The Specificity of Secretion through the Alpha and Beta Type II Secretion Systems of *Escherichia coli*

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By

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Abstract

The Type 2 Secretion System (T2SS) is a molecular apparatus that is found in many Gram negative bacteria. The T2SS system is used for the export of proteins from the cytosol to the extracellular space. A key element in secretion is the assembly of the secretin (GspD), during which monomeric GspD proteins must assemble into a multimeric structure in the outer membrane before secretion can occur. *Escherichia coli* possesses two different T2SS systems termed the alpha and the beta system. The beta T2SS system is normally active and has been shown to function in the secretion of heat-labile enterotoxin (LT) by *Enterotoxigenic (ETEC) E. coli* strains. The alpha T2SS system, present in both *ETEC* and K12 *E. coli* strains, is silenced during growth under standard laboratory conditions but has been used to study the secretion of LT when the T2SS system is overexpressed from plasmids.

The goal of this study was to express the chromosomal copies of the cryptic alpha T2SS system of *E. coli* and to observe its function in the secretion of LT and Chitinase (ChiA). Mutant strains of *E. coli* K12 were created in strain BW25113, these mutations consisted of deletions of *hns* and *stpA* which are known gsp_{α} operon repressors. No expression of the $GspD_{\alpha}$ secretin or secretion of LT or ChiA by the T2SS system were observed in the strains containing deletions in these genes. Additional mutant strains were created in *E. coli* K12 strain MG1655 in which the natural promoters of the gsp_{α} operons were replaced with inducible promoters, the lactosetryptophan fusion promoter (*ptac)* was used to control the gene expression of the $gspAB_{\alpha}$ operon while the arabinose promoter (*paraBAD)* was used to control the gene expression of the $gspC\text{-}O_{\alpha}$ operon. When the strains created were induced with IPTG and

arabinose, expression and assembly of the secretin multimer was observed, but this did not result in the secretion of LT or ChiA.

The GspAB complex is required for the assembly of the secretin in *Aeromonas hydrophila*. The requirements for the GspAB complex in secretin assembly was investigated in *E. coli* K12 strain MG1655. Mutants were created with a kanamycin (kan) resistance cassette inserted into *gspB,* while the natural promoters of *gspAB* and *gspC-O* were replaced with the inducible promoters *ptac* and *paraBAD,* respectively. Induction of these strains showed that $GspB_{\alpha}$ is not required for assembly of the secretin multimer.

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List of Abbreviations

T2SS – Type 2 Secretion System
LT – Heat-Labile Enterotoxin
ChiA – Chitinase
HNS – Histone-like nucleoid structuring protein
GSP – General secretion pathway
Tat – Twin Arginine Translocase
T4P – Type 4 Pilus
PppA – Prepilin peptidase A
Gm_1 – Monosialotetrahexosylganglioside
B subunit – Binding subunit
A subunit – Activity subunit
ABTS - 2, 2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt
FRT – Flippase Recognition Target
SDS – Sodium Dodecyl Sulfate
PCR – Polymerase Chain Reaction
IPTG - Isopropyl β-D-1-thiogalactopyranoside
FCKF – FRT-Cam-Kan-FRT
BSA – Bovine Serum Albumin

Hypothesis

The chromosomal copies of the alpha T2SS system can be activated allowing the expression and multimerization of $GspD_{\alpha}$. This activation will allow an examination of the substrate specificity of the T2SS.

Objectives

1. Delete the known (HNS and StpA) repressors of the *gsp* operon from *E. coli* K12.
2. Induce plasmids within *hns* or *stpA* cells that encode heat-labile enterotoxin or chitinase to see if they appear in the supernatants of these mutant cells
3. Examine the multimerization of $GspD_{\alpha}$ in *hns* or *stpA* mutants to see if the T2SS system is assembling.
4. Replace the promoters controlling the *gsp* operons in *E. coli* K-12 with ptac or pAra promoters.
5. Determine if heat-labile enterotoxin or chitinase can be secreted by *E. coli* strains that contain ptac and pAra promoters controlling the expression of the *gsp* operons.
6. Determine if $GspD_{\alpha}$ can form the secretin multimer in the absence of $GspB_{\alpha}$ in *E. coli* K12.
7. Examine Enterotoxigenic *E. coli* with ptac and pAra promoters controlling the expression of the *gsp* operons for the assembly of the alpha T2SS system.

