembly gels were transferred using GD program (2.5 A midi, 1.3 A mini; up to 25 V, 13 min).

Blots were immediately equilibrated in TN buffer (0.1 M Tris-HCl pH 7.5 0.15 M NaCl). After equilibration the membranes were blocked in TN containing 2% ECL Prime Blocking Agent, then exposed to the primary antibody (Table 1**)** in 2% block TNT (TN containing 1% Tween-20). Membranes were washed 3x 10 min with TNT before incubating Goat anti-Rabbit horse radish peroxidase in 2% block TNT and washed 3x10 min after incubation.

Samples were imaged using a chemiluminescent substrate, Lumigen ECL Ultra (GE Healthcare), and detected using a Chemidoc XLT (Bio-Rad).

Table 1: Antibody Dilutions

Primary Antibody	Working Dilution
α-EpsD	1:100,000
α-CT	1:20,000
α-MYC	1:20,000
Goat α-Rabbit HRP	1:100,000

4.6 Enzyme Assay of Culture Supernatant

Supernatant was harvested by centrifugation of 15 ml of cell culture at 31,000 x g for 15 minutes and 10 ml of supernatant was taken. Alternatively 2 ml of broth culture was centrifuged at 13,000 RPM on a desktop centrifuge for 3 minutes and 1.5 ml of supernatant was taken. Samples were frozen and stored at -20˚C until the time of assay.

Lipase activity of the culture supernatant was measured in a continuous colorimetric assay measuring the release of p-nitrophenol (pNP) from 4-nitrophenyl octanoate (pNPO) (Aragon *et. al.*, 2000). The assay was performed by mixing 500 µl of supernatant with 500 µl of buffer containing 200mM Tris-Hcl pH 8.0, 0.4 % Triton X-100, 2mM pNPO. The reaction was incubated at 25˚C and the absorbance at 405nm was recorded at 30 second intervals for twenty

minutes. One unit of lipase activity equals 1 nmol pNPO hydrolyzed per minute. All enzymatic activities are expressed as units/ml supernatant per OD_{600} of culture. Concentration of pNPO was determined to be adequate by testing several substrate concentrations of pNPO to determine saturation.

Protease activity of the culture supernatant was measured in a continuous colorimetric assay measuring the release of p-nitrophenol (pNP) from L-Leucine-4-nitroanilide (pNPL) (Aragon *et. al.*, 1976). The assay was performed by mixing 500 µl of supernatant with 500 µl of buffer containing 200mM Tris-Hcl pH 8.0 and started with the addition of 1µl of 0.5 M pNPL dissolved in methanol. The reaction was incubated at 25˚C and the absorbance at 405nm was recorded at 30 second intervals for 8 minutes. One unit of protease activity equals 1 nmol pNPL hydrolyzed per minute. All enzymatic activities are expressed as units/ml supernatant per OD_{600} of culture. PNPL was present at the highest soluble concentration.

Leakage of the outer membrane was tested by assaying beta lactamase with nitrocefin. To test leakage cell cultures were split in two distributions, upon which one sample had its supernatant harvested by centrifugation at 15,000 x g for 15 minutes while the other sample had the cells burst by use of a French Press at a 1000 PSIG, and cell debris was removed by centrifugation at 15,000 x g for 15 minutes. The assay tested the breakdown of nitrocefin and the corresponding increase in absorbance at 510 nm (O'Callaghan *et. al.,* 1972). The assay was performed by mixing 50 µl of supernatant or burst cell sample with 950ul of buffer containing 50mM Tris-Hcl pH 8.0 and 0.2mg/ml of nitrocefin. The reaction was incubated at 25˚C and the absorbance at 510 nm was recorded at 30 second intervals for 5 minutes. All mutant strains that did not contain the plasmid were tested, and found to have no appreciable enzyme activity over 24 hours.

4.7 Statistical Analysis

An unpaired two-sided Student's T-test was used for all statistical analysis. Values were considered significantly different at $P < 0.05$. Samples analyzed by T-test contained 4 biological replicates with each replicate tested 3 times. Error bars represent standard error of the mean (SEM) for biological replicates, and standard deviation for technical replicates.

4.8 Bioinformatics

To better understand the roles *epsAB* and *epsS* play in the T2SS the evolutionary history was studied by means of phylogenomic analysis. Ten genomes were selected, from the gamma proteobacteria, for analysis based on known data about their T2SS; *Escherichia coli* ETEC

H10407, *Escherichia coli* str. K-12 substr. MG1655, *Aeromonas hydrophila subsp. hydrophila* ATCC 7966, *Candidatus Hamiltonella defensa* 5AT, *Acyrthosiphon pisum, Klebsiella oxytoca* KCTC 1686, *Pseudomonas aeruginosa* PAO1,*Vibrio cholerae* O1 biovar El Tor str. N16961, *Vibrio vulnificus* YJ016, *Xanthomonas campestris pv. campestris* str. ATCC 33913, *Yersinia pestis* CO92.

Another 20 genomes were selected to ensure a diverse selection; *Acinetobacter baumannii* MDR-ZJ06*, Alteromonas macleodii* ATCC 27126*, Buchnera aphidicola* str. APS *Acyrthosiphon pisum, Dichelobacter nodosus* VCS1703A*, Haemophilus ducreyi* 35000HP*, Haemophilus influenzae* Rd KW20*, Hahella chejuensis* KCTC 239*, Halorhodospira halophila* SL1*, Idiomarina loihiensis* L2TR*, Legionella pneumophila* subsp*. pneumophila* str. Philadelphia 1*, Marinobacter aquaeolei* VT8*, Methylococcus capsulatus str. Bath,Pectobacterium atrosepticum* SCRI1043*, Pseudoalteromonas atlantica* T6c*, Pseudomonas syringae* pv. *syringae* B728a*, Psychromonas sp.* CNPT3*, Saccharophagus degradans* 2-40*, Salmonella enterica* subsp*. enterica serovar Typhimurium* str. T000240, *Shewanella baltica* OS155, *Shewanella loihica* PV-4.The protein sequences for all conserved ribosomal proteins were extracted, concatenated, aligned with Muscle (Edgar 2004), and uninformative sites removed with BGME (Criscuolo and Gribaldo, 2010).

After this process a maximum likelihood tree was constructed with PhyML (Guindon *et. al.,* 2010). Presence and absence of T2SS proteins was determined by an exhaustive HMM (Finn *et. al.,* 2011) search of the genomes, combined with selection from clustered genes.

Self targeting GspD was determined by the presence of two subdomains within the N domain (Viarre *et. al.,* 2009). All secretins were aligned with Muscle and those that contained the L1 domain at the N terminus and the L2 domain between the N3 domain and the secretin domain were defined as self targeting. The self targeting is purely sequence based and this should not be construed as definitive evidence.

5.0 RESULTS

5.1 Transposon Library

A transposon library was previously created in the Howard laboratory (Strozen 2012). This library was constructed to determine the other factor necessary for assembly in *V. cholerae* as mutations in *epsAB* resulted in only a slight decrease in T2SS function. The library was constructed using the Bah-2*epsAB* strain and colonies were selected based on drastic decreases in protease secretion. In numerous attempts, Dr. Strozen was unable to locate the region of mutation within these strains. Since this time, the other factor has been determined, *epsS,* and new primers had been developed to clone the gene. Using these primers, I determined the location of the insert.

The two clones of interest are 32 and 38. The strains were re-streaked from freezer stock on selective plates and the genomic DNA was extracted for analysis. Initial PCR was performed using primers US636 and US637 to check for the predicted resistance insertion within *epsAB* (Figure 14**)**.

In an attempt to amplify the target region of *epsS* primers, US595 and US597, were used that amplified the ORF. These primers failed to produce any visible bands. The search was expanded and the forward primer was replaced with US699, which would amplify the entire operon. These primers produced bands demonstrating a 1200bp band shift corresponding to the mini-Tn5 used to generate the clones (Figure 15**)**.

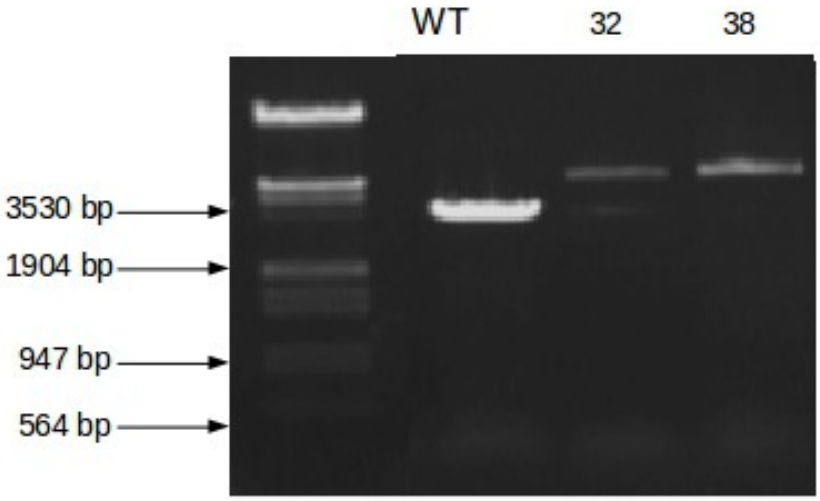


Figure 14. Confirmation that clones 32 and 38 contain an insert within *epsAB*. The region of the *V. cholerae* Bah-2 genome encompassing gene *epsAB* was amplified with primers US636 and US537 and whole genomic DNA samples from wild-type and transposon mutagenesis clones 32 and 38. This test confirms the strains identities by the expected band shift of 2000bp resulting from the CAT insertion.

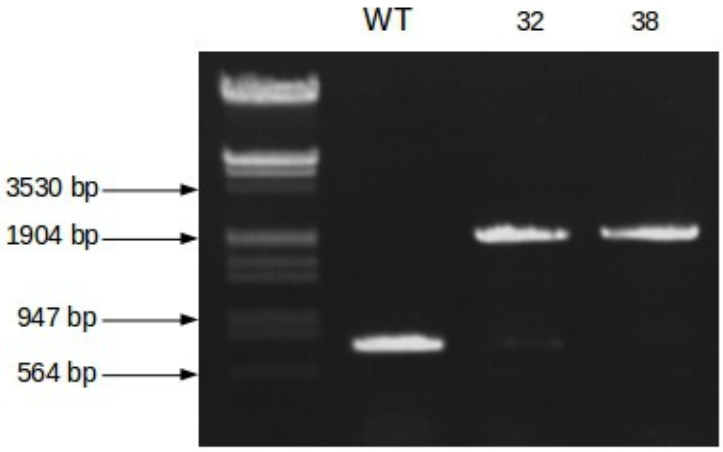


Figure 15. PCR amplification of the region of the genome encompassing the operon of *epsS* in transposon mutagenesis clones 32 and 38. Clones demonstrate the expected 1200bp band shift corresponding to the transposon insertion. The region of the *V. cholerae* Bah-2 genome encompassing gene *epsS* was amplified with primers US699 and US597 and whole genomic DNA samples from wild-type and transposon mutagenesis clones 32 and 38.

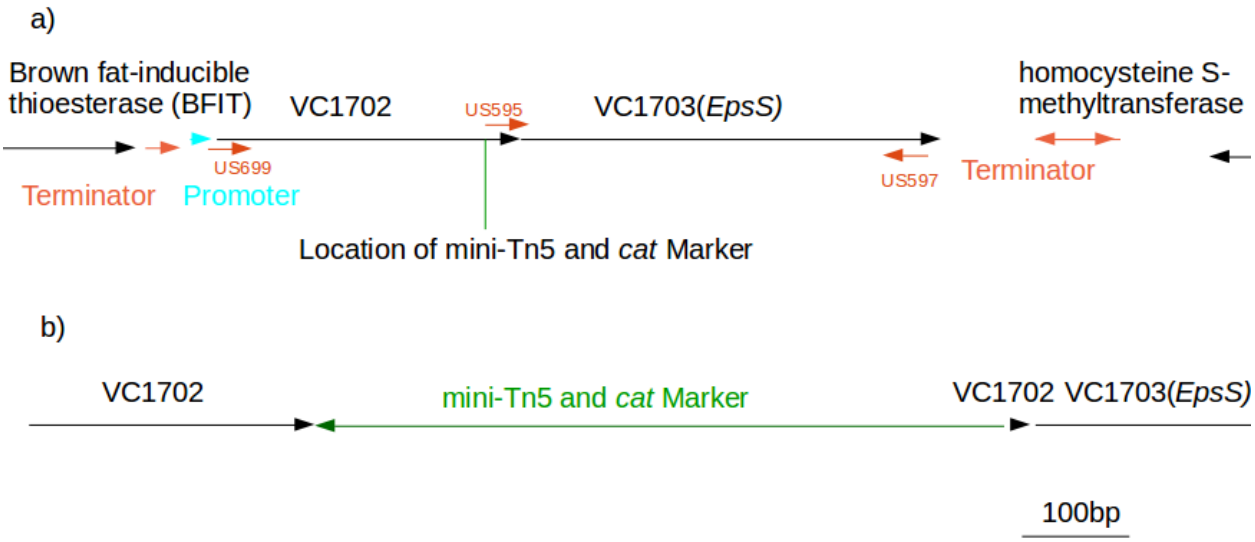


Figure 16. **Location of Transposon insertion within clones 32 and 38.** a) shows the local genomic area and the point at which the transposon inserted. b) demonstrates the size of insertion within VC1702. Primers, terminators, and promoters are not to scale.

The PCR reaction was repeated and the DNA product was sequenced. The sequence did not provide full coverage of the mini-Tn5 and the *cat* gene, but was sufficient to accurately determine the position of the insertion (Figure 16**)**. The insertion was located 14 bp upstream of the start of *epsS* and 13 bp upstream from the end of VC1702; both clones had an identical insertion. This demonstrated why the original PCR failed, as it used a primer that hybridized with an area split by the transposon.

5.2 Mutant Phenotypes

To determine the role EpsS and EpsAB contribute to T2SS activity and secretin assembly, strains were created or obtained that had mutations for the complex. The full list of strains can be found in Appendix C. Two strains were utilized because of their distinct phenotypes. Bah-2 was safer to work with because it was missing the toxin gene, but was *recA* deficient resulting in difficulty constructing mutants. C6706 had the opposite problems.

5.2.1 Bah-2 Phenotypes

Previous work in Bah-2 (Strozen *et. al.,* 2011) had demonstrated that the EpsAB protein complex was not required for secretin assembly, but the effect of *epsS* had not been tested in *V. cholerae.* Recent work (Strozen *et. al.,* 2012; Dunstan *et. al.,* 2013) had demonstrated it to be absolutely required in ETEC and EPEC, but neither of these systems contain an EpsAB complex. Strains were tested by standard physiological growth conditions.

Secretin assembly of Bah-2 mutants (Figure 17) showed a relatively constant level of EpsD monomer present in all strains. The assembled secretin was slightly reduced in *epsA*, while *epsS* showed a drastic decrease in secretin assembly. The double mutant demonstrated an almost undetectable level of secretin.

Function of the type 2 secretion apparatus in Bah-2 was measured through activity of known substrates of the T2SS, protease and lipase (Figures 18 and 19). Both the lipase and protease activities were slightly, but significantly, reduced in the *epsAB* mutant. The pilotin mutant had a drastic reduction in enzyme secretion while the double mutant had the lowest activity.

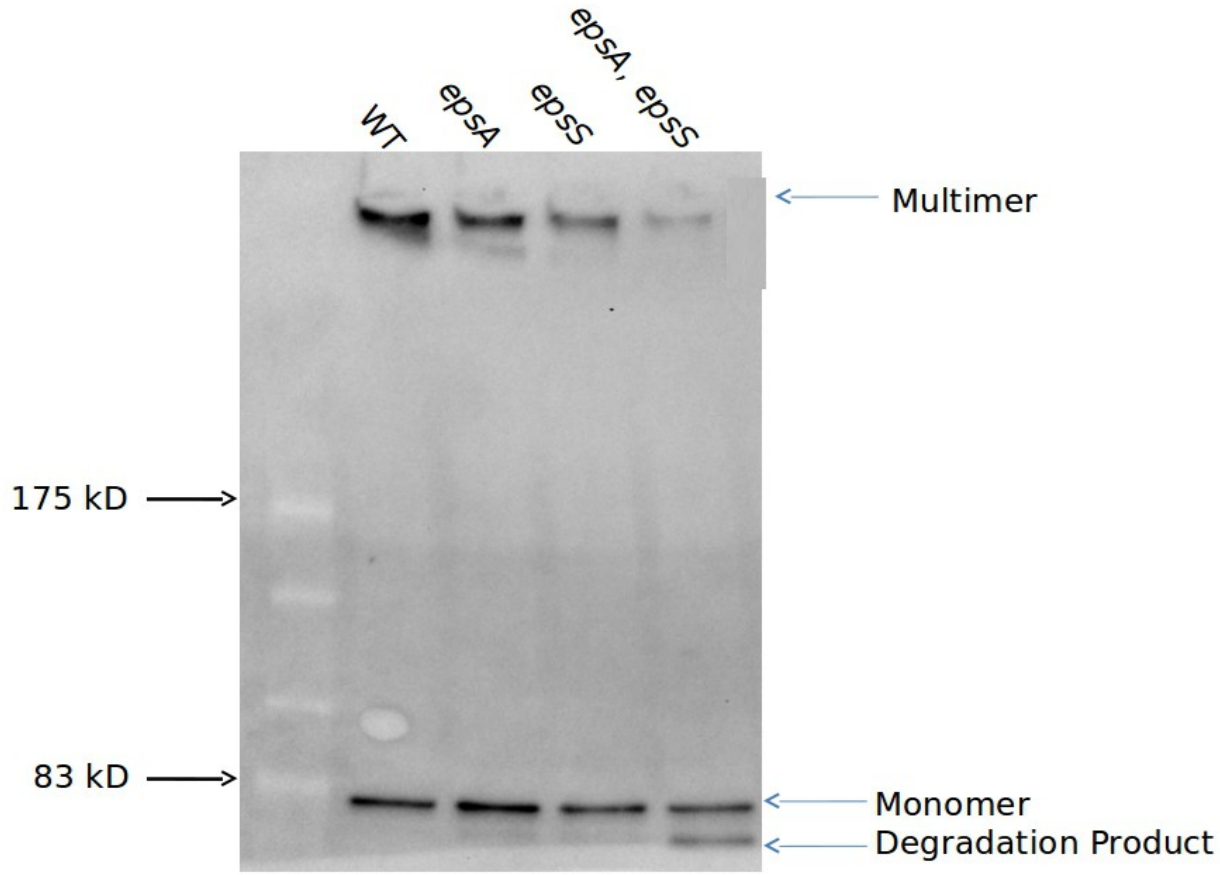


Figure 17. Assembly of the Secretin from EpsD monomers in Bah-2 mutant strains. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and whole cell samples were electrophoresed and immunoblotted with anti-EpsD. MW of ladder bands is given.

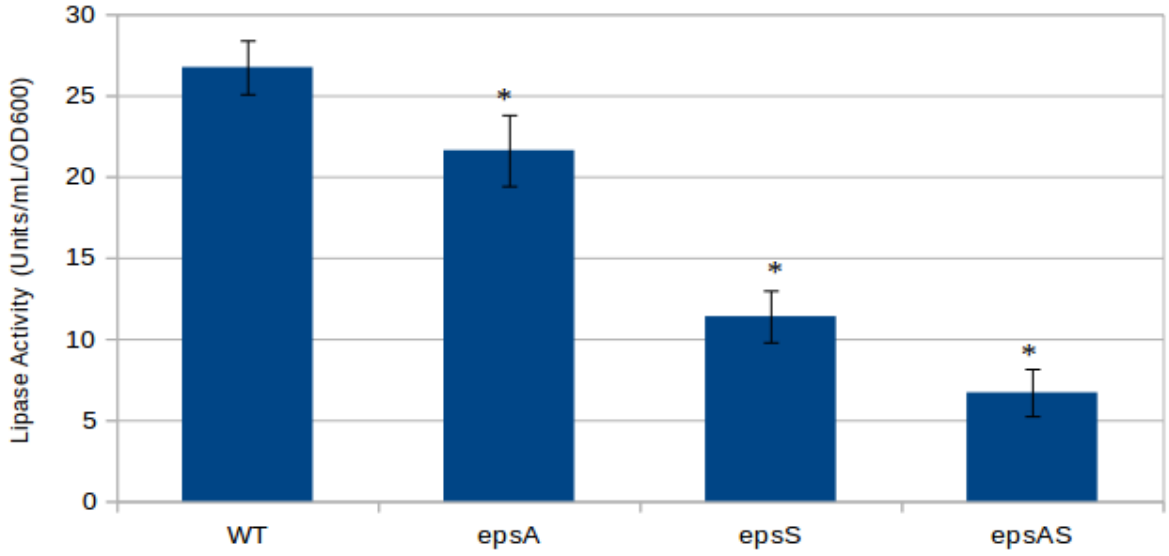


Figure 18. Lipase activity detected in culture supernatant of wild-type and mutant Bah-2 strains. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was harvested and tested. Data represents the average of four biological replicates. Error bars signify SEM. *Significantly different from WT levels.

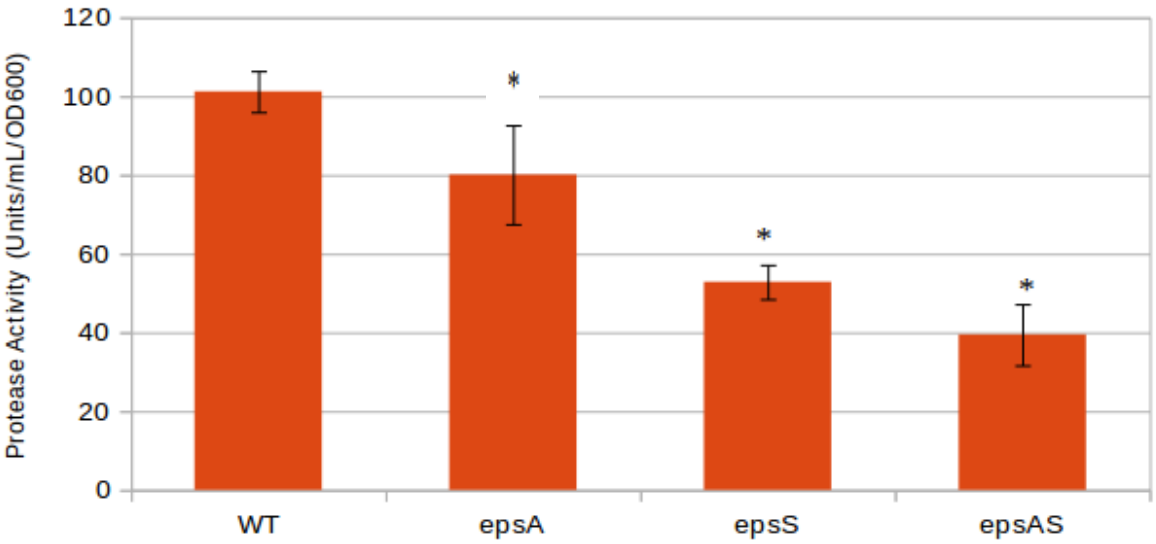


Figure 19. Protease activity detected in culture supernatant of wild-type and mutant Bah-2 strains. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was harvested and tested. Data represents the average of four biological replicates. Error bars signify SEM. *Significantly different from WT levels.