1 Introduction

1.1 Pathogenesis

1.1.1 *Escherichia coli*

Escherichia coli is considered a gut-associated microbe being found in the digestive tracts of humans and other warm blooded animals (Katouli, 2010). More than 400 species of bacteria have been found in the feces of a single person (Gorbach, 1996; Qin et al., 2010). *E. coli* and *Vibrio cholerae* are known to colonize the upper intestines (Gorbach, 1996). Colonization by strains of *E. coli* can cause severe diarrhea resulting in water loss through the production of toxins. *E. coli* is a Gram negative organism composed of many strains, some of which cause illness such as enterotoxigenic *E. coli* (*ETEC)* and some that do not including *E. coli* K12 strain MG1655. ETEC *E. coli* is the most significant cause of travellers' diarrhea in the third world (Qadri et al., 2005). There are six pathotypes of *E. coli* associated with diarrhea including shigatoxin producing *E. coli*, *ETEC E. coli,* enteropathogenic *E.coli*, enteroaggresive *E.coli*, enteroinvasive *E. coli* and diffusely adherant *E. coli* *(*http://www.cdc.gov/ecoli/general/index.html*)*. ETEC *E. coli* is the leading cause of diarrhea in children in developing countries and those that visit these areas (Gaastra and Svennerholm, 1996). For the years during the development of microbiology, *E. coli* was thought by most people to be the major inhabitant of the gastrointestinal system of animals. It is now understood that *E. coli* is not a major inhabitant but actually a minor inhabitant of most gastrointestinal systems (Savage, 1977).

1.1.2 Gram Negative Bacterial Secretion

A Gram negative bacteria has an inner membrane and an outer membrane (Figure 1 top) separated by a thin layer known as the periplasm (Sandkvist, 2001). Gram positive bacteria only have a single membrane which makes secretion much easier (Figure 1 bottom). Most Gram negative bacteria are coated by lipopolysaccharide (LPS). LPS is a large immunostimulatory molecule (for a review see Alexander and Rietschel, 2001). LPS is made up of a lipid anchor known as Lipid A, core sugars and a serotype specific O antigen (Horstman et al., 2004). Secretion through the outer membrane is solved by the assembly of the type 2 secretion system (T2SS system).