5.2.2 C6706 Phenotypes

C6706 provided unique opportunities for study that were unavailable in Bah-2. A complete transposon library was available and mutants were easily obtained. As such three other mutant strains were studied for their phenotypes, *epsB, epsBS,* and *epsC*. In this background *epsB* and *epsBS* provide a control for the *epsA* and *epsAS* mutants as *epsA* and *epsB* make a complex, but confirmation is needed that *epsB* alone does not increase EpsD assembly as it does in *Erwinia chrysanthemi* (Condemine, G. et, al,. 2000). *epsC* provides a positive control for secretin assembly, as it is not involved in secretin assembly, but also provides a negative control for T2SS activity as it has been shown that EpsC is absolutely required for secretion (Kortokov, K.V. *et al*., 2006). Unfortunately no *epsD* mutant was obtained from the extensive transposon library.

Secretin assembly of C6706 mutants (Figure 20) was reduced in *epsA* and *epsB*, while *epsS* showed a drastic decrease in secretin assembly. The double mutant had no detectable level of secretin. *epsC* showed full secretin assembly. The image also demonstrates an increase in the amount of EpsD monomer in *epsA, epsB, epsAS,* and *epsBS*. It is also observable that all of the strains containing *epsS* mutations show a second band corresponding to degraded monomer.

Function of the type 2 secretion apparatus in C6706 was measured through activity of known substrates of the T2SS, protease and lipase (Figures 21 and 22). Enzyme activity was much lower in C6706 when compared to Bah-2 in similar conditions. Lipase and protease activity had a slight but significant drop in *epsA* and *epsB* with a further decrease in *epsS.* There was no significant difference between *epsAS* or *epsBS* and *epsC* for either the protease or lipase assays. This demonstrates that either another secretion system is secreting proteases and lipases or the harvest method resulted in cell lysis. The cell lysis was tested (Table 2**)** and is discussed in section 5.5.

C6706 provides the unique opportunity of measuring cholera toxin transport through the T2SS and as such, we can measure its levels. WT, *epsA,* and *epsB* had similar levels (Figure 23). No detectable CT was secreted in the *epsAS*, *epsBS,* or *epsC* strains, while *epsS* had a drastic decrease in levels.

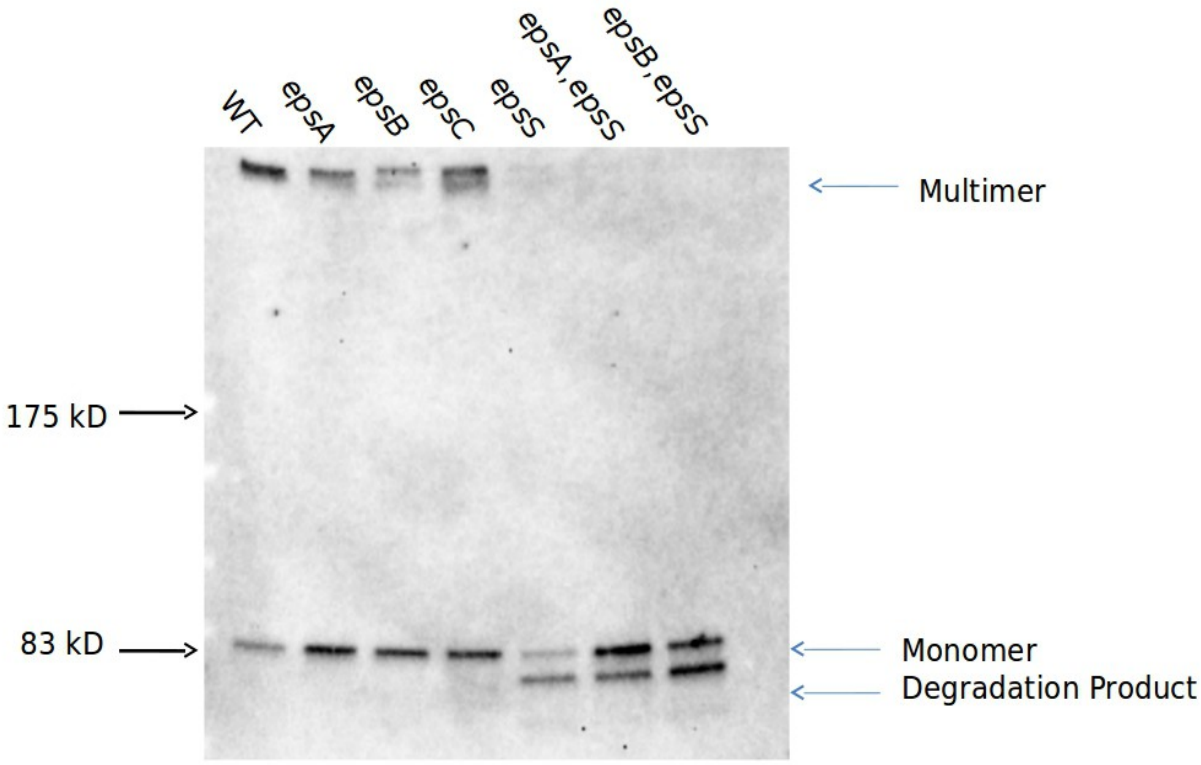


Figure 20. Assembly of the Secretin from EpsD monomers in C6706 mutant strains. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and whole cell samples were electrophoresed and immunoblotted with anti-EpsD. MW of ladder bands is given.

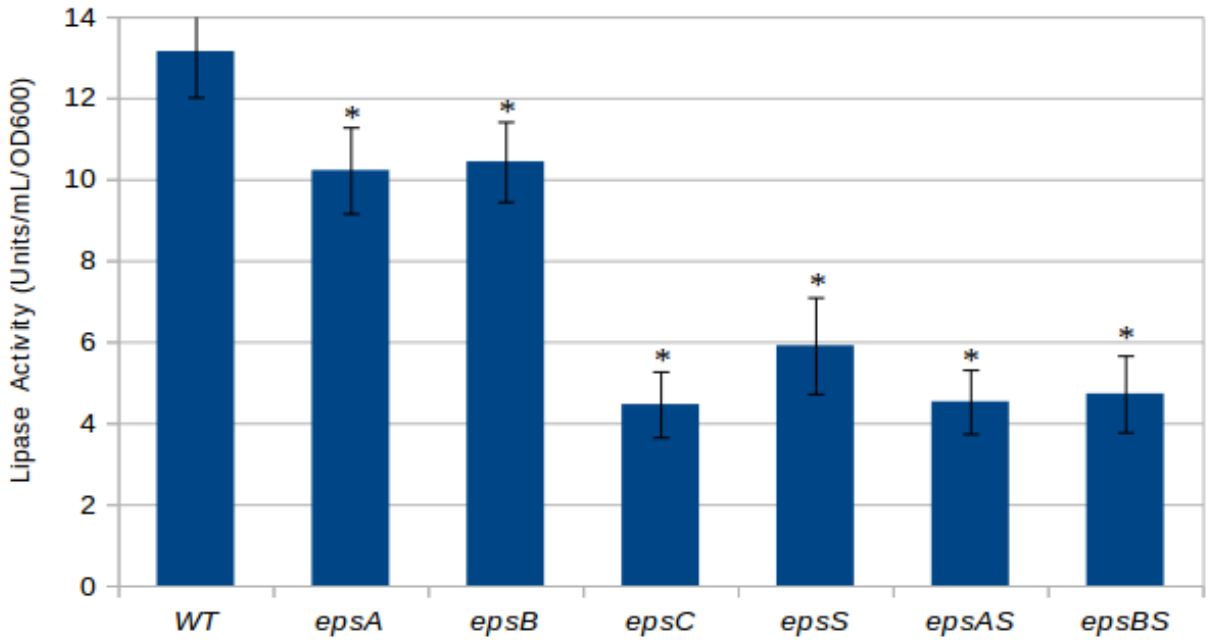


Figure 22. Lipase activity detected in culture supernatant of wild-type and mutant C6706 strains. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was harvested and tested. Data represents the average of four biological replicates. Error bars signify SEM. *Significantly different from WT levels.

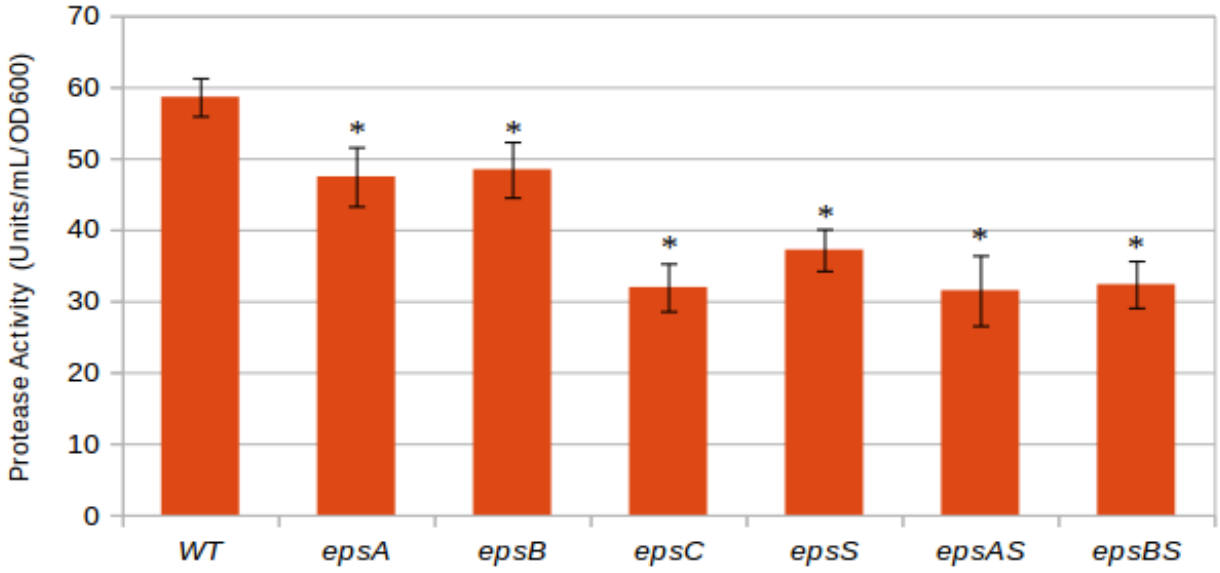


Figure 23. Protease activity detected in culture supernatant of wild-type and mutant C6706 strains. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was harvested and tested. Data represents the average of four biological replicates. Error bars signify SEM. *Significantly different from WT levels.

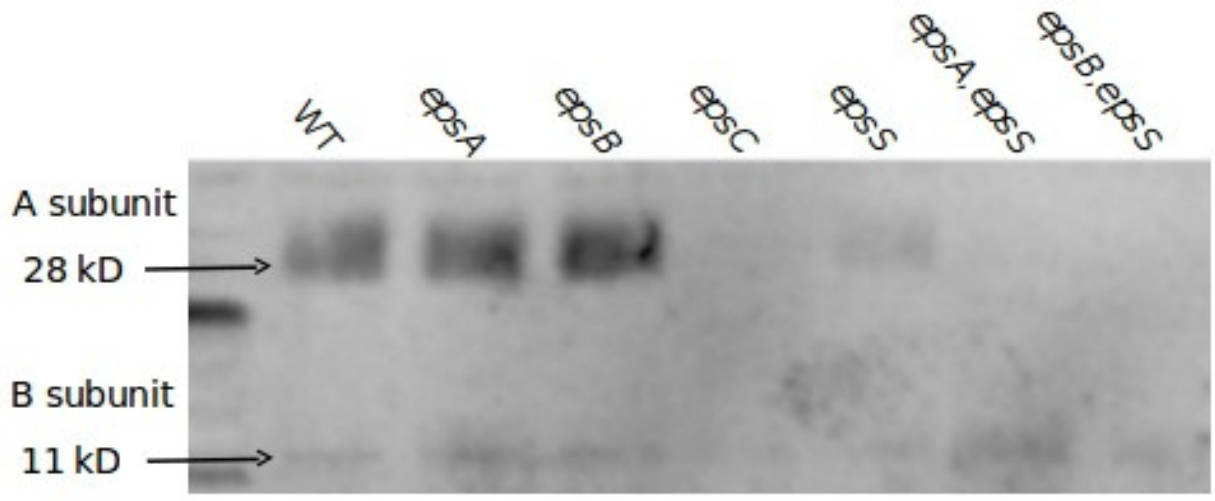


Figure 24. Assay of Cholera Toxin secretion in C6706 strains. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was collected and filtered through a 0.22μm filter. Samples were electrophoresed and immunoblotted with anti-CT. MW of toxin subunits are presented.

5.3 Cross-complementation

To test if EpsS and EpsAB performed redundant actions within the cells they were tested by complementation. Traditionally complementation is used to confirm that a phenotype is the result of the induced mutation. If the plasmid expressing the WT gene restores WT phenotype in the mutant strain, the mutation was wholly responsible for the loss in activity. This is an important control experiment to be done in all of these strains, but by over expressing EpsS in an *epsA* strain, and *vice versa*, it can be determined if the two serve redundant functions, or are distinct.

All clones were constructed using the vector pMMB67HE (Figure 24) and constructed as stated in Section 4.3. Plasmid specifications are listed in Appendix B. The mutant strains containing the vector were induced with 1mM IPTG or as otherwise stated and then tested for secretin assembly and T2SS function.

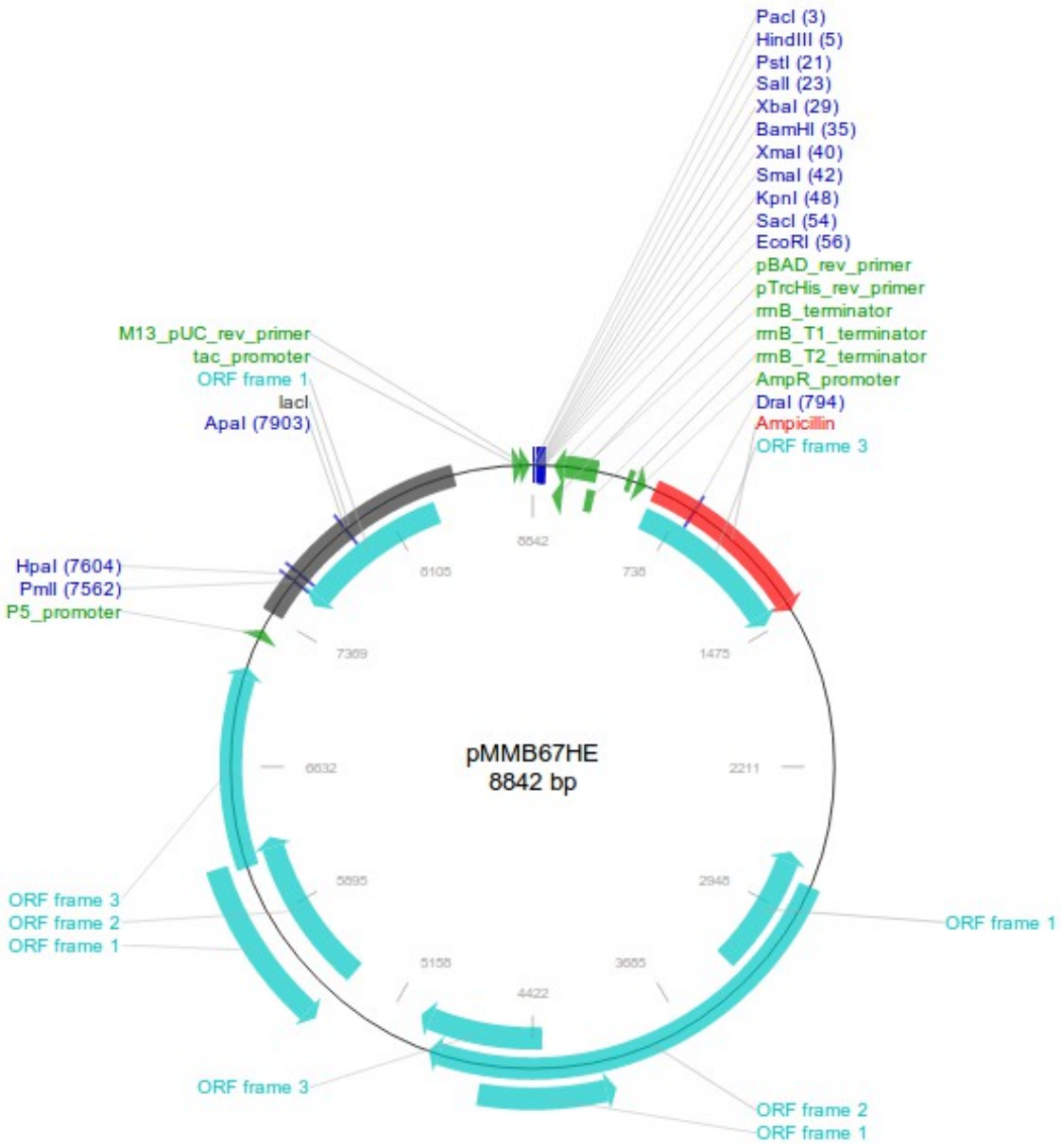


Figure 24. Plasmid Map of pMMB67HE.Plasmid map illustrating key features. (www.addgene.com 2014) All inserts were cloned between the XbaI and EcoRI sites.

5.3.1 Cross-complementation with EpsAB

Cross-complementation of *epsAB* was tested with vector pMMB67HE*epsAB*. It contained the *epsA* and *epsB* operon.

5.3.1.1Cross-complementation with EpsAB in Bah-2

Secretin assembly was complemented in the *epsA* mutant. No difference could be seen for *epsS* mutants between strains containing induced pMMB67HEepsAB and the mutants carrying an empty plasmid (Figure 25).

Complementation was more visible in the lipase and protease results. For both the lipase (Figure 26) and protease (Figure 27), epsAB partially complemented *epsS* at 1mM IPTG. The results also showed successful complementation of both *epsA* to WT levels and *epsAS* to *epsS* levels.

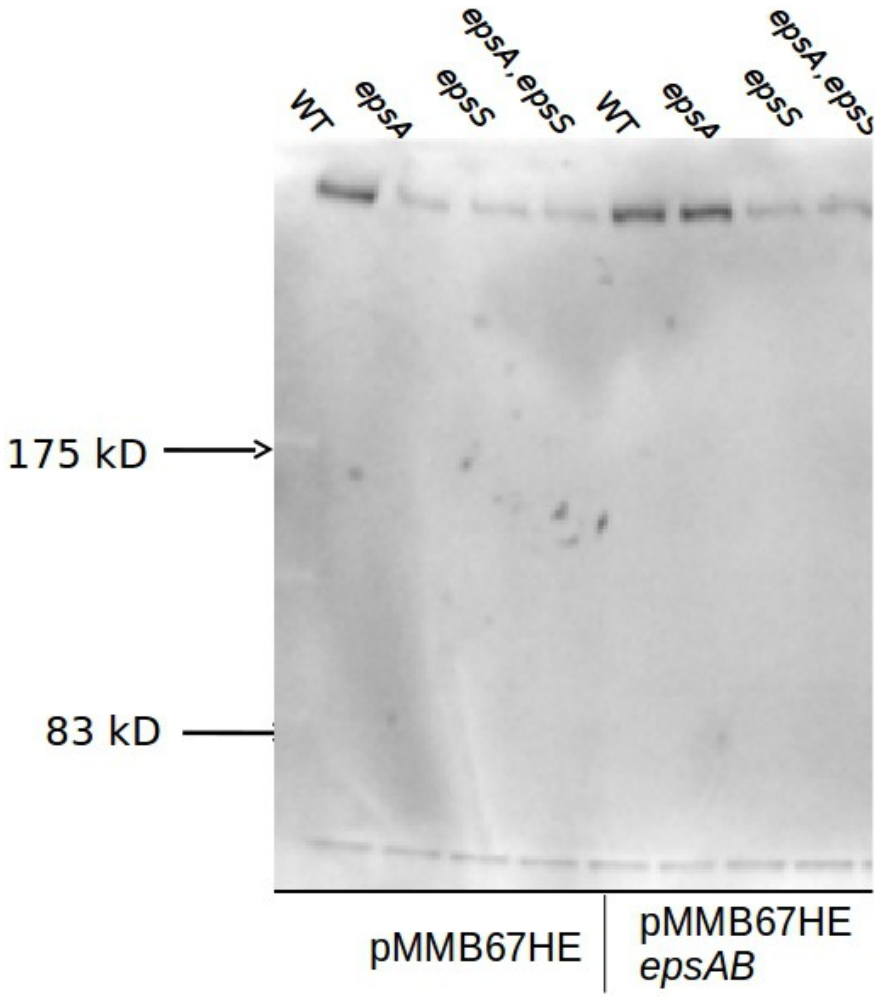


Figure 25. Assembly of the Secretin from EpsD monomers in Bah-2 mutant strains shows complementation of *epsA* with pMMB67HE*epsAB*. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and whole cell samples were electrophoresed and immunoblotted with anti-EpsD. MW of standard protein markers are given. Samples were compared which contained the empty vector (pMMB67HE) and those that contained pMMB67HE*epsAB* induced with 1mM IPTG.

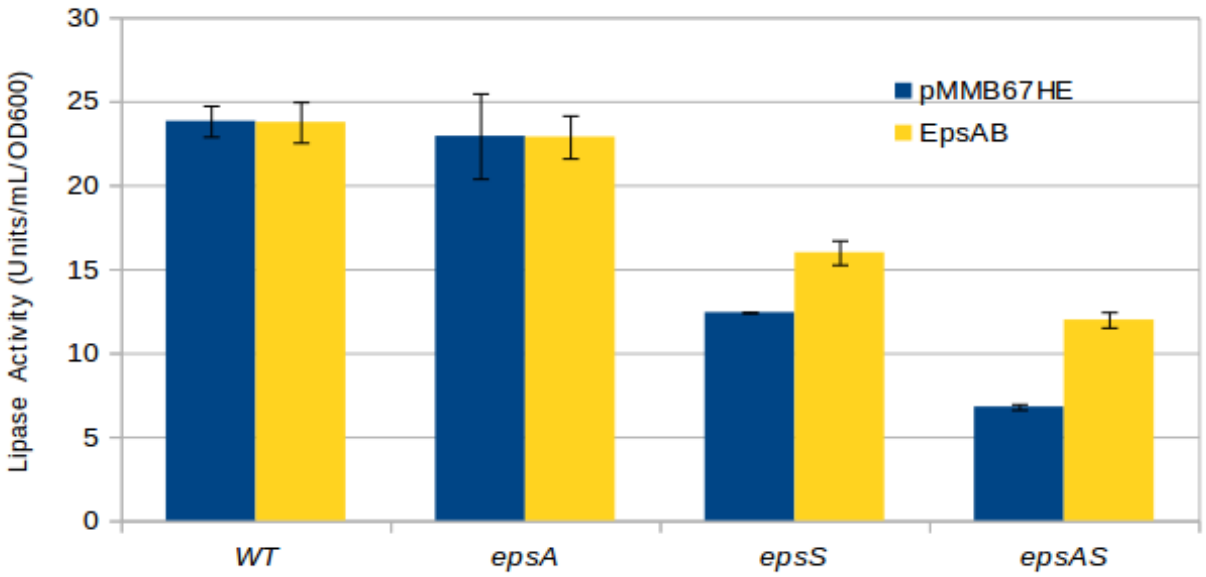


Figure 26. EpsAB can restore lipase activity in *epsA* mutants of Bah-2 to WT levels . Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 in the presence of 1mM IPTG and supernatant was harvested and tested. Data represents a single biological sample with three technical replicates.