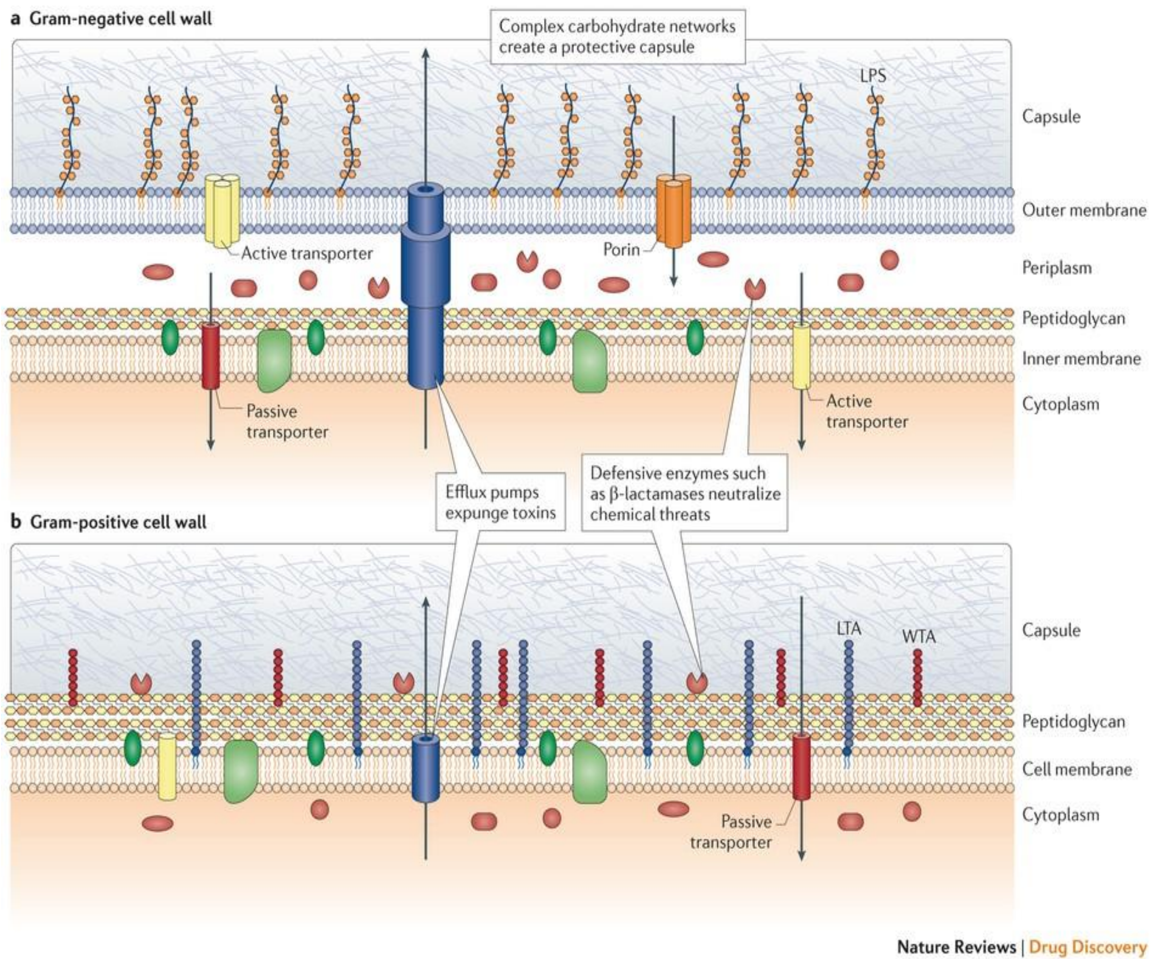


Figure 1: Gram Negative (top) vs Gram Positive (bottom). Tommasi et al., 2015. Reprinted by permission from Nature Publishing Group.

1.2 The Type 2 Secretion System

1.2.1 Introduction of the T2SS

The T2SS system, previously known as the main terminal branch of the general secretory pathway (GSP), is one of several secretion systems that exports proteins from within the cell to the extracellular milieu (Pugsley, 1993; Cianciotto, 2005). The T2SS in *E. coli* was first discovered by Tauschek et al (2002) for the secretion of factors that cause the diarrheal illness. Mutations in this secretion system inhibited secretion while the complementation of secretory machinery components in trans restored secretion. The T2SS system (Figure 2) is a molecular apparatus composed of 12 subunits of the *gsp* operon including the conserved 'core' genes known as *gspC-O;* the T2SS system may contain accessory proteins encoded by the genes *gspAB*, *gspN* or *gspS* (Douzi et al., 2012). The T2SS is composed of an inner membrane platform, a large secretin, an energy source and pre-pilin subunits that aid in the secretion of proteins (Nivaskumar and Francetic, 2013).

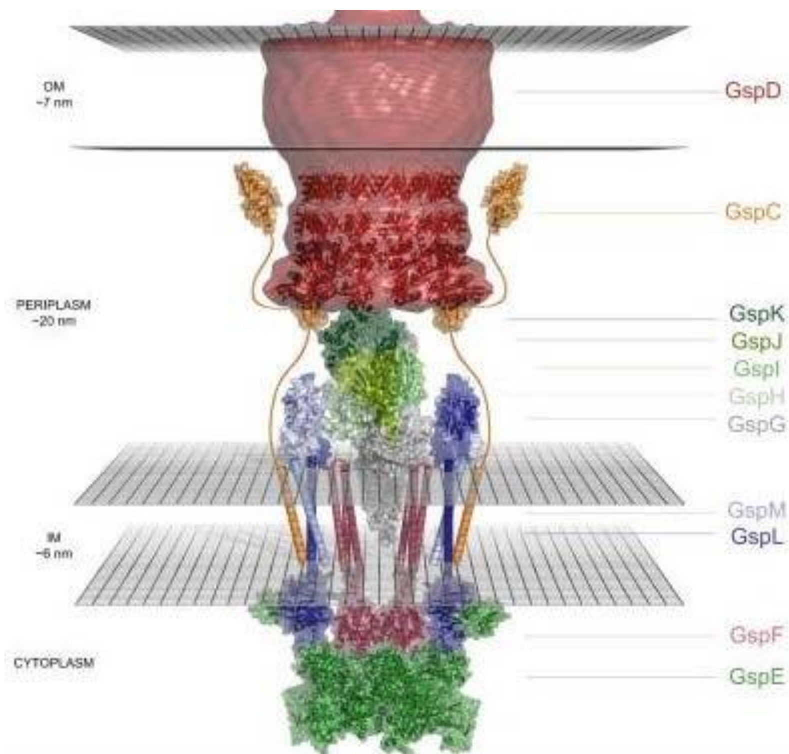


Figure 2: The T2SS system. The T2SS System is composed of many subunits of *gspC-O* and may contain accessory proteins such as *gspAB, gspN* and *gspS.* (McLaughlin et al., 2012) Reprinted with permission from Elsevier

1.2.2 Inner membrane transport

The T2SS system transports proteins that have crossed the inner membrane. The crossing of the inner membrane requires the use of either the sec (general secretion) system (Pugsley, 1993) for unfolded proteins or the tat (twin-arginine translocase) pathway (Voulhoux et al., 2001) for folded ones (Lee et al., 2006; Jermy, 2012). In the sec system, unfolded proteins in the cytoplasm are recognized by either a chaperone protein known as SecB or a Signal Recognition Particle (SRP). The chaperone, in the first step, carries the unfolded protein to a receptor (SecA). The second sec pathway includes the use of a SRP that passes the unfolded protein to FtsY, the SRP receptor. The signal sequence is then cleaved by SPase after being passed through the inner membrane pore. The pore consists of SecY, E and G and includes several additional protein components that assist in protein translocation, including SecA, D, F and YajC. Proteins that are destined for the periplasm pass through the inner membrane before they are folded. Folded secretory proteins in the periplasm are recognized by either the T2SS system or YidC. YidC aids in the assembly and folding of proteins that reside within the inner membrane (Scotti et al., 2000). Proteins destined for secretion then come in contact with the T2SS system in the periplasm and are secreted out of the bacterial cell.

1.2.3 The Inner Membrane Platform of the T2SS system

Assembly of the T2SS system (Figure 2) begins with *gspC, gspL* and *gspM* which form a platform in the inner membrane (Korotkov et al., 2012). The inner membrane platform's purpose is to allow the assembly of other T2SS system components (Py et al., 2001), not to secrete proteins into the periplasm (Tseng et al., 2009). Energy is required for the process of translocation which is supplied by GspE. GspE is an ATPase within the cytoplasm that takes stored energy in the form of ATP and converts it into energy that is utilized by the T2SS system (Lu et al., 2013). Lu et al (2014) have shown that the T2SS system in *Vibrio vulnificus* requires an interaction between the inner membrane platform (GspL) and the energy source (GspE) for proper secretion.

1.2.4 The Pseudopilus of the T2SS system

GspO is a peptidase that cleaves preGspG-K subunits before their assembly into the pseudopilus (Francetic et al., 1998). A second prepilin peptidase gene (*pppA*) was discovered in *E. coli* K12 which can be used as the only peptidase in the organism. PppA requires a glycine amino acid at the -1 amino acid for processing while the other doesn't. PppA was used in complementation studies in which it was able to complement the cleavage of pre-pilin subunits in the GSP system (Francetic et al., 1998; Yang et al., 2007). *ETEC E. coli* is known to possess only the PppA protein for pre-pilin subunit processing of the beta T2SS system. It has been proposed that the pseudopilus aids in the translocation of proteins from the periplasm through the outer membrane to the extracellular space (Filloux, 2004).

1.2.5 Outer Membrane Secretin

GspD subunits form a multimer known as the secretin composed of 12 subunits which insert into the outer membrane (Chami et al., 2005; Tauschek et al., 2002), this pore is essential for the export of proteins. GspC is a protein that interacts with the secretin, GspC is the link between the inner membrane platform including the energy source and the outer membrane pore. The secretion by Gram negative bacteria that possess this system cannot take place unless there is multimerization of GspD. The proteins of the T2SS system can be seen in Figure 2 and Figure 3. The GspD protein is stable enough to be boiled in SDS and remain folded. Localization of the secretin must be in the outer membrane of bacteria for the secretion of proteins. The N-terminal domain of the protein is made up of four different subdomains including N0, N1, N2 and N3 (Figure 3; Korotkov et al., 2009). The structure has a diameter of 155 Å with a length of 200 Å (Figure 3). The opening of the secretin is approximately 70 Å which narrows down to approximately 55 Å (Figure 3; Reichow et al., 2010). The N0-N1-N2 domain could be mapped into the wall of the periplasmic chamber (Reichow et al., 2010). According to this model, the N3 subdomain may be responsible for conformational changes during the secretion of proteins (Korotkov et al., 2011).