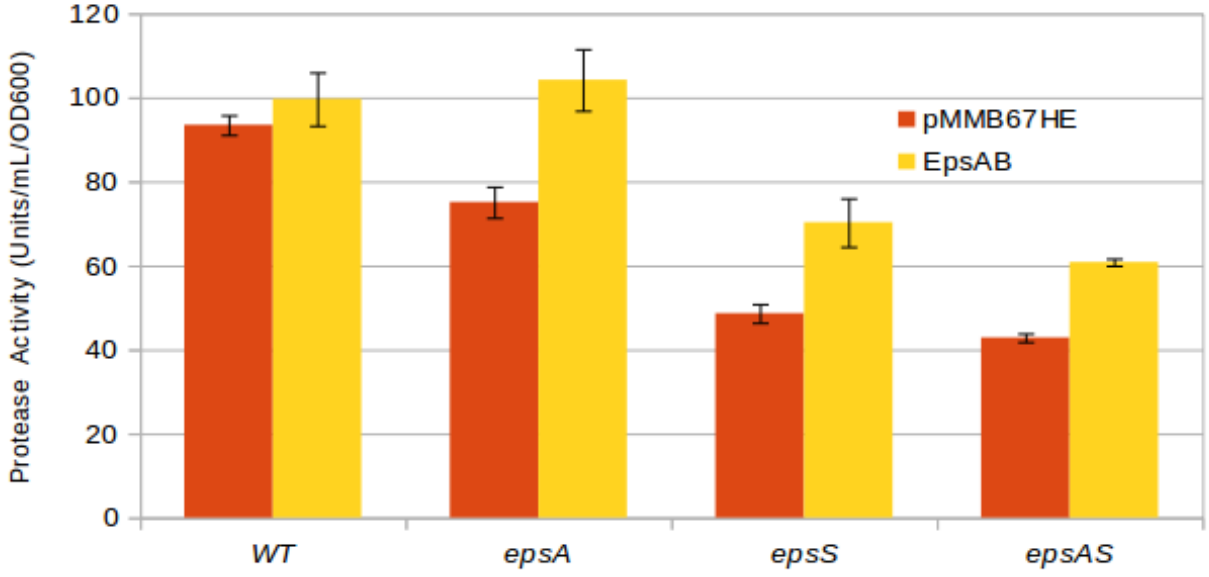


Figure 27. EpsAB can restore protease activity in *epsA* mutants of Bah-2 to WT levels . Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 in the presence of 1mM IPTG and supernatant was harvested and tested. Data represents a single biological sample with three technical replicates.

To determine if complementation of *epsS* by pMMB67HE*epsAB* was the result of over-expression 4 levels of IPTG were tested. A secretin assembly blot (Figure 28) was used to assay the strains. All induction levels increased the amount of secretin multimer in WT and *epsA* strains, but no visible complementation of the *epsS* mutation was observed.

Lipase (Figure 29) and protease (Figure 30) data was also obtained, and demonstrates that the *epsS* mutation was partially complemented at the highest expression level. All levels of induction successfully complemented the *epsA* mutation in the *epsA* and *epsAS* strains.

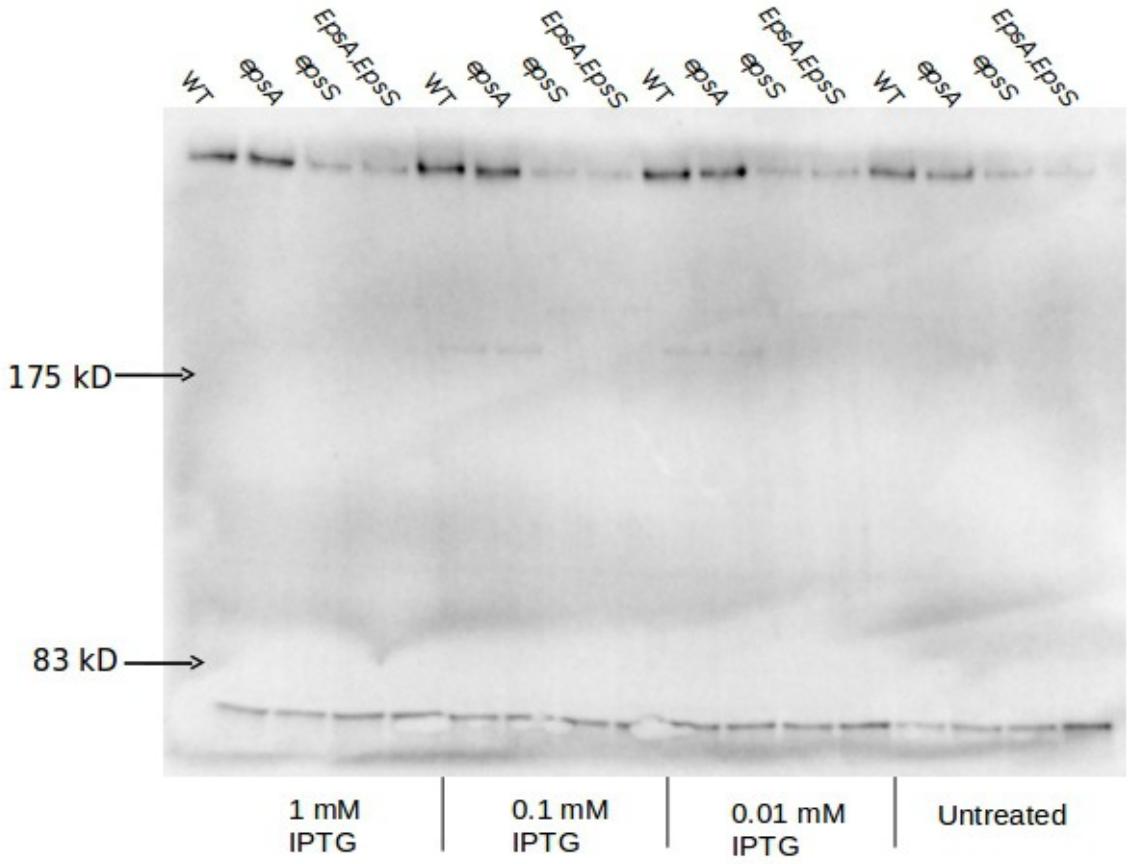


Figure 28. *epsA* is complemented for secretin assembly in Bah-2 with pMMB67HE*epsAB*. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and whole cell samples were electrophoresed and immunoblotted with anti-EpsD . Locations of standard protein markers are given. Levels of IPTG are given.

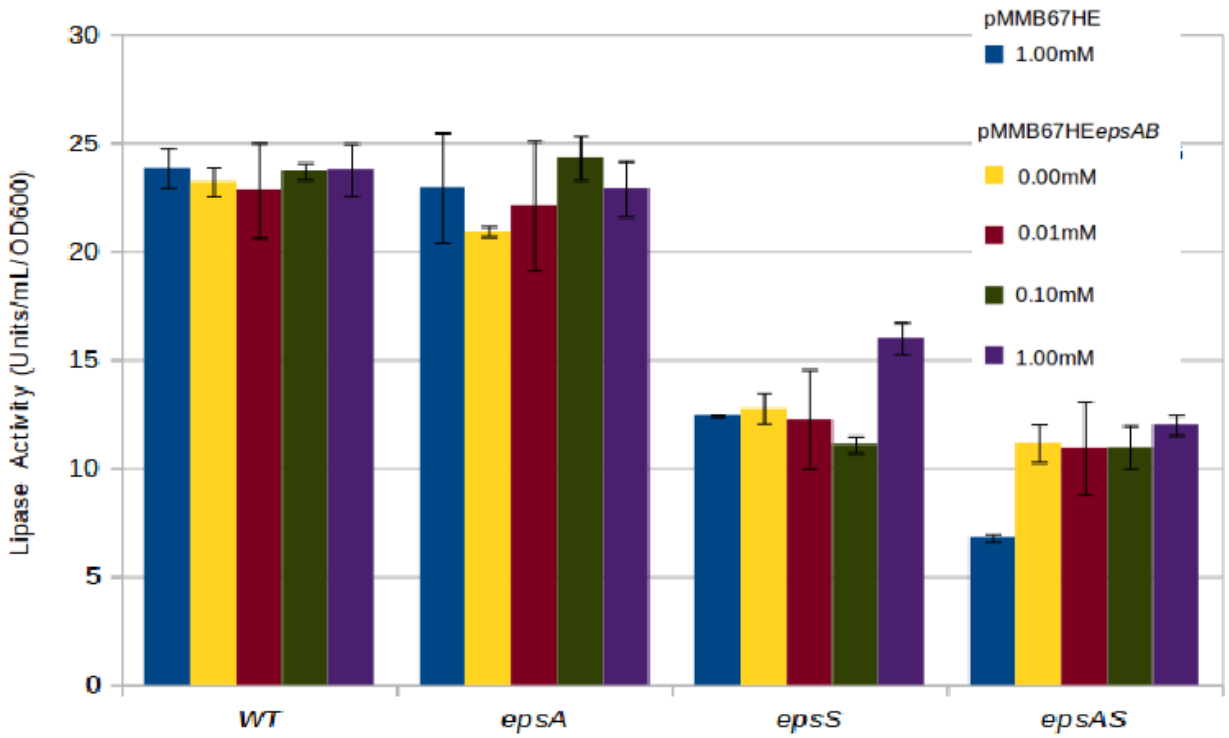


Figure 29. EpsAB can restore lipase activity in *epsA* mutants of Bah-2 to WT levels. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 in the presence of IPTG and supernatant was harvested and tested. IPTG levels are given. Data represent a single biological sample with three technical replicates. Error bars represent standard deviation.

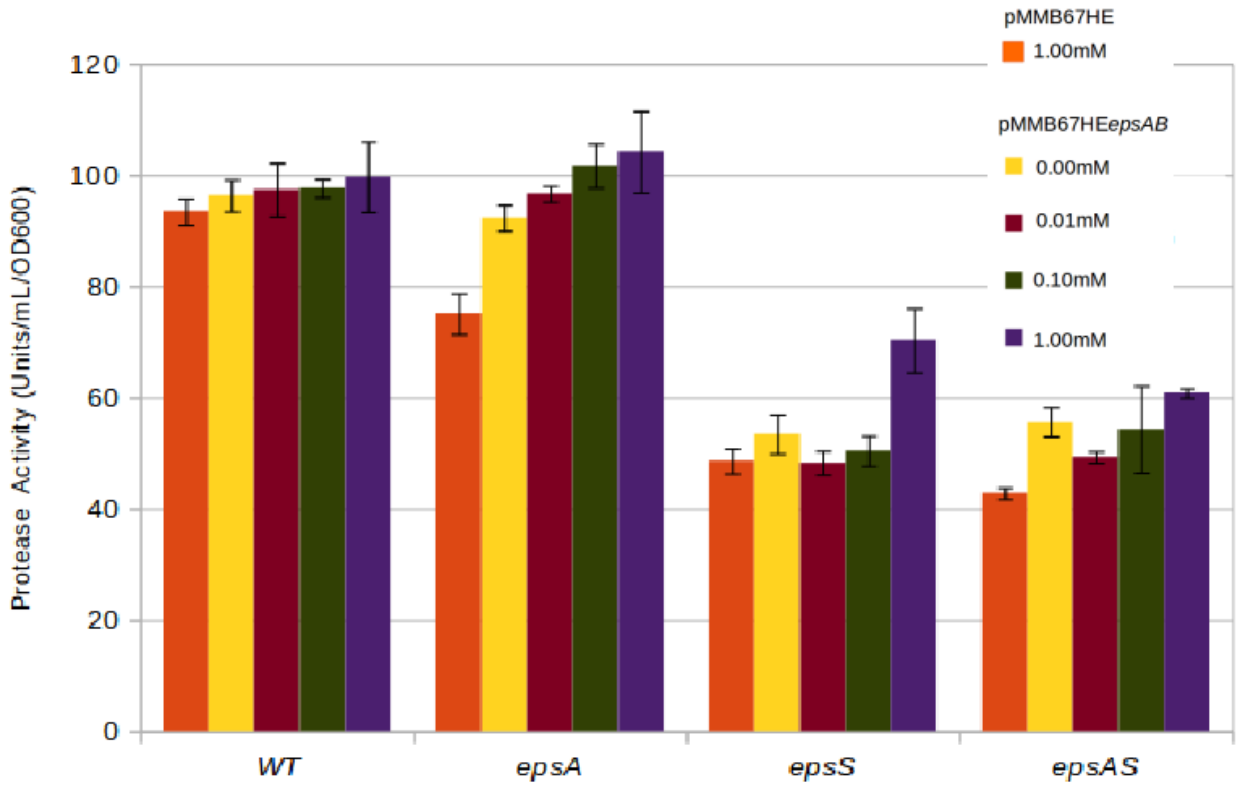


Figure 30. EpsAB can restore protease activity in *epsA* mutants of Bah-2 to WT levels. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 in the presence of IPTG and supernatant was harvested and tested. IPTG levels are given. Data represent a single biological sample with three technical replicates. Error bars represent standard deviation.

5.3.1.2 Cross-complementation with EpsAB in C6706

C6706 was tested more intensively and four biological replicates were tested for *epsAB* complementation.

The strains containing *epsA* mutations were successfully complemented in this experiment. This is apparent from the multimer bands on the secretin assembly blot (Figure 31), as WT and *epsA* strains show similar bands when complemented. *epsS* and *epsAS* are similar and show reduced levels compared to WT. No visible difference is discernible between the two WT strains or the two *epsS* strains.

Complementation was more visible in the lipase and protease results. The lipase data (Figure 32**)** show complementation of the *epsA* mutation and partial complementation of the *epsS* mutation. The protease (Figure 33) results showed successful complementation of both *epsA* to WT levels and *epsAS* to *epsS* levels. Cholera toxin secretion was also complemented (Figure 34).

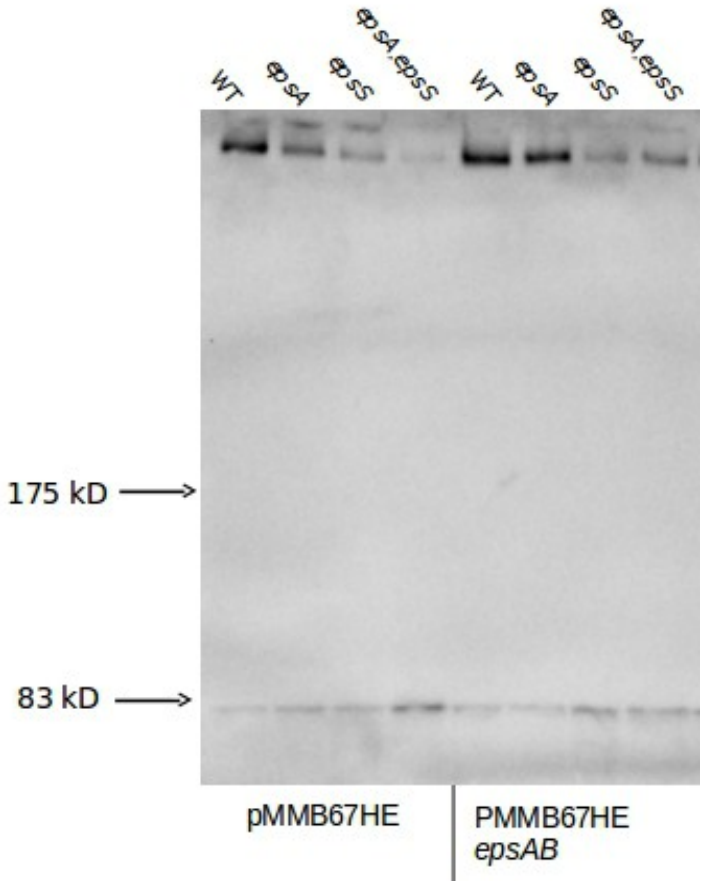


Figure 31. EpsAB can restore secretin assembly in *epsA* mutants to WT levels in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and whole cell samples were electrophoresed and immunoblotted with anti-EpsD . Locations of standard protein markers are given. Samples were compared which contained the empty vector (pMMB67HE) and those which contained pMMB67HE*epsAB* and induced at 1mM IPTG.

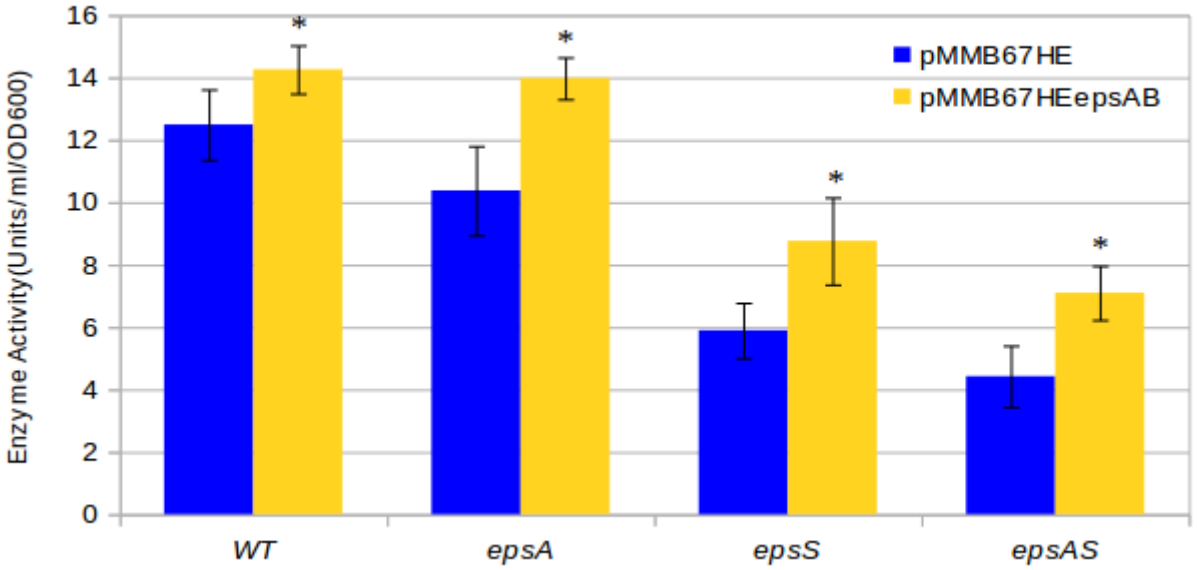


Figure 32. EpsAB can restore lipase activity in *epsA* mutants to WT levels in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was harvested and tested. Data represent the average of four biological replicates. Error bars signify SEM. *Significantly different from the same strain containing an empty vector.

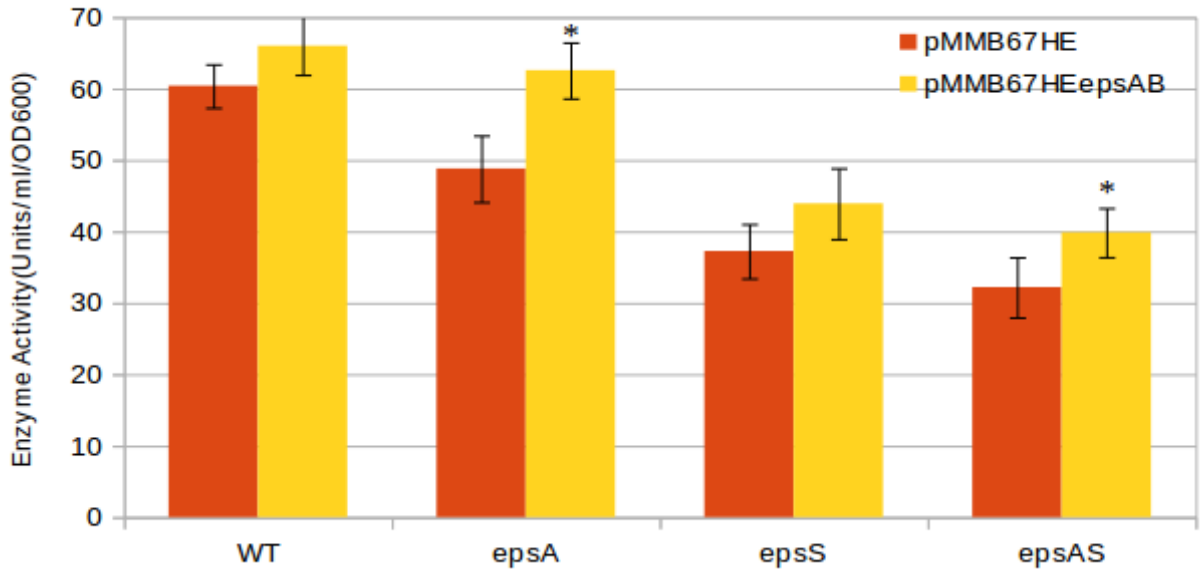


Figure 33. EpsAB can restore protease activity in *epsA* mutants to WT levels in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was harvested and tested. Data represent the average of four biological replicates. Error bars signify SEM. *Significantly different from the same strain containing an empty vector.

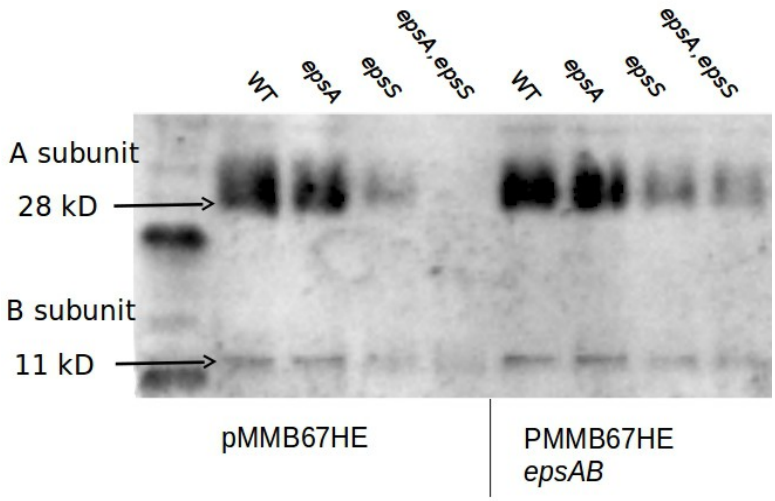


Figure 34 EpsAB can restore Cholera toxin secretion in *epsA* mutants to WT levels in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was collected and filtered through a 0.22µm filter. Samples were electrophoresed and immunoblotted with anti-CT . MW of toxin subunits is given.

A gradient of IPTG concentrations was performed in C6706 in an identical manner to Bah-2 and the results for secretin assembly (Figure 35) show complementation of *epsA* at all induction levels. The lipase activity defect was complemented in both *epsA* mutant strains at all induction levels (Figure 36). Interestingly WT levels of lipase secretion were increased and the *epsS* mutation was partially complemented by pMMB67HE*epsA* induction. Conversely the protease assay (Figure 37) demonstrated only complementation of the *epsA* mutation and the increase in the *epsS* mutant was not significant.

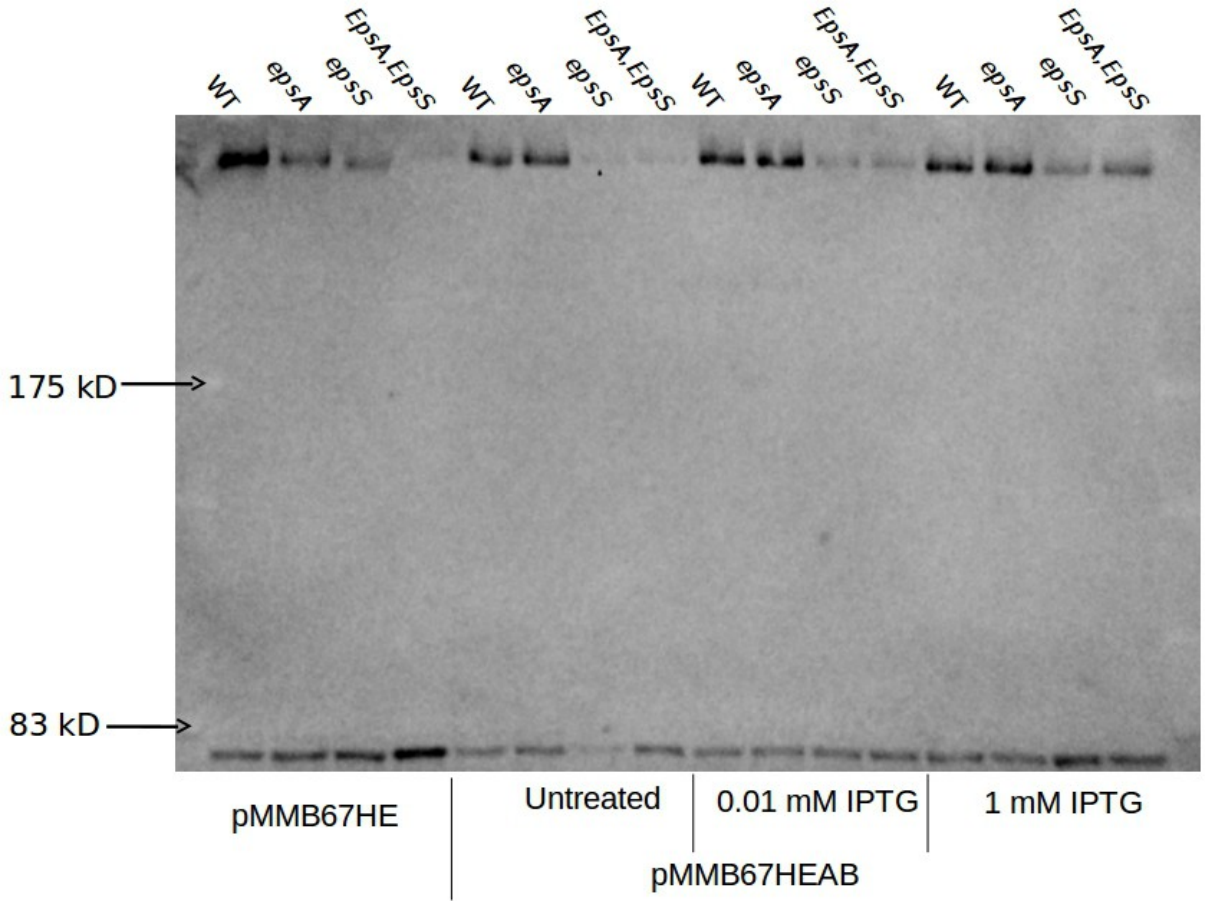


Figure 35. Gradient induction of pMMB67HE*epsAB* testing secretin assembly in C6706. The *epsA* secretin defects are successfully restored with pMMB67HE*epsAB* in C6706 at all induction levels. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and whole cell samples were electrophoresed and immunoblotted with anti-EpsD . Locations of standard protein markers are given. Samples were compared which contained the empty vector (pMMB67HE) and those that contained pMMB67HE*epsAB*. Concentration of IPTG is given. Blot is representative of 4 replicates

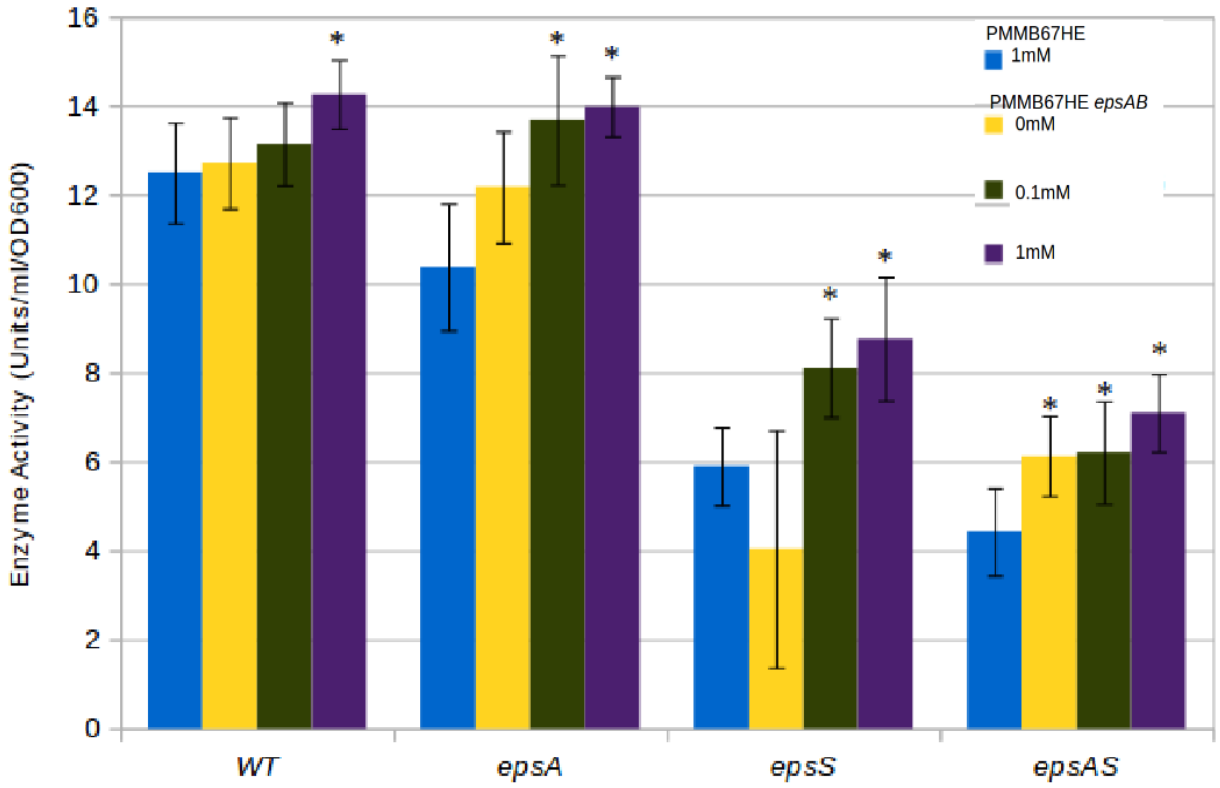


Figure 36. *epsA* lipase defects were restored in all strains with pMMB67HE*epsAB* and high induction levels partially complement the *epsS* mutation in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested Data represent the average of four biological replicates. Error bars signify SEM. *Significantly different from identical strains containing the empty vector.

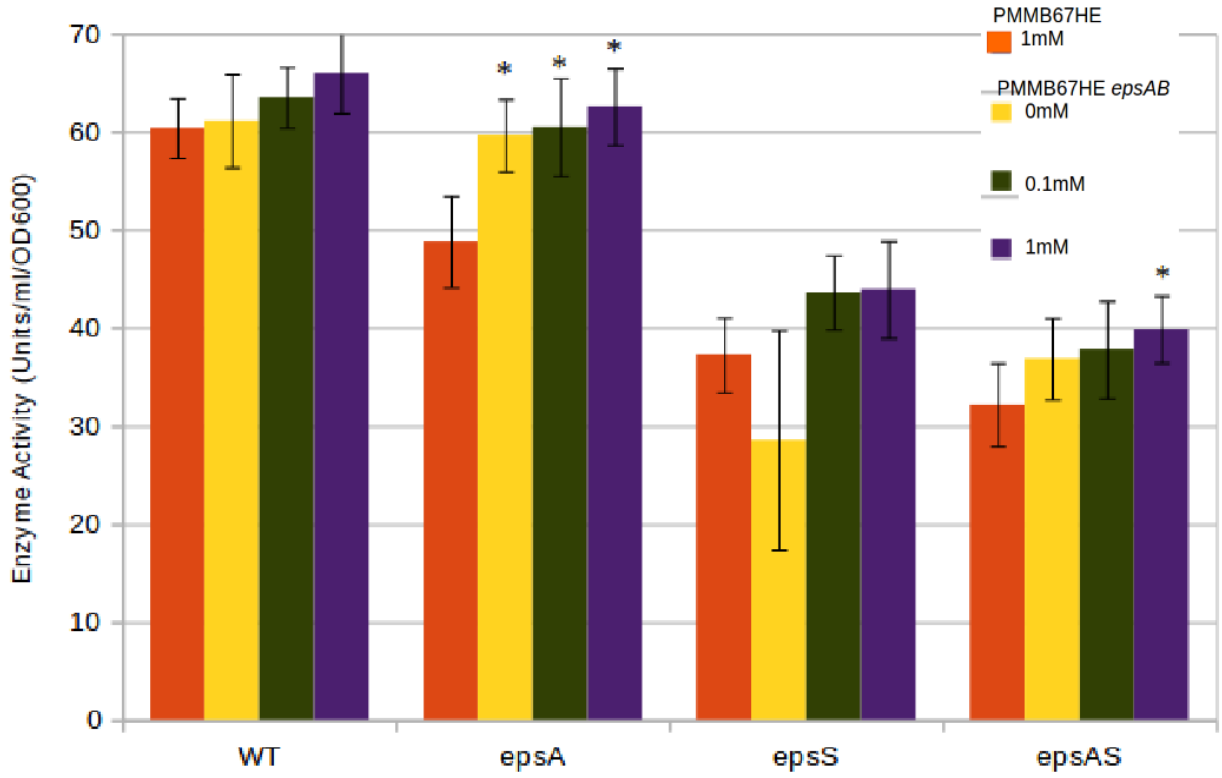


Figure 37. Protease activity was restored in all strains with pMMB67HE*epsAB* at all induction levels in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent the average of four biological replicates. Error bars signify SEM.
*Significantly different from identical strains containing the empty vector.

5.3.2 Cross-complementation with EpsS

Cross-complementation of EpsS was performed with several different variations of the *epsS* gene. None of these attempts were successful without an additional gene induced.

5.3.2.1 Cross-complementation with pMMB67HELC and pMMB67HELM clone

To complement *epsS,* a clone was constructed (pMMB67HELC) which contained the annotated start codon of VC1703. This start codon is within the ORF of the upstream gene, VC1702. For visualization please see Figure 13. Figures 38 and 40 demonstrate that neither a Myc tagged (pMMB67HELM), or native stop codon (pMMB67HELC) of this clone complement any mutants' lipase activity in either *Vibrio* strain. Figures 39 and 41 show the same with protease activity.

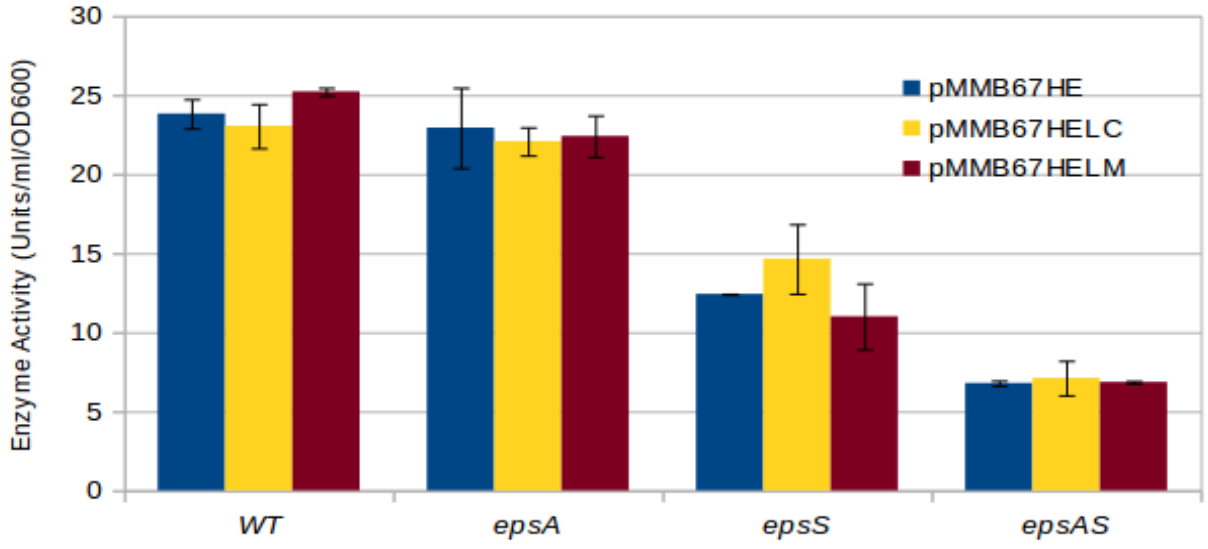


Figure 38. Lipase activity was unchanged with induction of pMMB67HELC or pMMB67HELM in Bah-2. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

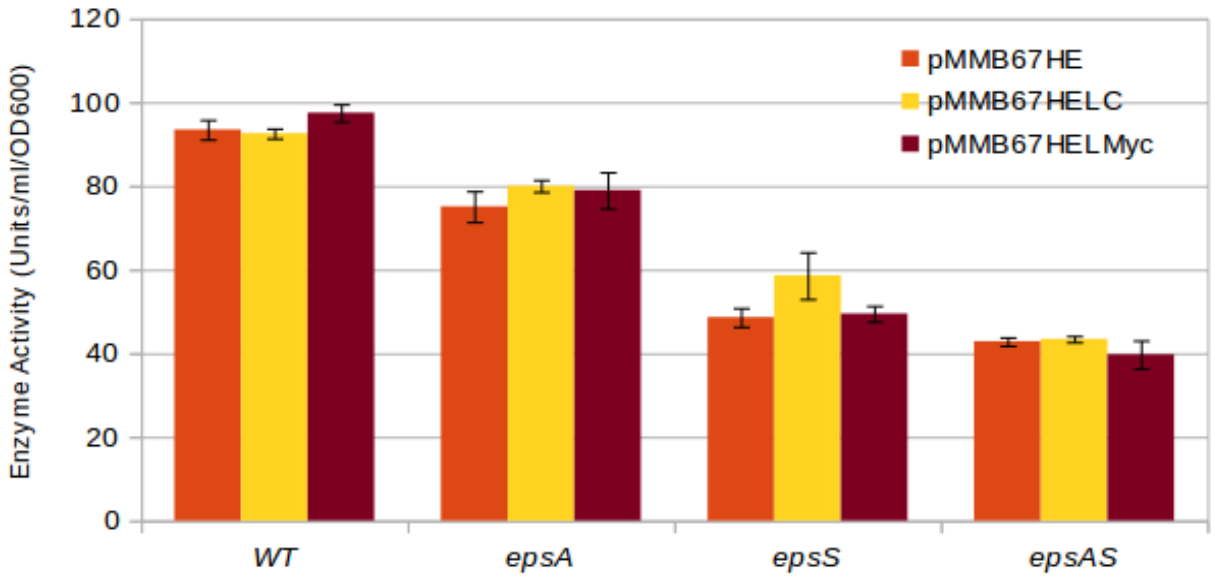


Figure 39. Protease activity was unchanged with induction of pMMB67HELC or pMMB67HELM in Bah-2. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

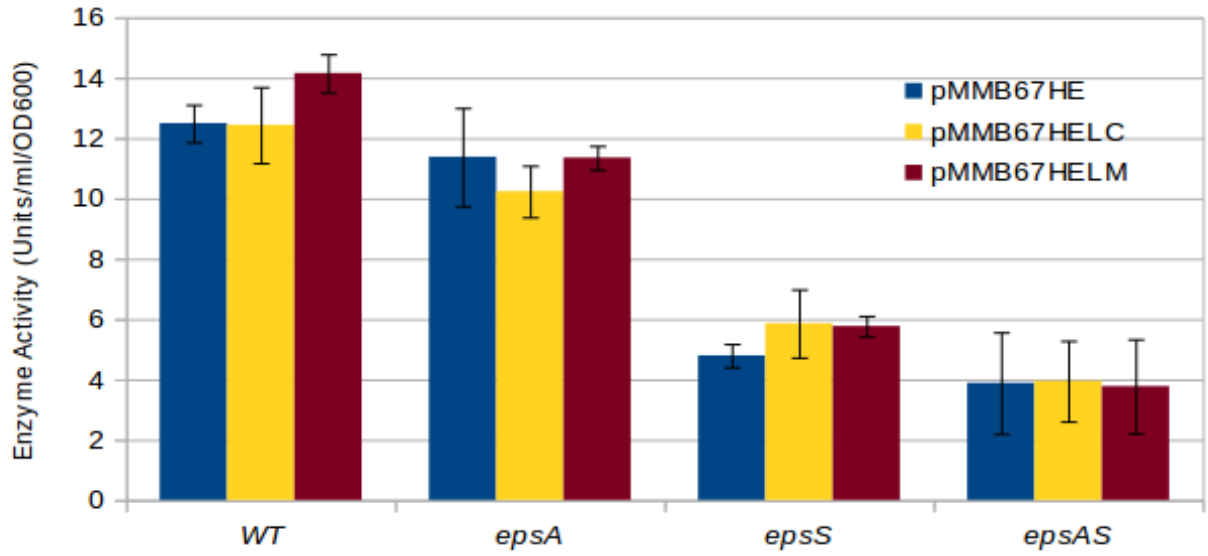


Figure 40. Lipase activity was unchanged with induction of pMMB67HELC or pMMB67HELM in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

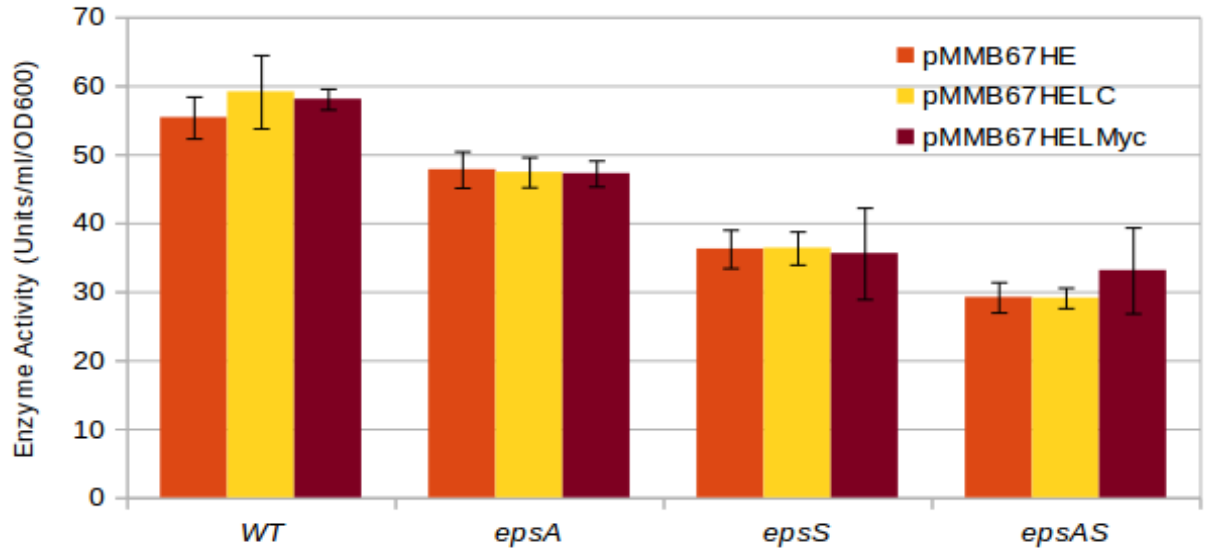


Figure 41. Lipase activity was unchanged with induction of pMMB67HELC or pMMB67HELM in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

5.3.2.2 Cross-complementation with pMMB67HESC and pMMB67HESM clone

As the annotated genbank start codon failed to complement, it was thought a shorter construct may complement as it had previously been shown that the length of the protein corresponded to a later start codon (Rininsland *et. al.* 2012).

As such clones were constructed with this shorter ORF in the same manner as the pMMB67HELM and pMMB67HELC clones. The region of cloned DNA is shown in Figure 13. These clones also failed to complement the mutant strain deficiencies in lipase and protease secretion for Bah-2. (Figures 42 and 43)

C6706 was tested with 4 biological replicates, but no complementation of the deficiency in secretin assembly (Figure 44**),** lipase secretion (Figure 45**)**, or protease secretion (Figure 46) was observed.