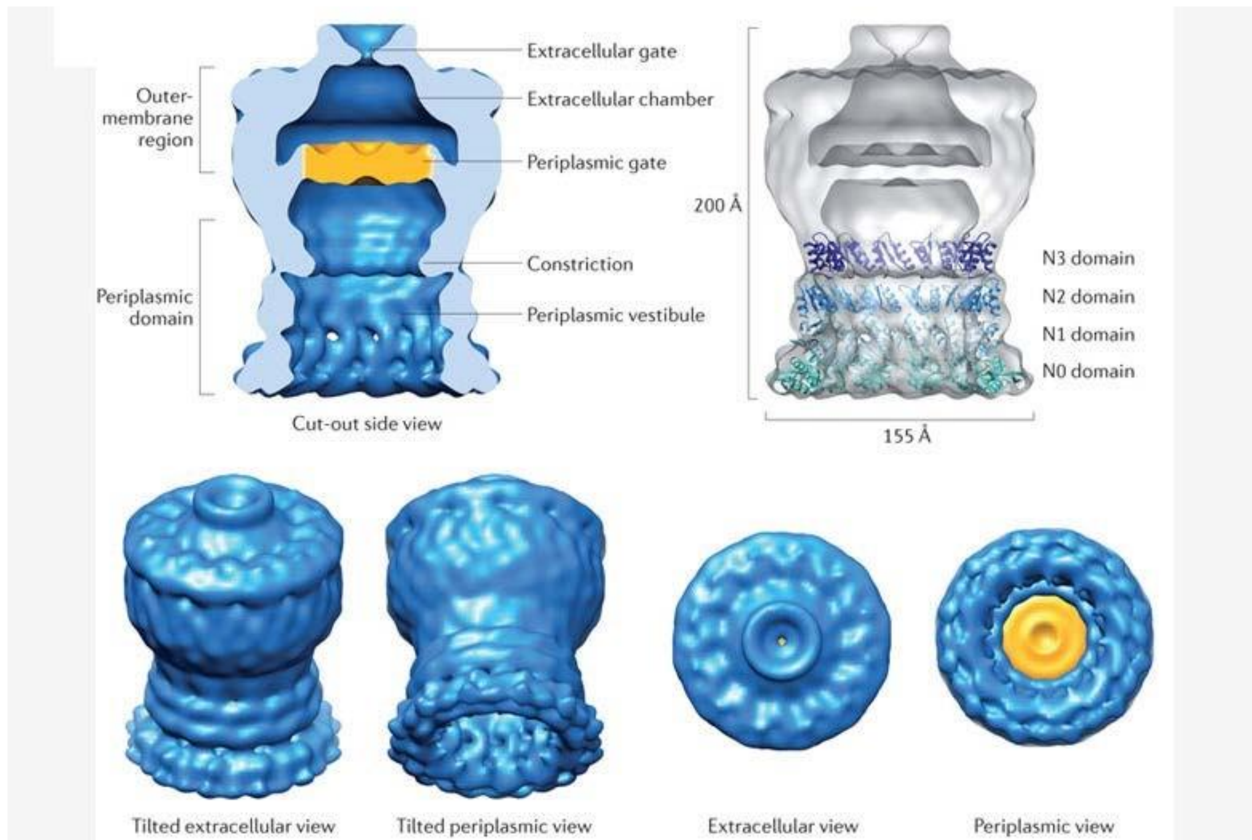


Figure 3: A model of the *Vibrio cholera* T2SS system obtained through cryo-EM. A slice of the GspD protein from *V. cholerae* in the top left panel. The top right panel shows the organisation of N-terminal domains of this protein and the bottom shows different views of the GspD secretin multimer from *Vibrio cholerae*. Konstantinin et al, 2012. Reprinted with permission from Nature Publishing Group.