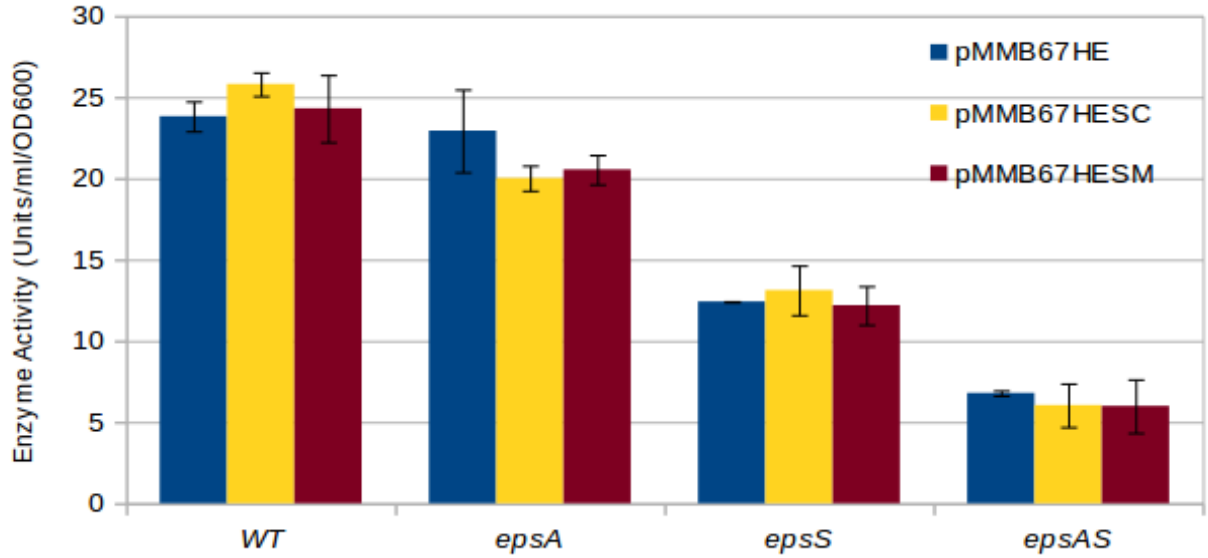


Figure 42. Lipase activity was unchanged with induction of pMMB67HESC or pMMB67HESM in Bah-2. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

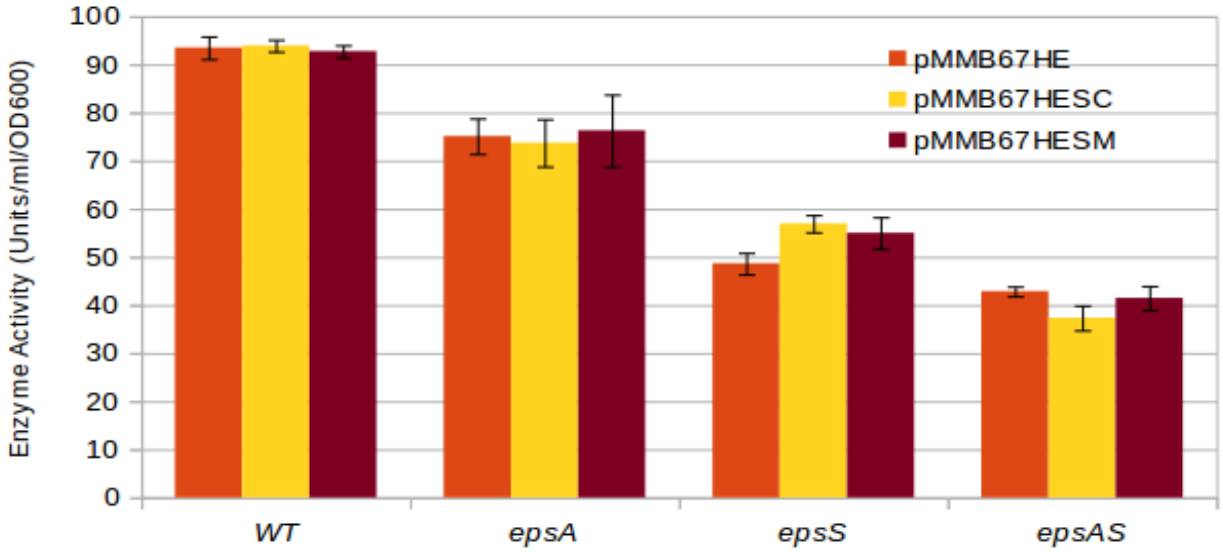


Figure 43. Protease activity is unchanged with induction of pMMB67HESC or pMMB67HESM in Bah-2. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

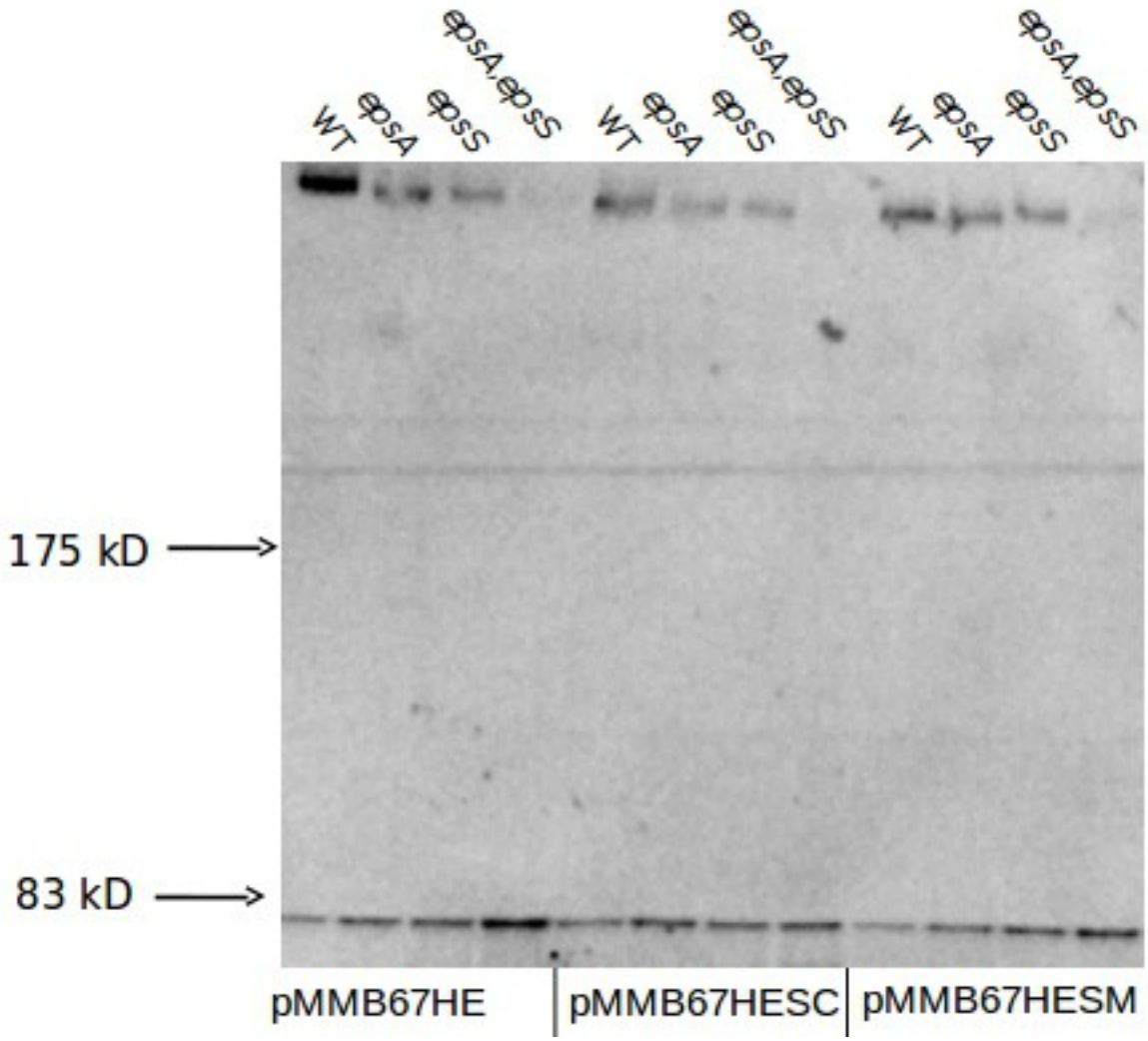


Figure 44. Secretin assembly was unchanged with induction of pMMB67HESC or pMMB67HESM in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and whole cell samples were electrophoresed and immunoblotted with anti-EpsD. Locations of standard protein markers are given.

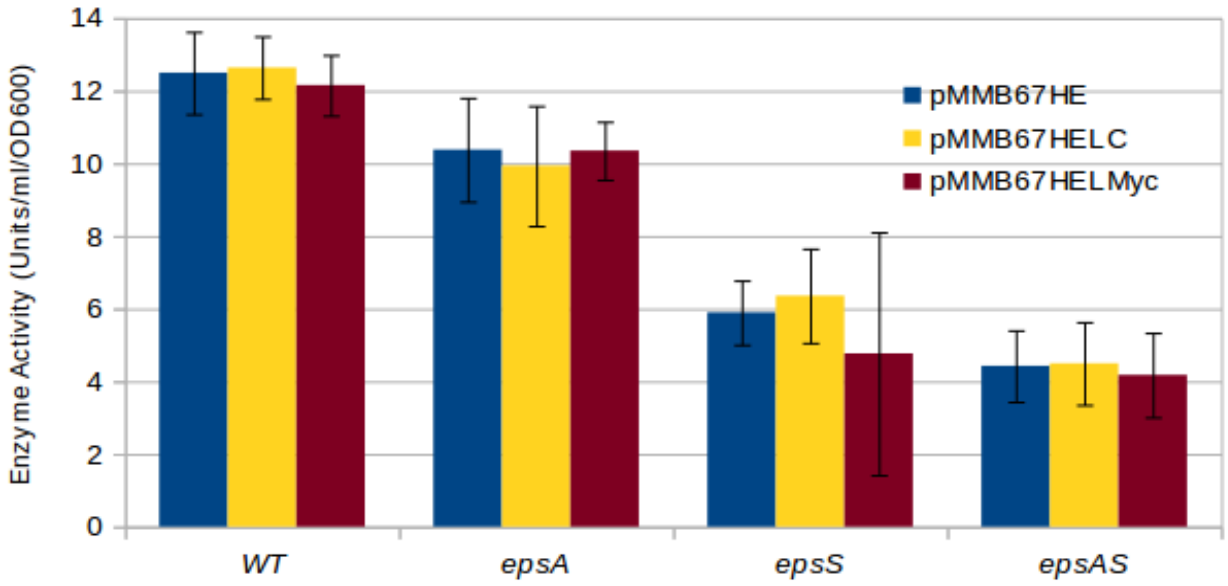


Figure 45. Lipase activity was unchanged with induction of pMMB67HESC or pMMB67HESM in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent the average of four biological replicates. Error bars signify SEM.

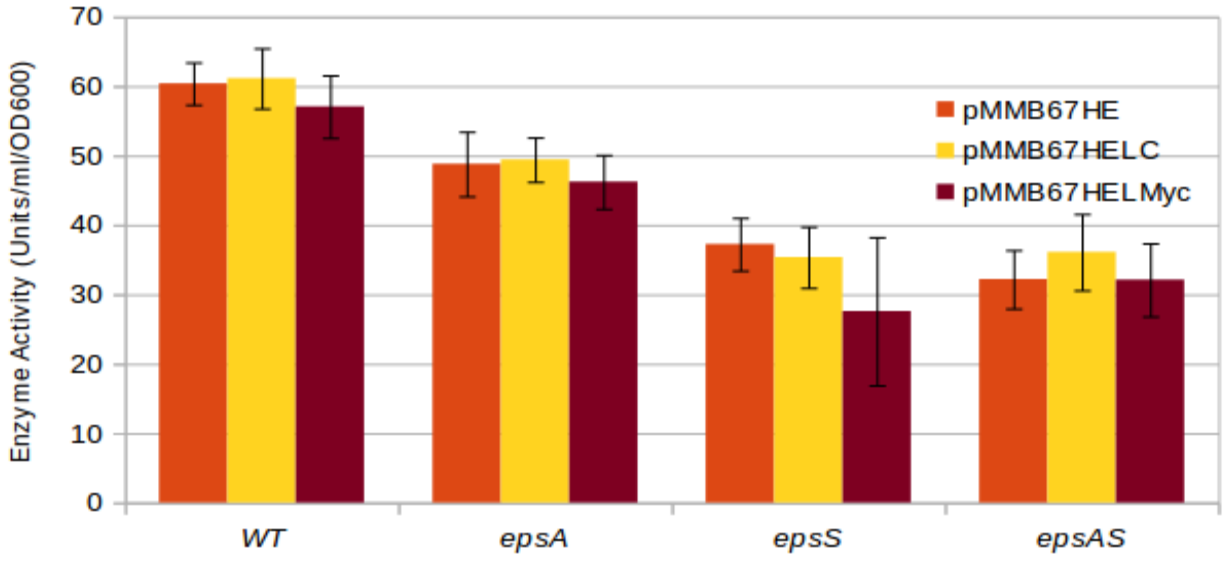


Figure 46. Protease activity was unchanged with induction of pMMB67HESC or pMMB67HESM in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent the average of four biological replicates. Error bars signify SEM.

5.3.3 Cross-complementation with *epsS* and VC1702

In another attempt to complement *epsS* a new construct was made. This construct, pMMB67HEL2C contained the operon containing VC1702 (unknown function) and VC1703 (e*psS*). See Figure 13 for representation of the genomic region cloned. This clone successfully complemented all mutations.

5.3.3.1 Cross-complementation with pMMB67HEL2C in Bah-2

In an identical method to that used with the epsAB clones, the operon containing the pilotin was first tested against an empty vector. The secretin assembly blot (Figure 47) not only showed complementation, but assembly which was increased from the WT phenotype. The same samples also had increased lipase (Figure 48) and protease (Figure 49) above wild-type levels in all strains.

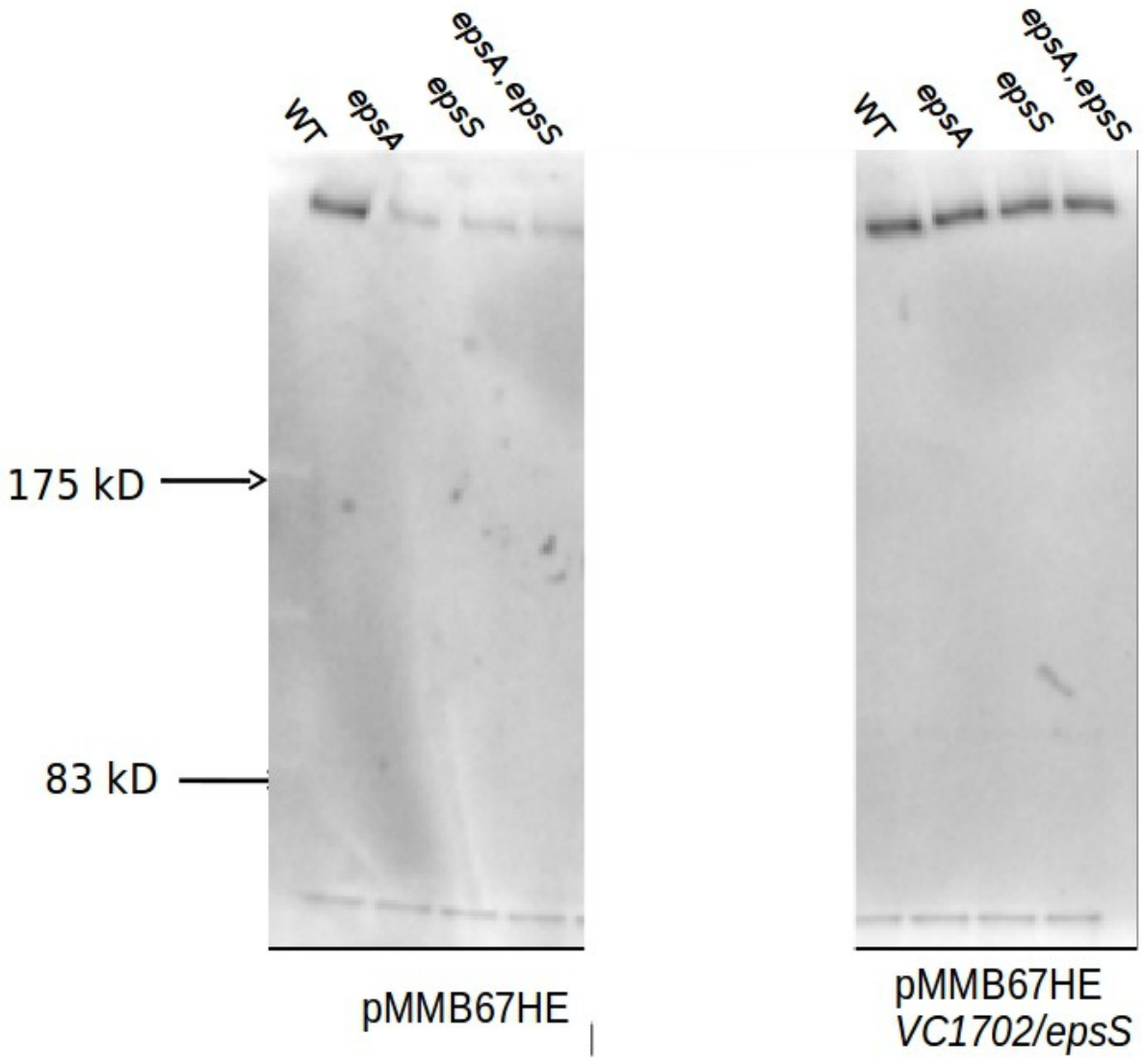


Figure 47. Induction of the VC1702/EpsS operon restored secretin assembly in Bah-2. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 in the presence of 1mM IPTG and whole cell samples were electrophoresed and immunoblotted with anti-EpsD. Locations of standard protein markers are given. Samples were compared which contained the empty vector (pMMB67HE) and those that contained the L2C plasmid.

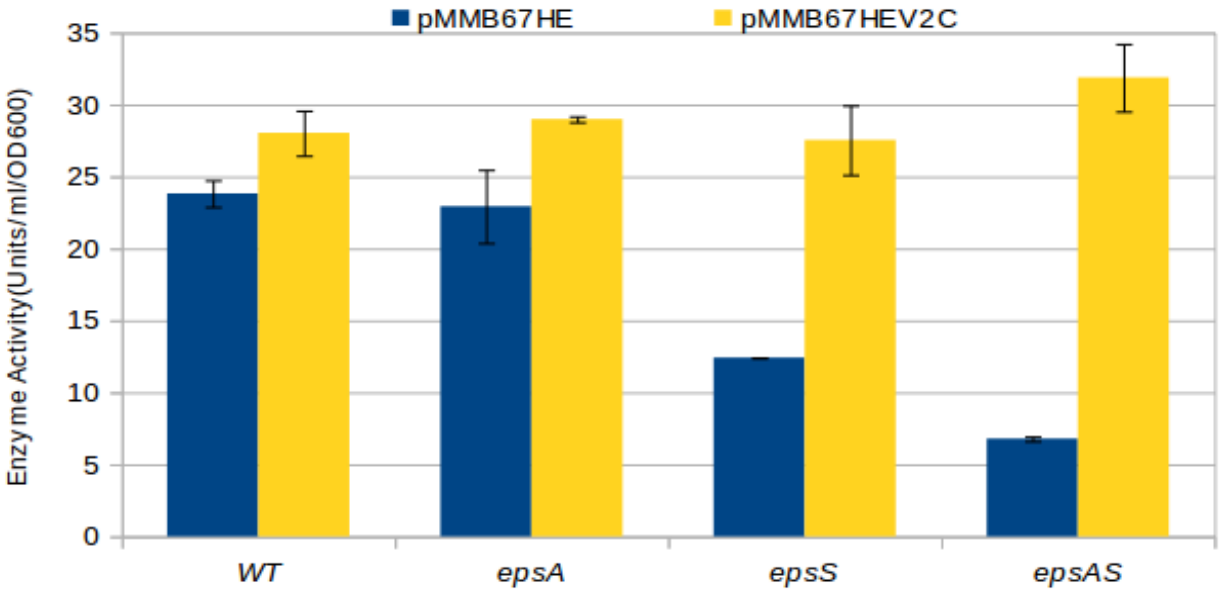


Figure 48. Induction of pMMB67HEL2C restored lipase secretion in all mutants of Bah-2. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

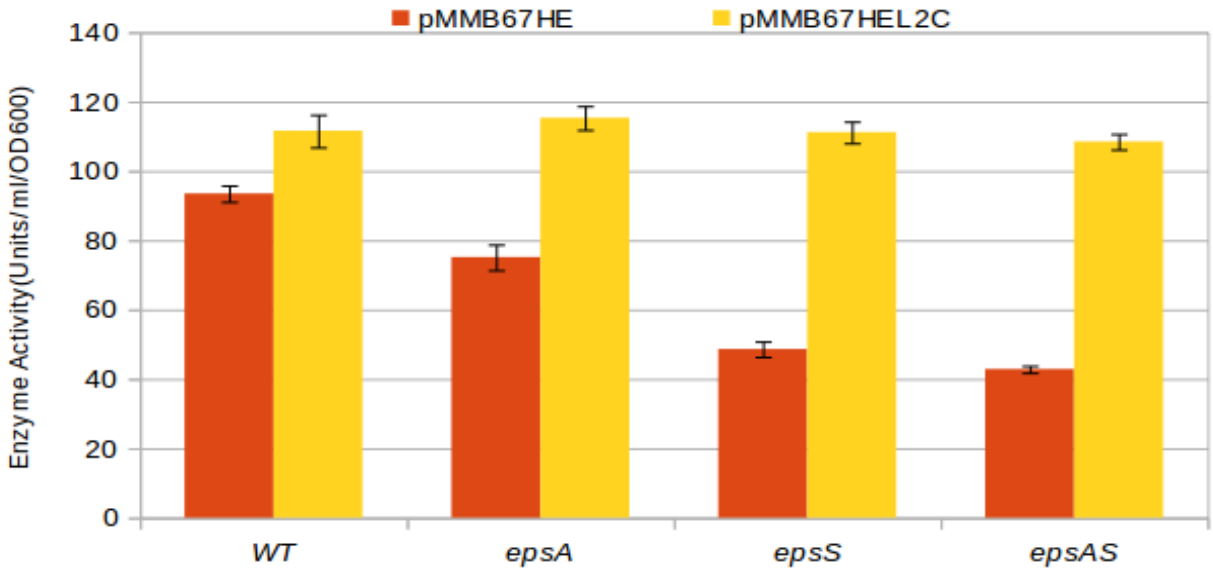


Figure 49. Induction of pMMB67HEL2C restored protease secretion in Bah-2. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

In a similar manner to the *epsAB* clones the pMMB67HEL2C clones were tested on a gradient of IPTG concentration. The secretin assembly blot (Figure 50) shows increased assembly in all induced strains in comparison to the uninduced plasmid. This matches well with the lipase (Figure 51) and protease assays (Figure 52).

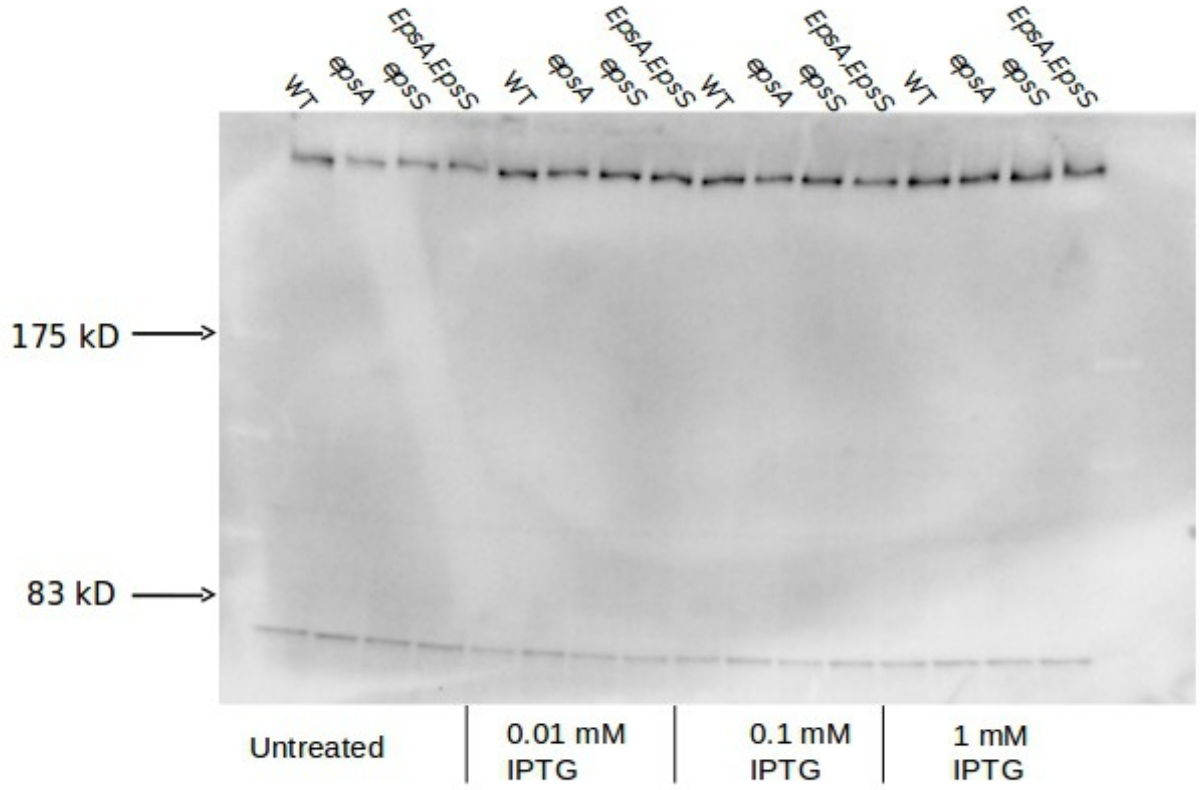


Figure 50. Induction of pMMB67HEL2C restored secretin assembly in all strains of Bah-2 at all induction levels. Presence of the pMMB67HEL2C restored the WT phenotype in all mutants. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and whole cell samples were electrophoresed and immunoblotted with anti-EpsD . Locations of standard protein markers are given. Levels of IPTG are given.

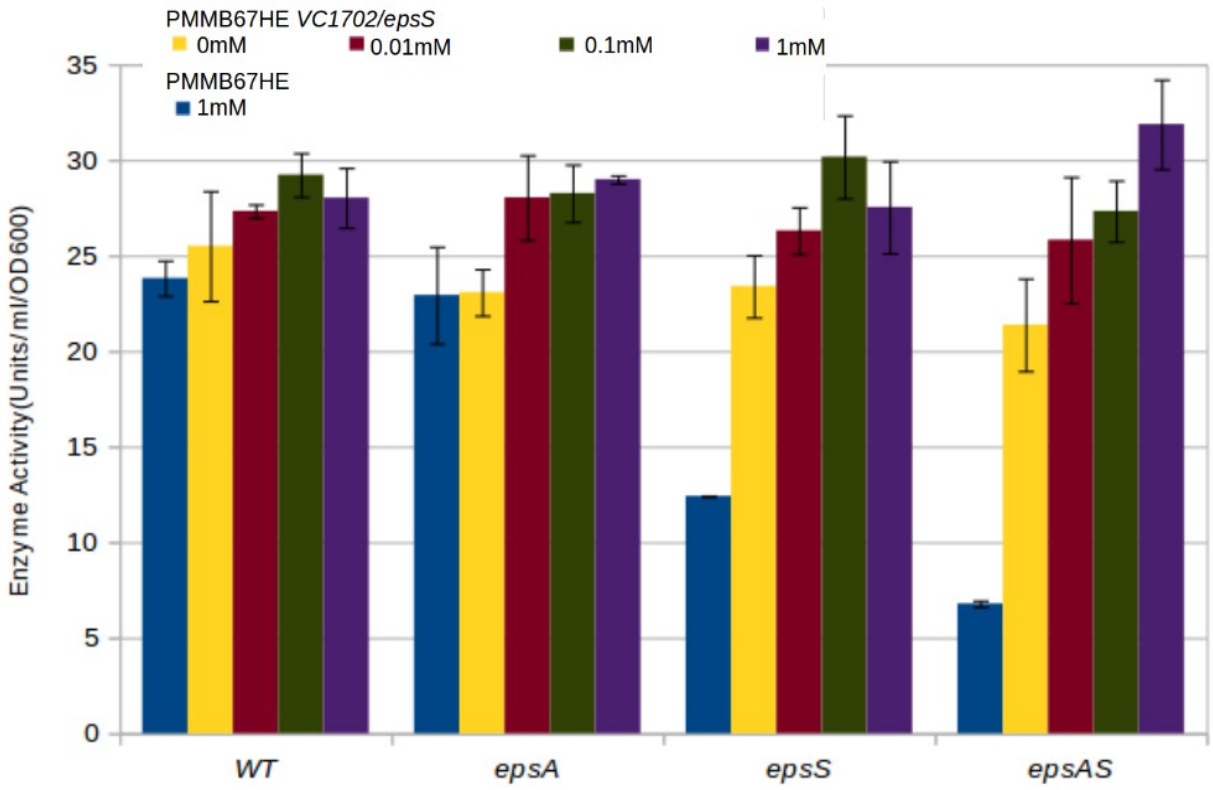


Figure 51. pMMB67HEL2C complemented lipase secretion in Bah-2 at all induction levels. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

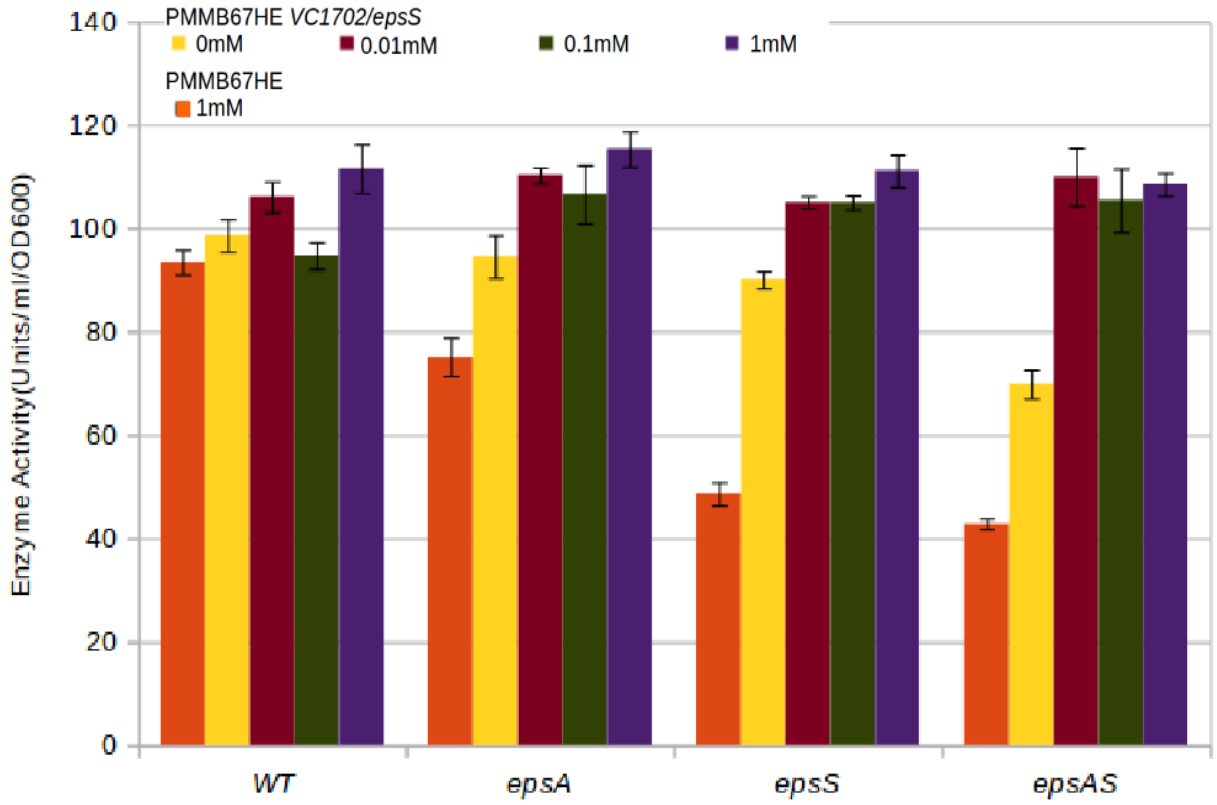


Figure 52. pMMB67HEL2C restored protease secretion in Bah-2 at all induction levels. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

5.3.3.1 Cross-complementation with EpsS and VC1702 in C6706

The C6706 strain was tested in the same manner as Bah-2 with pMMB67HEL2C. The secretin assembly blot (Figure 53) not only showed complementation of the mutation phenotype, but assembly which was increased from the WT phenotype. The same samples also showed increased lipase (Figure 54) and protease (Figure 55) above wild-type levels in all strains. Complementation was seen in cholera toxin secretion (Figure 56)

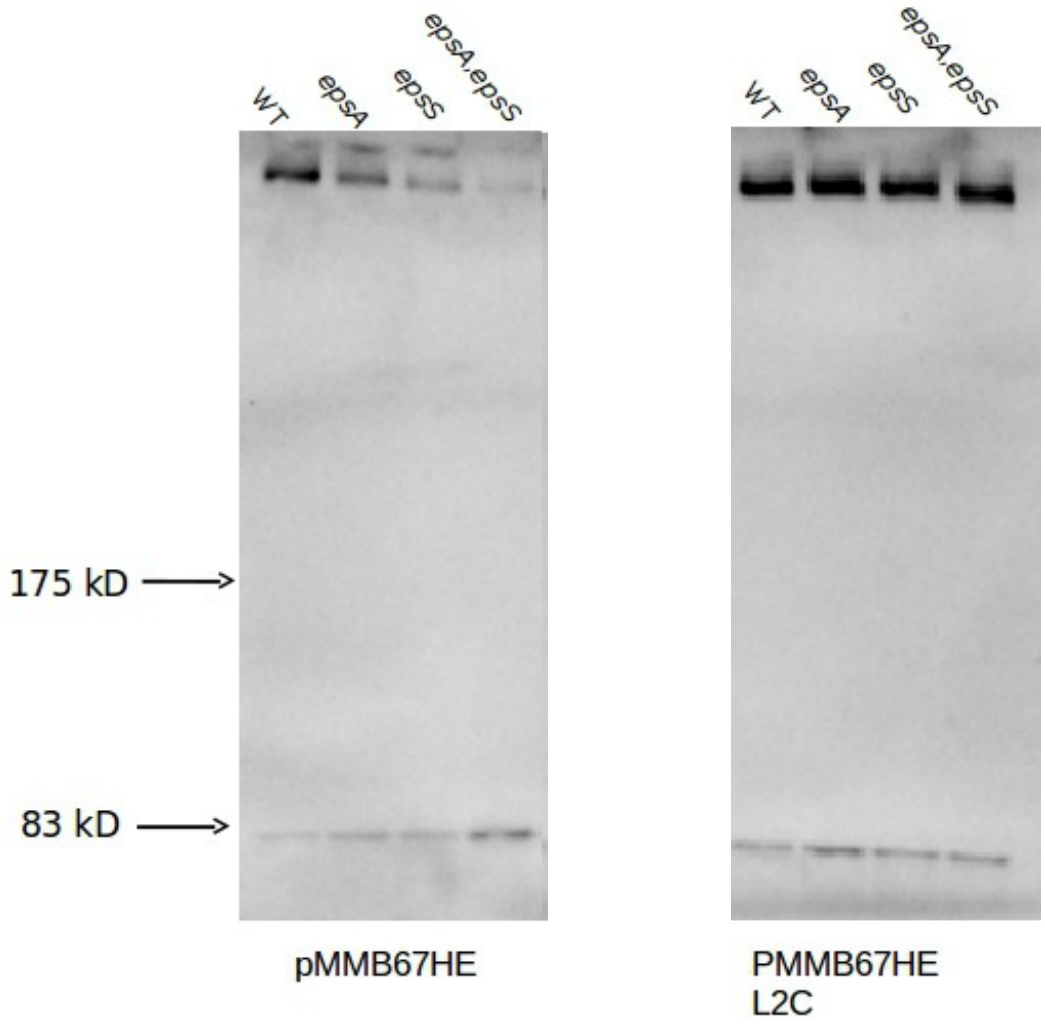


Figure 53. pMMB67HEL2C complemented and increased secretin assembly in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and whole cell samples were electrophoresed and immunoblotted with anti-EpsD. Locations of standard protein markers are given. Samples were compared which contained the empty vector (pMMB67HE) and those that contained the L2C plasmid.

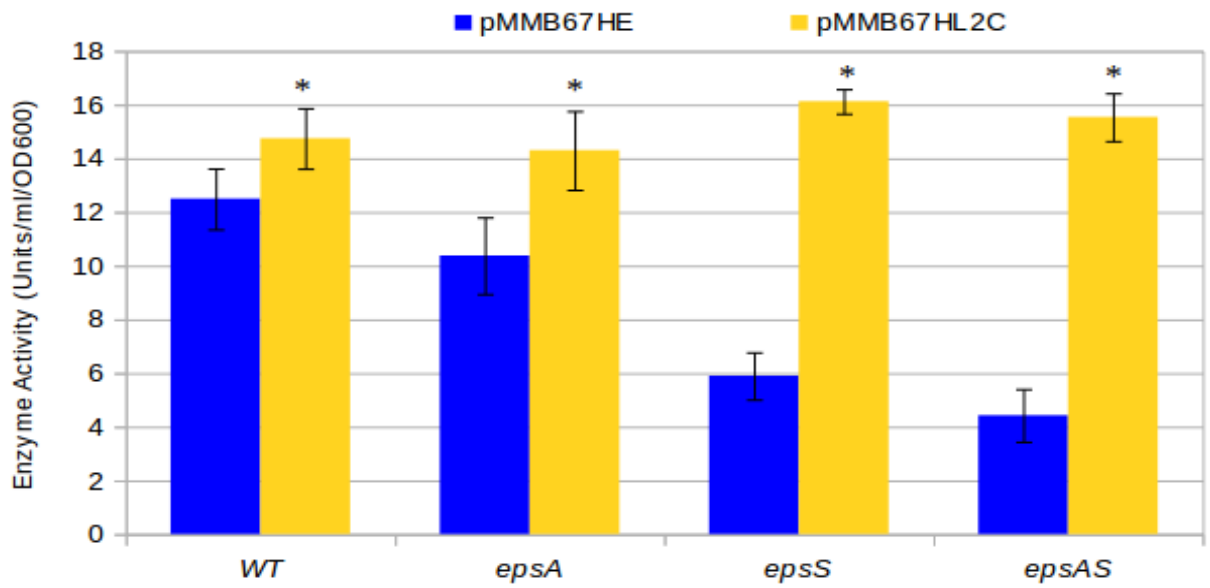


Figure 54. pMMB67HEL2C complemented lipase secretion in all strains of C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was harvested and tested. Data represent the average of four biological replicates. Error bars signify SEM. ***S**ignificantly different from the same strain containing an empty vector.

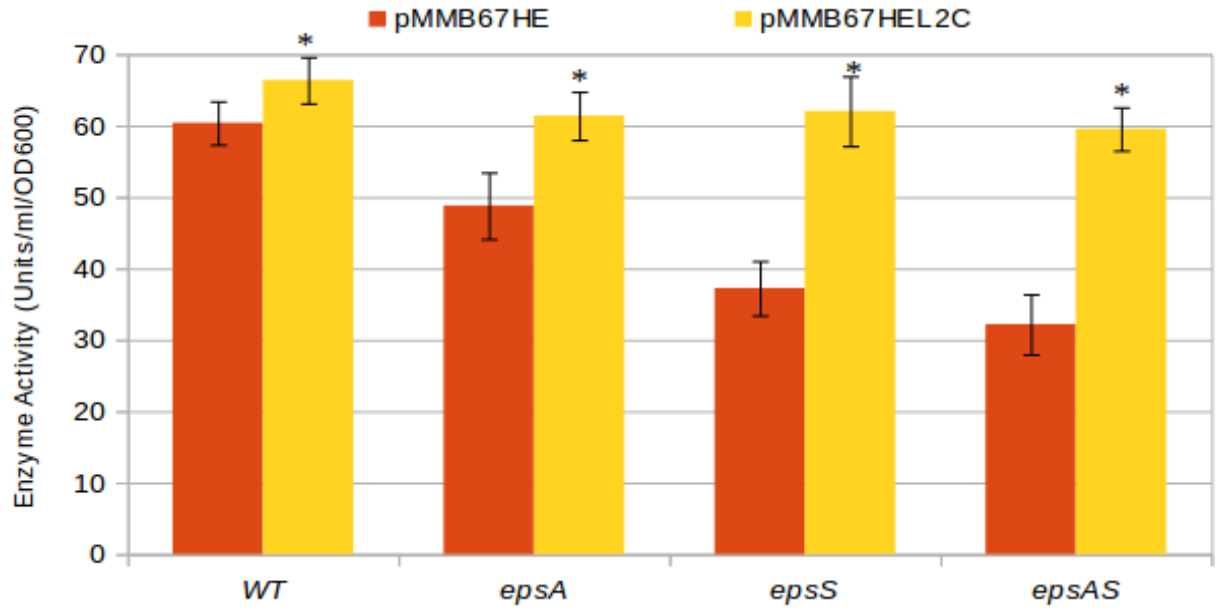


Figure 55. pMMB67HEL2C complemented protease secretion in strains all of C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent the average of four biological replicates. Error bars signify SEM. *Significantly different from identical strains containing the empty vector.

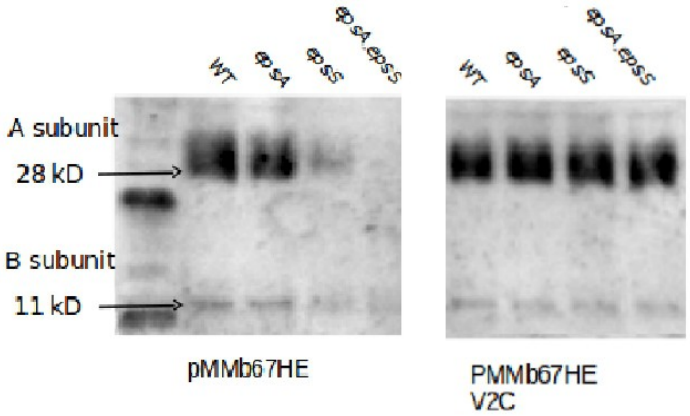


Figure 56. Cholera Toxin secretion was restored by pMMB67HEL2C in all C6706 strains. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was collected and filtered through a 0.22µm filter. Samples were electrophoresed and immunoblotted with anti-CT. MW of toxin subunits is listed.

As in the Bah-2 clones the C6706 clones were tested on a gradient of IPTG. The secretin assembly blot (Figure 57) shows assembly above WT levels at all induction levels. The lipase data (Figure 58), and the protease assay (Figure 59) only show increased secretion at higher induction levels, yet all induction levels at least restored the WT phenotype.

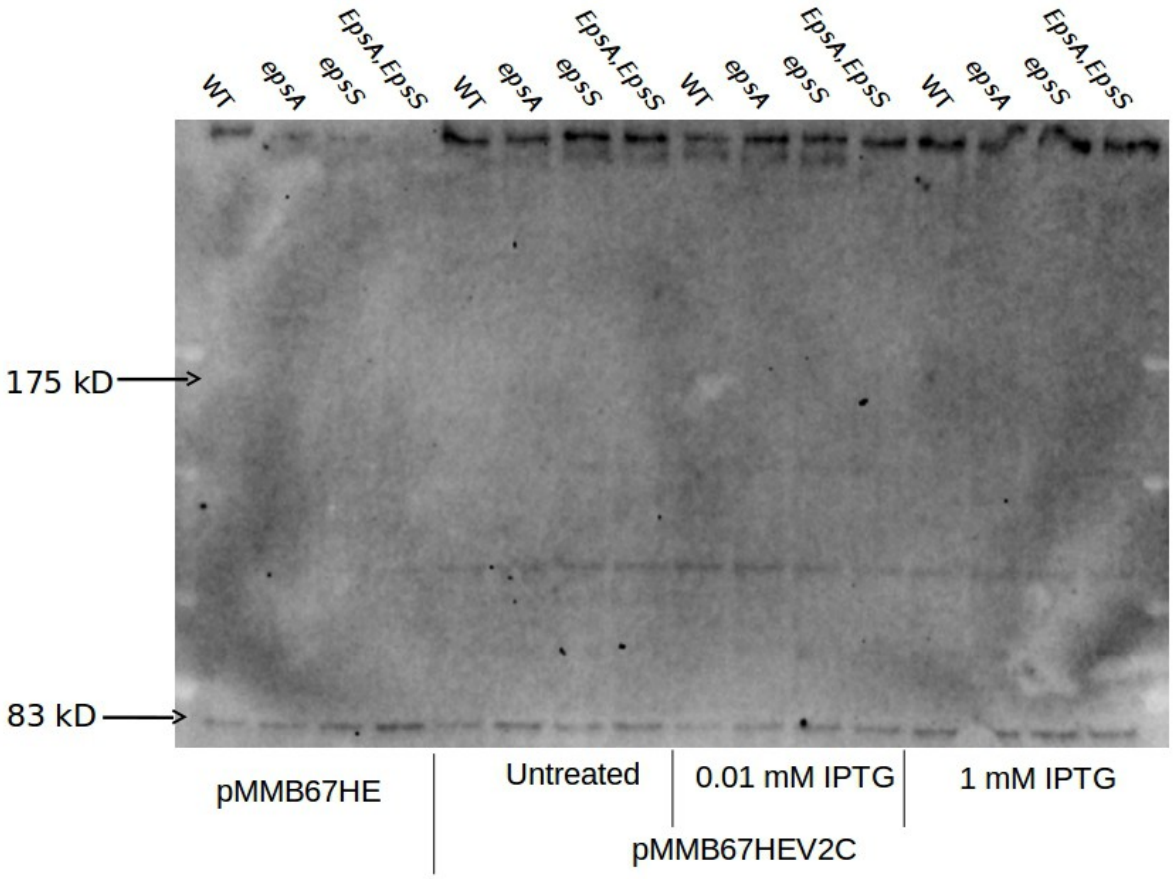


Figure 57. pMMB67HEL2C complemented and increased secretin assembly in C6706 at all induction levels. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and whole cell samples were electrophoresed and immunoblotted with anti-EpsD. MW of standard protein markers are given. Samples were compared which contained the empty vector (pMMB67HE) and those that contained the L2C plasmid. Image is representative of 5 replicate blots.