1.2.6 T2SS system Accessory Proteins

The GspA and GspB requirement for secretion by *E. coli* has not been fully characterized. The requirement of these accessory proteins have been studied in *Aeromonas hydrophila* and *Vibrio species* being called ExeAB and EpsAB respectively. Mutations were created in *A. hydrophila* such that the cells did not secrete proteins (Jiang and Howard, 1991). These mutations were mapped to the *exeAB* or *exeC-N* regions. ExeA contains a single peptidoglycan binding domain (Howard et al., 2006; Li and Howard, 2010). ExeA (GspA) is an ATPase that functions to supply energy for assembly of the secretion system. Mutations in this conserved ATP binding site cause defects in complex formation (Schoenhofen et al., 1998). The ExeB protein (a *gspB* homolog) was shown to interact with the ExeD (a *gspD* homolog) protein upon analysis using yeast and bacterial two hybrid systems. Hybrid studies were performed by Elizabeth Vanderlinde et al (2014) to show this binding interaction. GspD was split into four segments containing domains N0, N1N2N3, N0N1 and N2N3. Only N0N1 fusions had growth on Trp-Leu-His media upon its interaction with GspB. The result suggested that ExeB and ExeD interact (Vanderlinde et al 2014). ExeA forms multimers upon binding to peptidoglycan (Li and Howard, 2010). It has previously been shown that ExeAB in *A. hydrophila* forms a complex (Ast et al., 2002). ExeB is degraded without the presence of ExeA and vice versa. The complex is required for the assembly of ExeD and the secretion of proteins in *Aeromonas salmonicida* and are involved in the assembly of EpsD in *Vibrio* species. The presence of the EpsAB complex was shown not to be required in the secretion of proteins by *V. cholerae* (Strozen, 2012). The T2SS outer membrane secretin is localized to the inner membrane when the complex is not present

in *A. hydrophila (*Ast et al., 2002). Sucrose density fractionation was used to separate the membranes followed by a western blot detecting the amount of ExeD in membranes (Ast et al., 2002). Both *E. coli* K12 and *ETEC E. coli* have these proteins in the alpha T2SS system but function in these *E. coli* systems is yet to be determined.

A third accessory protein is known as GspS. GspS is also involved in secretin assembly by delivering the outer membrane protein (GspD) to the outer membrane (Strozen et al., 2012). Deletion of the EpsS protein and the EpsA protein causes severe defects in secretin assembly in *V. cholerae* (Poppleton, 2013). The function of EpsA and EpsS may be redundant. (Poppleton, 2013).

1.3 Gram negative organisms that possess a T2SS system

The T2SS system is found in many Gram negative organisms including *V. cholerae* in the human digestive tract for the secretion of cholera toxin (Dustan, 2013; Korotkov et al., 2011; Sandkvist et al., 1993). *V. cholerae* is known to cause life threatening secretory diarrhea characterized by watery stools. The watery stools often lead to vomiting resulting in hypovolemic shock (Finkelstein, 1996). *V. cholerae* has the *eps* system which is a T2SS which allows the secretion of cholera toxin (Sandkvist et al., 1997).

The T2SS system can be found in *A. hydrophila* in the human body for the secretion of toxins such as aerolysin (Ast et al., 2002; Bo and Howard, 1991; Schoenhofen et al., 1998). *A. hydrophila* causes sporadic disease in humans but can cause epidemic disease in organisms such as fish (Tekedar et al., 2015). The pathogen poses a significant threat to the fish farming industry. The *A. hydrophila* T2SS system is known to consist of the same components as the

T2SS system in *E. coli* but is known as the Exe system instead of the Gsp system.

Klebsiella species are known to secrete different proteins through the T2SS. The two major known organisms of this genus are *Klebsiella oxytoca* and *Klebsiella pneumoniae. K. oxytoca* is the second most causative agent of bacteremia in the world (Korvick et al., 1992) while *K. pneumoniae* accounts for a significant portion of hospital acquired urinary tract infections, pneumonia, septicemias, and the infection of soft tissues (Podschun and Ullman, 1998). One of the secreted proteins from *K. oxytoca* is known as pullulanase (Francetic and Pugsley, 2005). Pullulanase is a debranching enzyme that specifically hydrolyzes α(1-6) glycosidic linkages (Michaelis et al., 1985; Xu et al., 2014). It has been shown that the T2SS is required for evasion of the immune system by *K. pneumonia* (Tomàs et al., 2015). The T2SS system in this organisms is composed of *pul* genes and was the first T2SS system studied being found in *K. oxytoca* (Russel, 1998).

Pseudomonas aeruginosa has been studied for many years and is responsible for many infections (Joyt et al., 2011). *P. aeruginosa* contains many different T2SS systems with different functions. One system known as the Xcp system was the first T2SS system studied in this organism. The system is responsible for the secretion of many virulence factors including elastase, lipase, exotoxin A and alkaline phosphotase (Akrim et al., 1993). Studies show that the presence of the T2SS from *P. aeruginosa* plays a role in the lethality of pneumonia (Jyot et al. 2011). It has been shown that the secretion of proteins requires the interaction between XcpP and XcpQ. These two proteins must be required for the recognition of secretion substrates (de Groot et al., 2001). The second T2SS system discovered in this organism was the Hxc system which stands for homologous to *xcp* genes. It was found to be responsible for the secretion of

low molecular weight alkaline phosphatases (Ball et al., 2002). The third secretion system found in this organism is the Txc system which stands for the third homolog to *xcp*. Not many studies on this secretion system have been done but the system is known to secrete chitin-binding protein E which functions to bind chitin (Cadoret et al., 2014).

1.4 Suggested Evolution from the Type 4 Pilus (T4P)

It has been hypothesized that the T2SS is evolutionarily similar to T4P based on the similarities of the pilus structures (Gu et al., 2012). The systems even show homologs in Grampositive bacteria and archaebacteria (Hazes and Frost, 2008). The T2SS is used for the secretion of proteins while the T4P is important for many functions including adhesion, motility, microcolony formation and the secretion of proteins including proteases and colonization factors (Craig and Li., 2008). The T4P and the T2SS system both have five inner membrane proteins that interact (Karuppiah et al., 2013). The assembly of inner membrane proteins is required for assembly of other components of the systems. The T4P uses one pseudopilin to form the pilus fiber while the T2SS system has five different pseudopilin proteins that form the pseudopilus for secretion. It has been shown that the formation of a pilus or pilus-like structure is a hallmark for the function of both systems (Hazes and Frost, 2008). Both systems and archaeal flagella consist of pilin-like proteins that hold specific functions within the system (Peabody et al., 2003). The T4P and the T2SS system are required in their respective organisms for processes such as adherence and pathogenesis. The removal of a single component of the systems can drastically affect function (Kulkarni et al., 2009). Both systems are known to be composed of similar protein components (Figure 4).

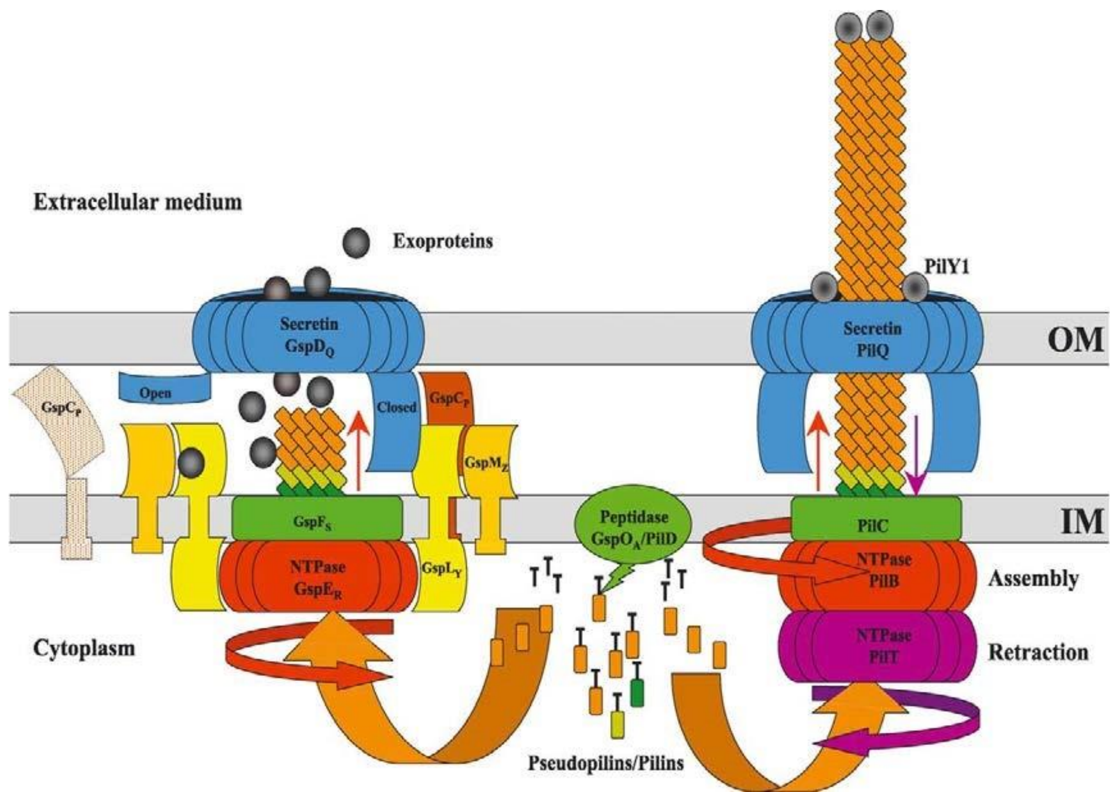


Figure 4: The T2SS system (left) and its evolution from the type 4 pilus (right). The T2SS System is composed of several subunits of *gspC-O* while similar proteins have been found to be required for function by the Type 4 pilus. The grey circles are secreted proteins that move through the T2SS system. Filloux, 2004. Reprinted with permission from Elsevier.