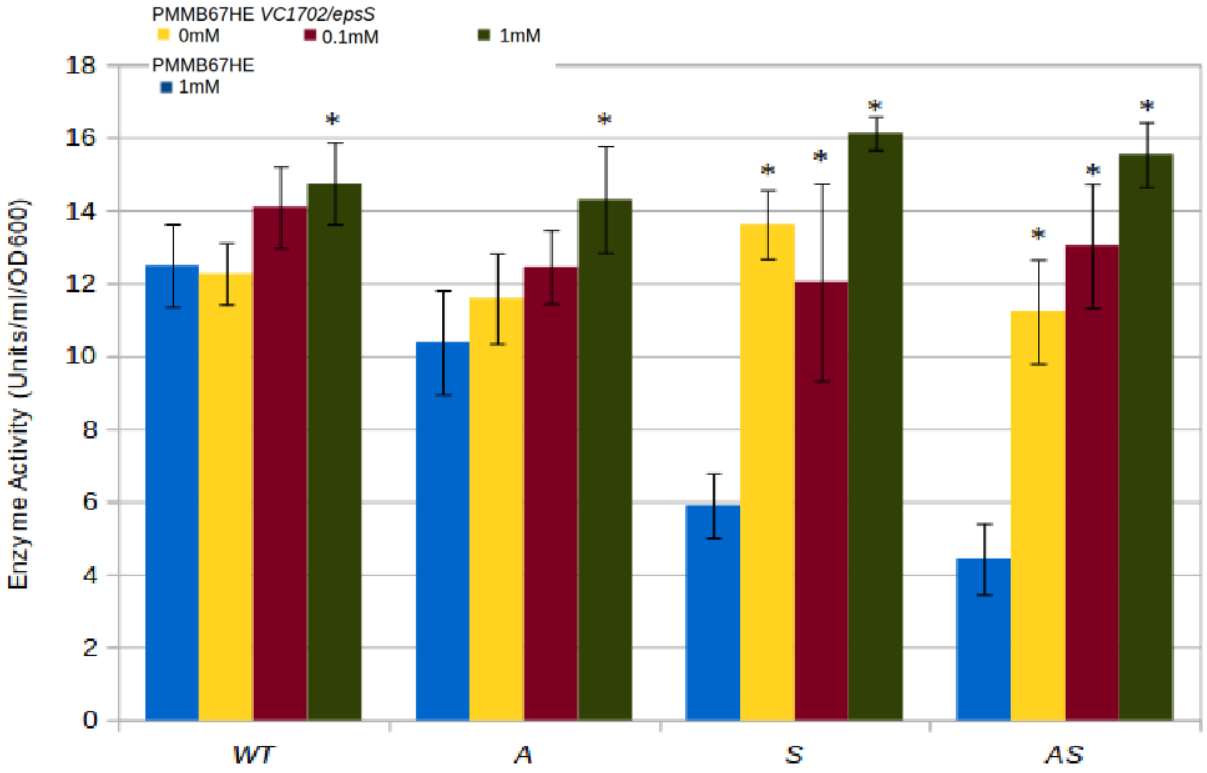


Figure 58. All induction levels of pMMB67HEL2C complemented lipase secretion in C6706. All induction levels restored the WT phenotype. pMMB67HEL2C successfully complemented the mutants without any induction. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was harvested and tested. Data represent the average of four biological replicates. Error bars signify SEM. *Significantly different from the same strain containing an empty vector.

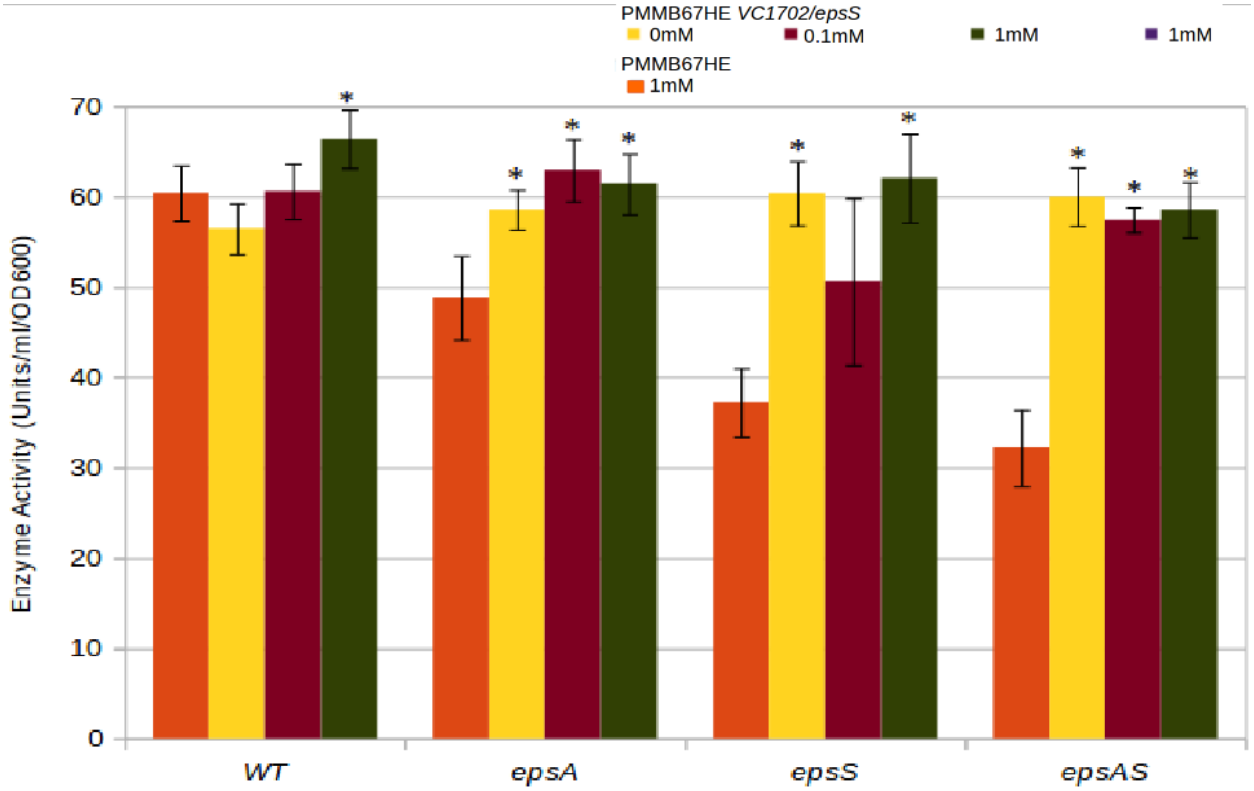


Figure 59. All induction levels of pMMB67HEL2C complemented protease secretion in C6706. All induction levels restored the WT phenotype. pMMB67HEL2C successfully complemented the mutants without any induction. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was harvested and tested. Data represent the average of four biological replicates. Error bars signify SEM. *Significantly different from the same strain containing an empty vector.

5.3.3.2 Cross-complementation with a Myc tagged construct

The inability to successfully complement an *epsS* mutant without VC1702 lead to key questions. Is VC1702 involved in assembly or is this simply a matter of expression? The simplest test is to determine if *epsS* is expressed in either pMMB67HELM or pMMB67HESM. As a control for Myc interference pMMB67HEL2C was constructed with a Myc tag and designated pMMB67HEL2M. The method was identical to the construction of pMMB67HELM. The lipase (Figure 60) and protease (Figure 61) data demonstrate complementation in Bah-2.

C6706 was again tested more thoroughly with four biological replicates. The results of the assembly blot (Figure 62), lipase assay (Figure 63), and the protease assay (Figure 64) are very similar to those of the non Myc tagged clone, pMMB67HEL2C (Section 5.3.3.1).

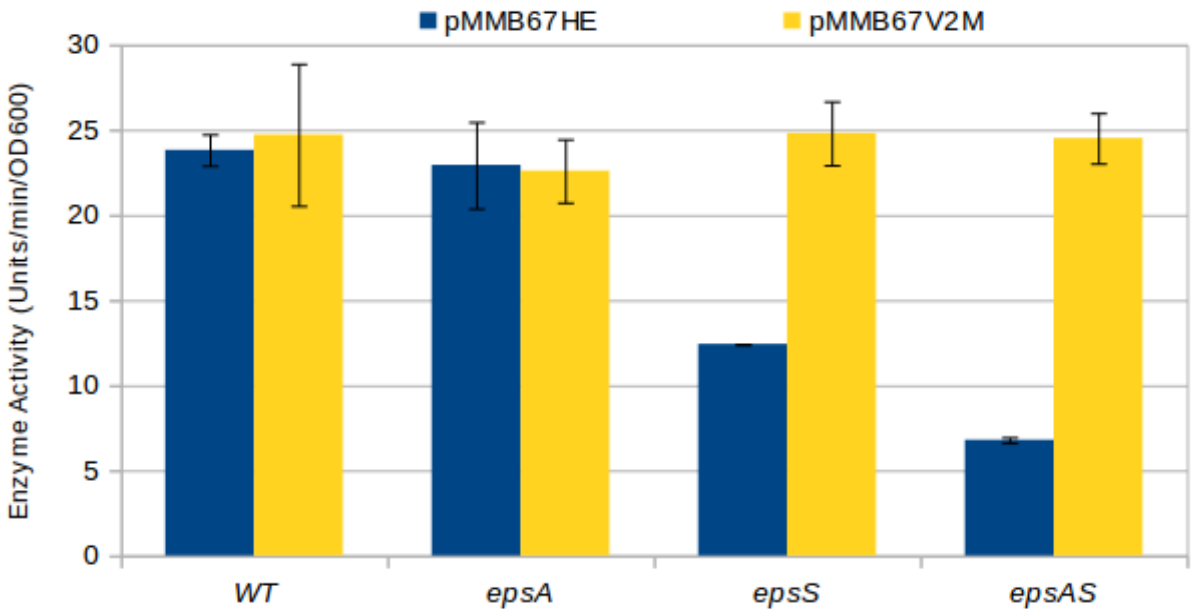


Figure 60 pMMB67HEL2M complemented lipase secretion in Bah-2. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

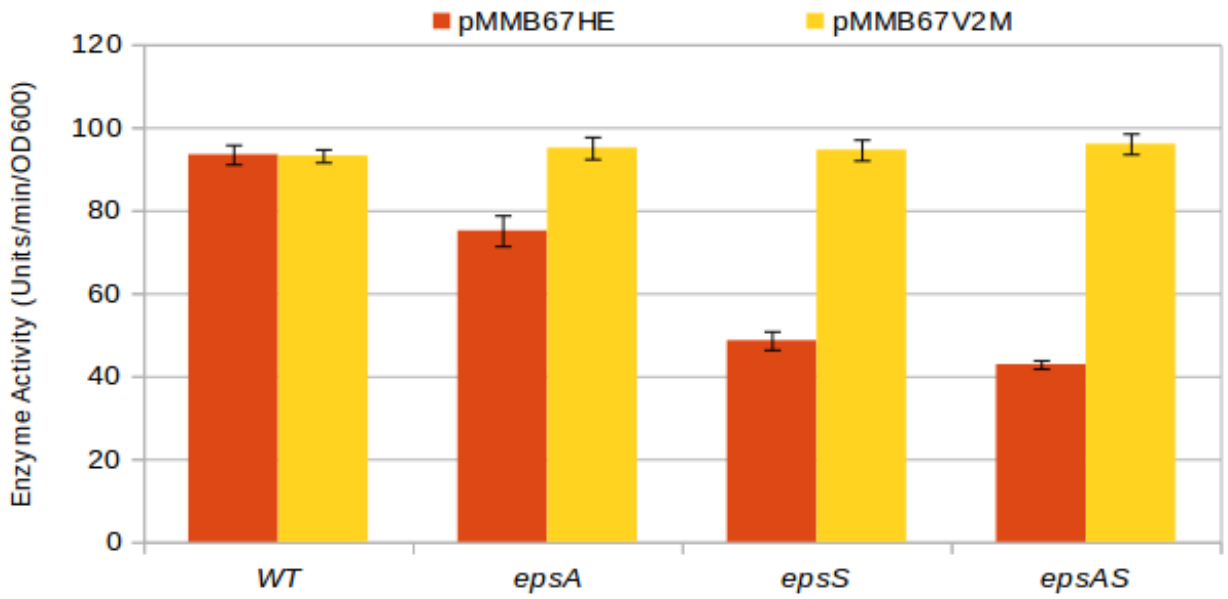


Figure 61. pMMB67HEL2M complemented protease secretion in Bah-2. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and supernatant was harvested and tested. Data represent one biological replicate with 3 technical replicates. Error bars represent standard deviation.

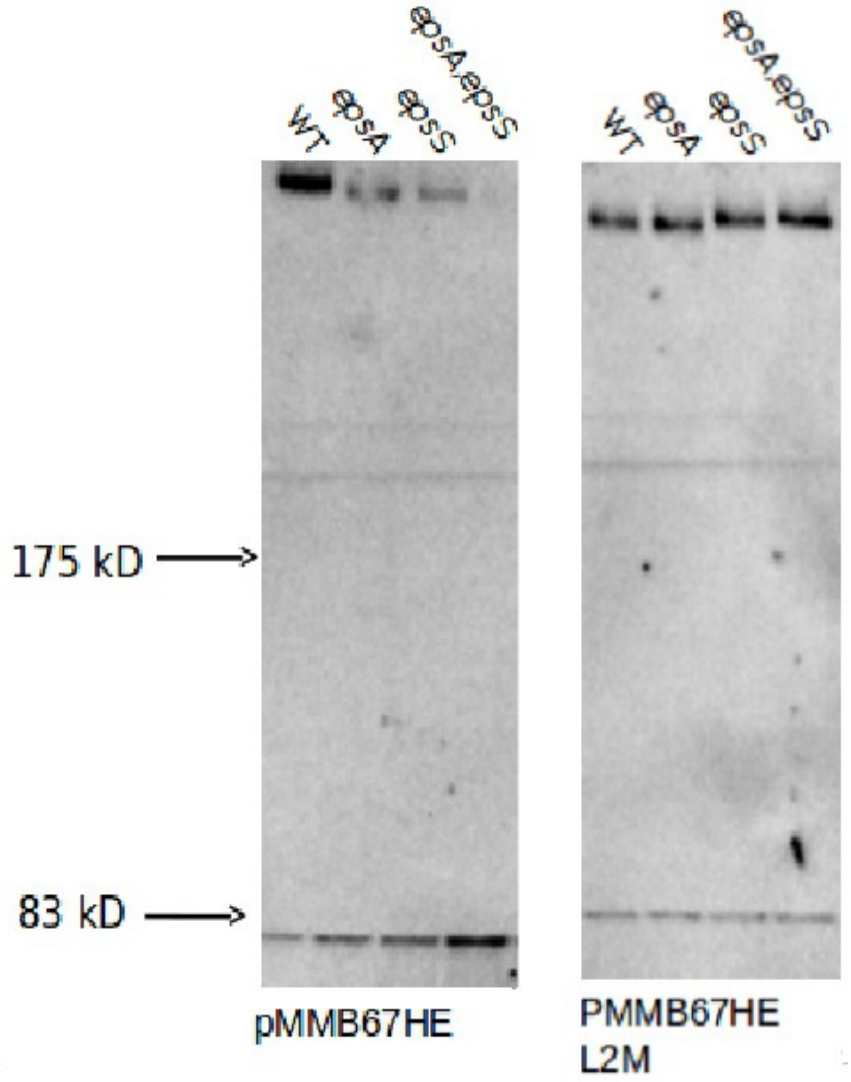


Figure 62. Secretin assembly was complemented with pMMB67HEL2M. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 with 1mM IPTG and whole cell samples were electrophoresed and immunoblotted with anti-EpsD . Locations of standard protein markers are given. Levels of IPTG are given.

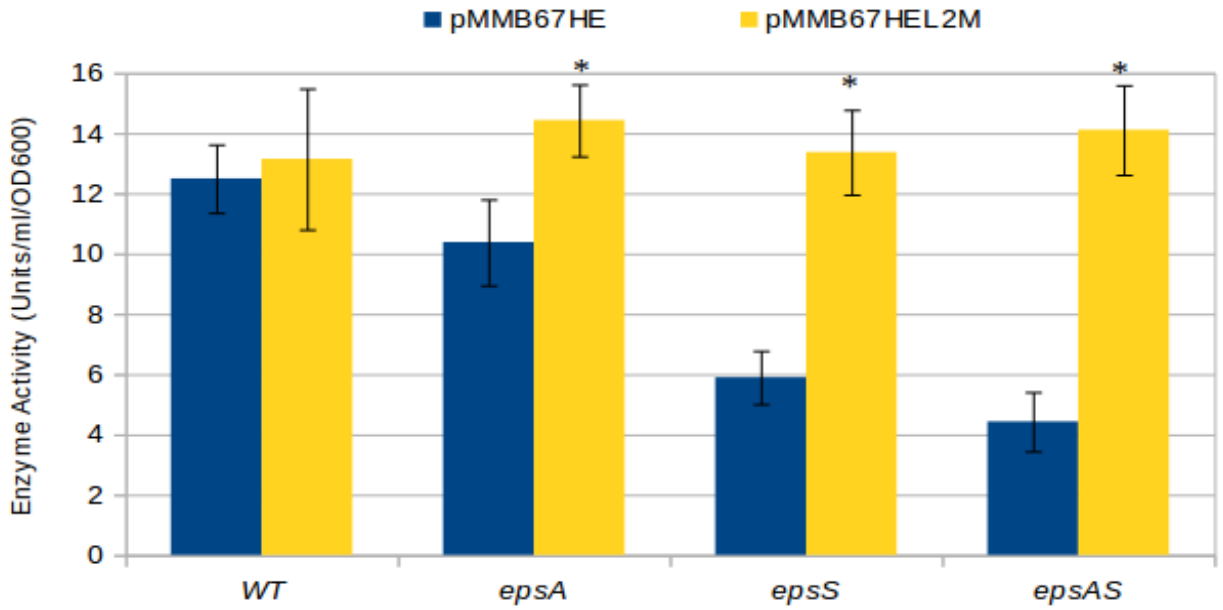


Figure 63. pMMB67HEL2M complemented lipase secretion in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0, with 1mM IPTG and supernatant was harvested and tested. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was harvested and tested. Data represent the average of four biological replicates. Error bars signify SEM. *Significantly different from identical strains containing the empty vector.

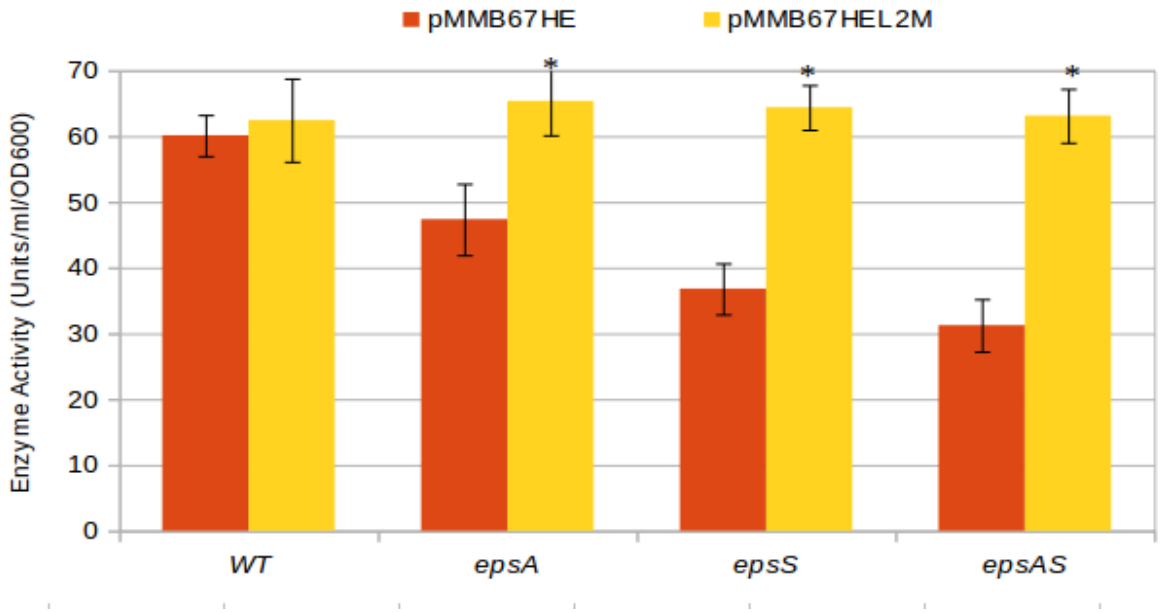


Figure 64.pMMB67HEL2M complemented protease secretion in C6706. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0, with 1mM IPTG and supernatant was harvested and tested. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and supernatant was harvested and tested. Data represent the average of four biological replicates. Error bars signify SEM. *Significantly different from identical strains containing the empty vector.

5.4 Testing Expression of EpsS clones

As only the clones containing the entire operon of EpsS complemented, the question arises, is this a problem in transcription, translation, or does VC1702 play a vital role in secretin assembly? To test this a Myc blot was performed with the *epsS* strains with pMMB67HEL2M, pMMB67HESM, and pMMB67HE at three separate induction levels (Figure 65). These results demonstrate that EpsS is produced in both the clones containing VC1702 as well as *epsS* (pMMB67HEL2M) and the clone containing just *epsS* (pMMB67HESM). The mutant without any plasmid and one which possessed only the empty vector were negative controls.
This demonstrates that VC1702 is involved in secretin assembly in some way in *V. cholerae*. This figure also demonstrates why gradients of IPTG failed to produce variable levels of phenotypes, as the gene is almost fully induced without any IPTG.

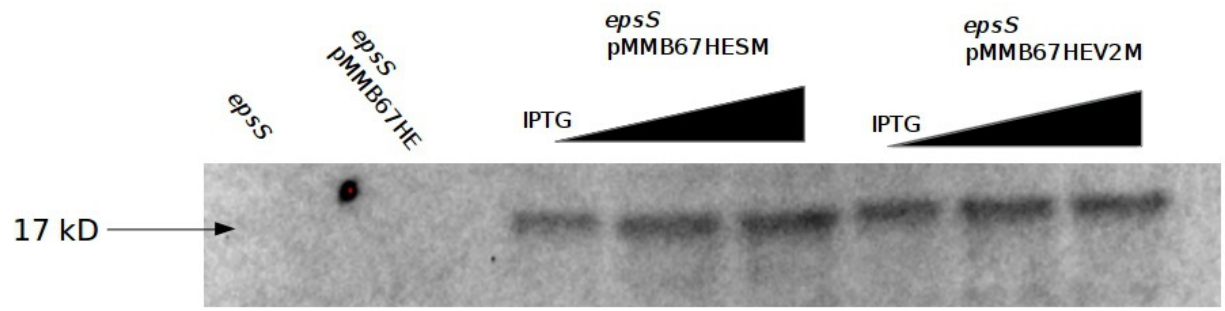


Figure 65. EpsS was expressed in all clones regardless of complementation success. Cultures of mutants containing the plasmid were grown to an OD 600 nm of 2.0 and whole cell samples were electrophoresed and immunoblotted with anti-Myc . The expected size of EpsS is 17kDa. Samples were induced with 0, 0.1, or1mM IPTG, except pMMB67HE which was induced at 1mM.

5.5 Leakage of Periplasmic Enzymes

The background enzymatic activity in the mutant phenotypes and the finding that activity exceeds WT levels raises a question: is the outer membrane leaking? The plasmid pMMB67HE encodes a known periplasmic enzyme, β-lactamase, which is easily assayed using nitrocefin (See method section for details). Each of the mutant strains without any plasmid were tested and found to have no activity within their cells, or in the supernatant. C6706 strains containing the empty vector, pMMB67HE*epsAB*, or pMMB67HEV2C were tested in each mutant strain with induction of 1mM IPTG. Table 2 shows the percentage of total activity that is present within the supernatant as compared to the total cell. In almost all cases it is under 5% and varies little between different plasmids. This demonstrates that leakage does not play a major role in the complementation observed.

Table 2. Periplasmic leakage is low in all strains.

Plasmids Strains	Cell Lysates (Units/ml/OD600)	Supernatant (Units/ml/OD600)	Percent of total activity present in supernatant
pMMB67HE			
WT	9.1 +/- 0.3	0.50 +/- 0.03	5.5
epsA	9.2 +/- 0.2	0.29 +/- 0.20	3.2
epsS	11.7 +/- 0.2	0.38 +/- 0.20	3.2
epsAS	13.1 +/- 0.1	0.16 +/- 0.08	1.2
pMMB67HEAB			
WT	9.6 +/- 0.1	0.59 +/- 0.53	6.1
epsA	7.6 +/- 1.03	0.48 +/- 0.07	6.3
epsS	11.4 +/- 1.6	0.44 +/- 0.07	3.8
epsAS	9.5 +/-0.9	0.27 +/- 0.4	2.9
pMMB67HEV2C			
WT	9.9 +/- 0.3	0.12 +/- 0.7	1.3
epsA	8.3 +/- 0.8	0.08 +/- 0.08	1
epsS	14.0 +/- 0.3	0.60 +/- 0.33	4.3
epsAS	11.1 +/- 0.7	0.32 +/- 0.24	2.9

5.6 Phylogenomic analysis of T2SS with focus on pilotin and gspAB acquisition, loss, and ancestry.

To better understand the role *epsAB* and *epsS* play in the T2SS, the evolutionary history was studied by means of phylogenomic analysis. Ten genomes were selected from the gamma proteobacteria for analysis due to previous physiological work on their T2SS. Another 20 genomes were selected to ensure a diverse representation of the Gammaproteobacteria (See section 4.8 for details about all methodology and programs used). The protein sequence for all conserved ribosomal proteins was extracted concatenated, aligned, and uninformative sites removed. After this process a maximum likelihood tree was constructed (Figure 66), and was annotated with the presence and absence of each protein.

This work demonstrates that the T2SS is present in most but not all gamma proteobacteria. It was lost in *D. nodus, S. enterica,* and the Pasteurellales. The tree also demonstrates that the assembly factors and pilotins are only found together in the Vibrionales. GspAB is the dominant assembly mode of the system, with pilotins being a later addition.

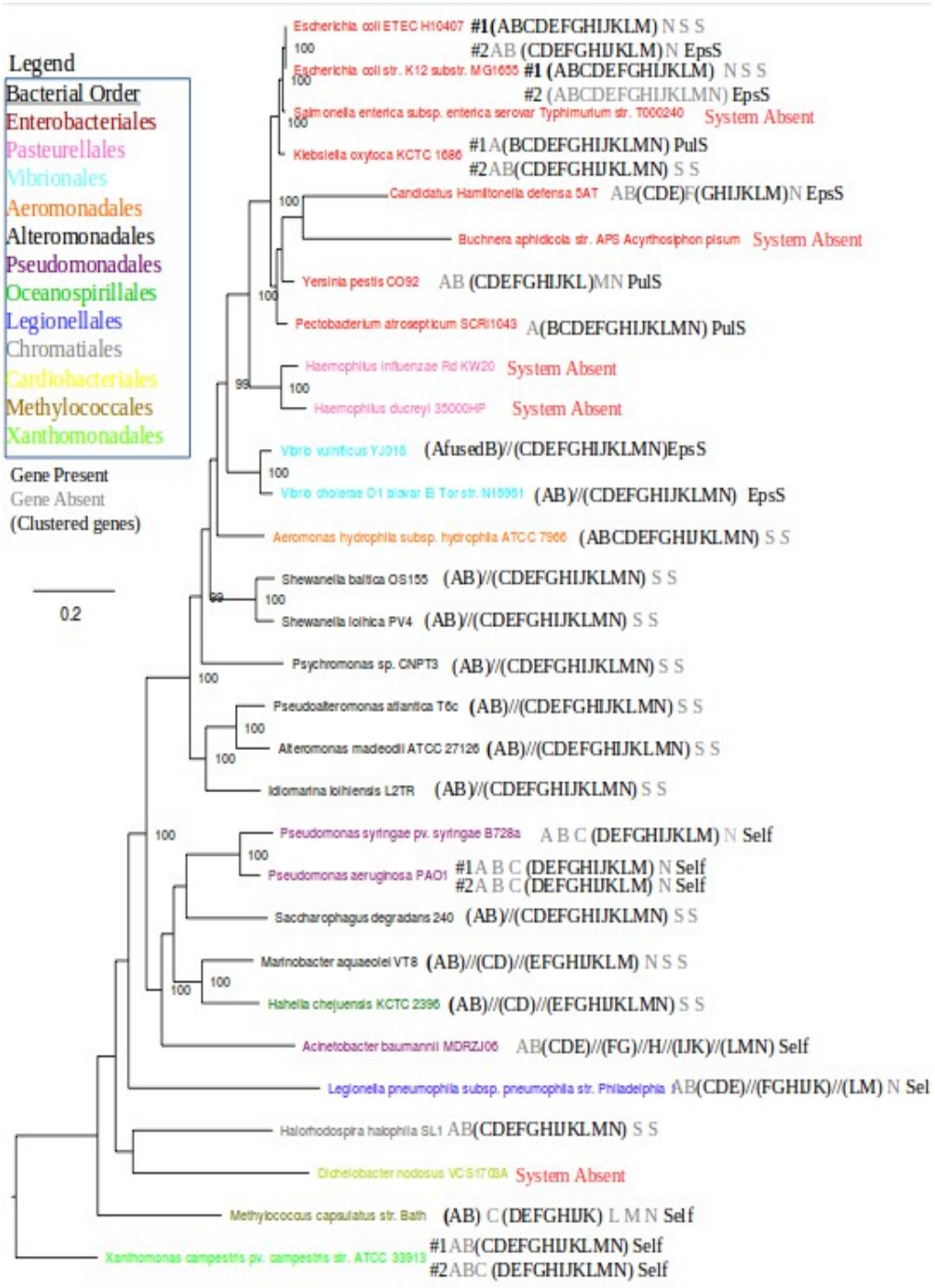


Figure 66. Ribosomal Tree of gamma proteobacteria. Phylogenomic tree represents the maximum likelihood of 6039 sites with only bootstraps greater than 99/100 shown. This tree shows presence of T2SS genes (black) and absence (grey). Each protein is represented by its single letter code (C = GspC). Self targeting secretins are marked as Self (See Section 5.6)

6.0 DISCUSSION

The study of secretin assembly proved to be difficult in *V. cholerae,* yet it yielded interesting results. The strains Bah-2 and C6706 had distinct and different problems but each had similar results in the end. These strains are biologically similar as they are both 01 El Tor biotypes, but they were isolated from two different patients (Thelin *et. al*., 1996; Pearson *et al*. 1993). The double species resulted in redundant data that strengthens conclusions and as such each mutation will be discussed individually in the scope of both strains.

Independent validation of the pilotin was achieved by the transposon library (Figure 14-16) . The interruption of protease activity and secretin assembly in Bah-2 supports the hypothesis that *epsS* is involved in the T2SS. The location of the insert is problematic as one can not determine if the phenotype is due to disruption of *epsS* or VC1702 activity.

The phenotypes of the *epsA* and *epsB* strains demonstrates that secretin assembly (Figures 17 and 20) and secretion (Figures 18, 19, 21, 22) are slightly reduced compared to WT, this matches well with previously published results (Strozen *et. al,* 2011). When *epsS* was mutated drastic decreases in secretion and secretin assembly were observed. Assembly of the secretin and secretion was further decreased in the *epsAS* double mutant, yet assembly and secretion were not abolished in this mutant. Secretion of lipases and proteases can be explained by the presence of enzymes secreted by other pathways. One such mechanism may be associated with the outer membrane via the attached N-terminal lipid moiety (Zuckert *et. al.,* 2014). The previous study on *epsS* had demonstrated that the secretin will form in the inner-membrane without the pilotin present, and this could account for the low level of secretin assembly observed in the double mutants of Bah-2 (Figure 17) (Dunstan *et. al.,* 2013).

Cholera toxin was not observed in the double mutant strain using standard analysis procedures (Figure 23), but overloading of the gel and overexposure of the blot showed some toxin present in the supernatant (data not shown). This can be accounted for in two ways, first some leakage of periplasmic contents was observed in all strains (Table 3). This level was around 5% and may be wholly responsible for the background CT. Conversely CT has been demonstrated to be exported *via* outer membrane vesicles (OMV) (Chatterjee & Chaudhuri 2011). These OMVs only account for a small percentage of secretion but may add to the background of lipases and proteases. OMVs have been shown to have increased production when proteins accumulate in the periplasm (Song *et. al.,* 2008) and this accumulation may have

led to a decrease in the difference between T2SS WT levels and those in the T2SS secretion knockout mutants (*epsC, epsAS, epsBS)*. Should future studies be conducted on this strain, enzymatic assays on isolates of OMVs by ultracentrifugation should be performed.

The *epsA* mutant had another phenotype, increased monomer abundance (Figure 17, 20), while the *epsS* mutants show the same or decreased levels as WT. The double mutants also show increased monomer abundance. This accumulation has been seen in *Aeromonas* when the *exeAB* genes are mutated (Ast *et. al.,* 2002), but it is not observed in pilotin mutants (Strozen *et al.,* 2012; Collin et al., 2011; Carbonelle *et. al.,* 2005; Schuch & Maurelli 1999).

The accumulation of monomers when *epsA* is mutated presents a question. Are these true monomers that did not assemble into a secretin or are they the result of improper assembly which resulted in a heat sensitive secretin? Should the monomers have assembled into heat sensitive secretin as in the T3SS (Schuch & Maurelli 2001), this would demonstrate that the *epsAB* complex is required not for assembling the secretin in the outer-membrane, but guiding *epsD* monomers to the outer membrane and ensuring proper assembly. Should the increased intensity of *epsD* monomers be from accumulation of unassembled monomers, this would demonstrate that *epsS* and *epsAB* are definitively partially redundant. Native PAGE may be able to test this, but the secretin runs poorly in acrylamide gels as it is so large. Membrane fractionation is another possible method, but secretin has been shown to self assemble under conditions induced by such an experiment and may prove to be futile (Strozen, 2012)

The pilotin mutants had another observed phenotype, degradation of the secretin monomer (Figures 17 and 20). This result matches well with the results from *Klebsiella* and ETEC, as a lack of pilotin has been shown to cause the monomers to be susceptible to degradation by DegP (Daefler *et al.,* 1997). DegP preferentially cleaves between paired hydrophobic residues, but our data is insufficient to determine the exact cleavage site. A problem lies in our EpsD antibody, which was created with the periplasmic region of the protein, and as such we are unable to determine where the cleavage has occurred, as we only have a *ca.* 75kDa fragment. This data proves that the presence of the pilotin does protect the secretin from degradation. All complementation blots do not show this fragment as the Criterion gels that were used could not show both degradation products and assembled secretin.

Complementation of the *epsA* mutant was successful with a plasmid carrying both *epsA* and *epsB* (Figures 25-34). The double mutant, *epsAB,* had its levels restored to that of the *epsS* mutant. Expression of this gene did not fully complement the pilotin mutant though,

demonstrating that the pilotin has functions that are not accounted for by the assembly factors EpsAB. When a gradient of induction levels were tested (Figures 36-38) slight complementation of *epsS was* seen at high induction levels. As the plasmid complemented *epsAB* with no induction, this demonstrates that only over-expression of *epsAB* will complement the function of *epsS.*

Complementation of the *epsS* mutant was only successful with a plasmid carrying the entire operon of VC1702 and *epsS* (Figures 47-58). This clone would complement with or without a myc tag, demonstrating that the C-terminus can be modified without a loss in activity (Figures 61-65). This complementation was successful in all mutant strains. The most visible result was in the CT blot (Figure 56), which showed strong bands for all strains expressing the plasmid. This result matches well with the previous work on EpsD (Dunsten *et. al.,* 2013), yet is difficult to compare to the work on the *Klebsiella* pilotin. Most work on PulS has been performed with the T2SS on a vector expressed in *E. coli* (d'Enfert and Pugsley, 1989; d'Enfert *et al.,* 1987). This work does not allow comparisons to true WT levels as components of the system may be over expressed.

The pilotin can compensate for *epsA* deficiencies. Unfortunately the vector was leaky and would over express the protein even when it was uninduced, so it is difficult to determine the level of redundancy between *epsAB* and *epsS.* Yet we can still infer that *epsAB* has a redundant function within the cell.

This pilotin may function in the same manner as PulS, as not only protection and assistance in proper assembly, but in regulation of secretin assembly in the outer membrane (Huysmans *et. al.,* 2014). *epsS* is in a separate operon from most of the other components of the T2SS system, and thus may be regulated separately. As the entire system is under control of a single operon, this independent regulation by degradation may prevent secretin forming without other components. Regulation of secretin assembly is essential, as on its own it forms a channel in the outer membrane which allows the passage of small molecules (Disconzi *et, al.,* 2014).

The requirement for VC1702 was originally thought to be one of expression, but a myc blot of constructs with and without the preceding gene demonstrates that the pilotin is expressed in both clones (Figure 65) indicating VC1702 must have an effect beyond expression. The gene does not appear to be part of the T2SS as it is highly conserved with 40% identity among the gamma proteobacteria, as opposed to the T2SS components which range from <10% to 25%. This suggests that this protein has a more global function. The previous study (John *et. al.,*

2005) located it in the inner-membrane and this is suggests that this protein is part of some integral pathway, perhaps the BAM or Lol complexes.

This gene needs to be expressed with *epsS* in *cis*. This is odd as in most other species this gene is found on its own unit, while the *Vibrio* species have it and *epsS* on the same putative operon. The expression of VC1702 would be disrupted by the *epsS* mutation which was generated by the suicide vector. The ORF for the chloramphenicol gene is in the opposite direction as the native *epsS* and may disrupt transcription of the VC1702 *epsS* operon in all cases.

This may have explained the necessity for VC1702 to be present for complementation success, however the gene should not have been disrupted in C6706 *epsS.* This strain was generated by transposon mutagenesis and had the insertion in the forward direction of *epsS* near the 3' end of the gene. This fact demonstrates that the genes may need to be in *cis* to be expressed. Due to the fact this gene is expressed individually in other bacteria suggests that this may be a matter of RNA stability and without *epsS* the transcript is degraded before it can be transcribed.

VC1702, and its homolog YciI, are of great interest and may offer insights into the system as a whole. To test this genes' function I would order an *E. coli* mutant from the Keio collection or mutate the gene in *Aeromonas* and see if it disrupted the T2SS.

A final observation of all *epsS* strains is that the colonies form a hard shell. Wild type Vibrio colonies are white-yellow, and have a consistency similar to that of *E. coli*, yet both the *epsS* and *epsAS* mutants of both strains produced colonies which were difficult to pierce. This could be due to disruption in VC1702 or may be the result of *epsS* playing a role in T4P.

The bioinformatic data (Figure 66) justify using *V. cholerae* as a model organism to study *epsAB* and *epsS* as pilotins and assembly factors were both present in just the Vibrionales. This computational study demonstrated that the *epsAB* genes are by far the most prevalent form of T2SS secretin assembly. This could be disproved if other pilotins are discovered.

The assembly factors GspAB were ancestral in the system and were present before the self targeting group split from the rest. The self targeting GspD proteins are only hypothesized, in fact the second T2SS present in *P. aeruginosa* was shown not to self target; nonetheless, they do form a distinct evolutionary clade of secretins and T2SS. The pilotins are a late addition to the system and appear to displace *epsAB* gene function. This is common with aggressive transposable elements (Kidwell & Lisch 2007). The physiological data from this study suggests

that *epsS* could completely displace *epsAB*, yet it has been retained in the Vibrionales for some unknown reason.

In conclusion EpsAB is redundant within *V. cholerae*, and they likely play a role in peptidoglycan remodeling. Conversely EpsS can fully restore secretion and secretin assembly and is the major factor in EpsD monomer protection and targeting to the outer-membrane.

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APPENDIX A
PRIMERS USED IN THIS STUDY

Table 3: Primer description and sequence. Restriction sites (RS) are indicated by underline

Name	Area of amplification & partner	Sequence 5'-3'
US159	Outward from transposon	TGGATTTCGATCACGGCACGATC
Us160	Outward from transposon	GCCACAGCCAAACTATCAGGTC
US595	Forward annotated start codon of VC1703 with Xba1 RS	GCGTGCTCTAGAATTTACTGGCTCAACCGTGA
US597	Reverse VC1703 with Ecor1 RS and Myc tag	GCCCGCGAATTCTTACAGATCCTCTTCTGAGATGAGTTTTTGTTCACCTT
US610	Forward VC1703 colony PCR mutant check	GATTGGCTGGGTATAAAGGGATGATCC
US611A	Reverse VC1703 colony PCR mutant check	GAACCCGCGATTCGTGAAGGTTTACC
US611B	Native reverse primer Vc1703 with Xba1 RS	CTGTAAGAATTCTTAACCTTGCAGCTCTCTTTC
US612A	Native forward primer Vc1703 with EcoR1 RS	GCCAACGTCATTTCTAGACCTTTCAAAAAAGTATTCTG
US636	Forward EpsAB with Xba1 RS	GGCTCTAGAATGTATCTGAACTTCTTTGGGTTTGACGAA
US637	Reverse EpsAB with EcoR1 RS	TATGAATTCTCATCGCATAGCAAAACGGTCACGCAAGTG
US699	Forward VC1702 with Xba1 RS	GCTCTAGAATGTGGTATGTGATTTTTGCGCAGGATGTCG
US670	Reverse VC1703 with EcorR1 RS	ATGAATTCCGTTAGAAACCGCAAAGGCCTCTTATGAGGC
US727	Outward from Tn CAM gene	GATCGTGCCGTGATCGAAATCCA
US728	Outward from Tn CAM gene	TGGATTTCGATCACGGCACGATC
US731	Reverse VC1703 with EcoR1 RS	GTAAGAATTCGCTTAACCTTGCTTGCAGCTCTC
US732	Reverse VC1703 Myc taged with EcoR1 RS	ATAGAATTCTTACAGATCCTCTTCTGAGATGAGTTTTTGTTCACCTTGC

APPENDIX B
PLASMIDS

Table 4: List of plasmids used in this study. All genetic loci refer to chromosome 1 of Vibrio cholerae O1 biovar El Tor str. N16961

Plasmid	**Short Designation**	**Relevant Phenotype/Genotype and Description**	**Source or Reference**
pMMB67HE		IncQ lacI bla(AmpR) Ptac rrnB	Morales *et. al.,* 1988
pMMB67HE-LM[e*epsS*(1834309..1834800)/MYC]	LM	Contains ORF of *epsS* with genbank annotated start codon with C-terminus MYC tag	This Study
pMMB67HE-SM[e*epsS*(1834401..1834800)/MYC]	SM	Native *epsS* ORF with C-terminus MYC tag	This Study
pMMB67HE-LV[e*epsS*(1834309..1834800)]	LV	Contains ORF of *epsS* with genbank annotated start codon	This Study
pMMB67HE-SV[e*epsS*(1834401..1834800)]	SV	Native *epsS* ORF	This Study
pMMB67HE*epsAB*[e*epsAB*(2625289-2622851]	AB	Contains the natural sequence of the *epsAB* operon	This Study
pMMB67HE-LV2[e*epsS*(1834309..1835000)]	LV2	Contains the *VC1702* and *epsS* operon	This Study
pMMB67HE-L2M[e*epsS*(1834309..1835000)/MYC]	L2M	Contains the *VC1702* and *epsS* operon with C-terminus MYC tag	This Study
pMRS101		oriR (ColE1), oriR (R6K), MCS, mobRK2, *sacBR*, ApR , SmR	Sarker and Cornelis 1997
pMRS101/*epsS*::cat		Suicide Vector for allele exchange of e*epsS*	Howard Laboratory

APPENDIX C
STRAINS

Table 5: List of strains used in this study

Strains	Relevant Phenotype/Genotype and Description	Source or Reference
Escherichia coli		
SM10 λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λpir	Miller *et al.*, 1988
S17-1 λpir	*TpR SmR recA, thi, pro*, hsdR-M+RP4: 2-Tc:Mu: Km Tn7 λpir	Simon *et al,.* 1983
DH5α	fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	New England Biolabs
XL-Blue	recA1 lac endA1 gyrA96 thi-1 hsdR17 supE44 relA1[F' proAB lacIq ZΔM15 Tn10]	Bullock *et al.,* 1987
Vibrio cholerae		
Bah-2	Vibrio cholerea 01 Eltor biovar, *recA, ctxB,* rif^R ,Sm^R	Pearson *et al*. 1993
Bah-2 *epsA*	Strain "Bah-2" *epsA*::Kn^R	Strozen et al. 2011
Bah-2 *epsS*	Strain "Bah-2" *epsS*::Cm^R	This study
Bah-2 *epsAS*	Strain "Bah-2 *epsA" epsS*::Cm^R	This study
C6706	Vibrio cholerea 01 Eltor biovar, *LacZ* ,Sm^R	Thelin *et. al*., 1996
C6706 *epsA*	Strain "C6706" *epsA*::TnFGL3 Kn^R	Cameron *et al.*, 2008
C6706 *epsB*	Strain "C6706" *epsB*::TnFGL3 Kn^R	Cameron *et al.*, 2008
C6706 *epsC*	Strain "C6706" *epsC*::TnFGL3 Kn^R	Cameron *et al.,* 2008
C6706 *epsS*	Strain "C6706" *epsS*::TnFGL3 Kn^R	Cameron *et al.,* 2008
C6706 *epsAS*	Strain "C6706 *epsA*" *epsS*::Cm^R	This study
C6706 *epsBS*	Strain "C6706 *epsB*" *epsS*::Cm^R	This study