1.5 Alpha vs Beta Type 2 Secretion Systems

1.5.1 The Beta T2SS system

Two different T2SS systems have been found in *ETEC E. coli* known as the alpha and beta systems (Strozen et al., 2012). The systems are separated based on genetic comparisons (figure 5). These two systems upon further study can be separated into either Vibrio-type or Klebsiellatype systems (figure 6) based on phylogenic analysis (Dunstan et al., 2013). The alpha T2SS system in *E. coli* is classified as a Klebsiella-type while the beta T2SS system in *E. coli* is classified as a Vibrio-type system (Dunstan et al., 2013).

The beta T2SS system is composed of a single operon that consists of *gspC-M* preceded by *yghG, pppA* and *yghJ* (figure 6). This system does not contain *gspO* but contains a gene with a similar function, pre-pilin peptidase (*pppA)*. The PppA protein functions to cleave pre*gspG-K* before the components can assemble into the pseudopilus (Francetic and Pugsley, 1996). One protein that is unique to the beta T2SS system is *yghG* ($gspS_{\beta}$), which has been shown to be the pilotin of this T2SS system aiding in the assembly of $GspD_{\beta}$ (Strozen et al., 2012). The beta T2SS system is expressed when *E. coli* strains containing this system are grown under standard laboratory conditions. However another T2SS system is present in *ETEC E. coli.* This second T2SS system shares a large portion of genetic similarity with the beta T2SS system (figure 6). This second T2SS system was designated the alpha T2SS system which is not expressed during growth under standard laboratory conditions (Strozen, 2012).

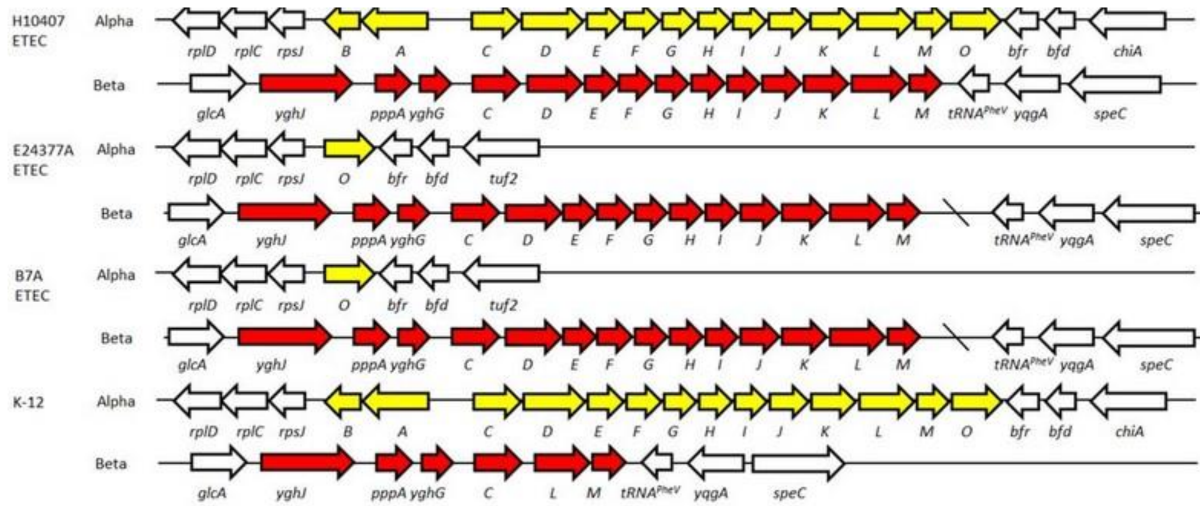


Figure 5: Differentiation of the T2SS in *E. coli*. *ETEC E. coli* has an active beta and a cryptic alpha T2SS system while *E. coli* K12 strain MG1655 has a cryptic alpha T2SS system and a truncated beta T2SS system (Strozen, 2012).

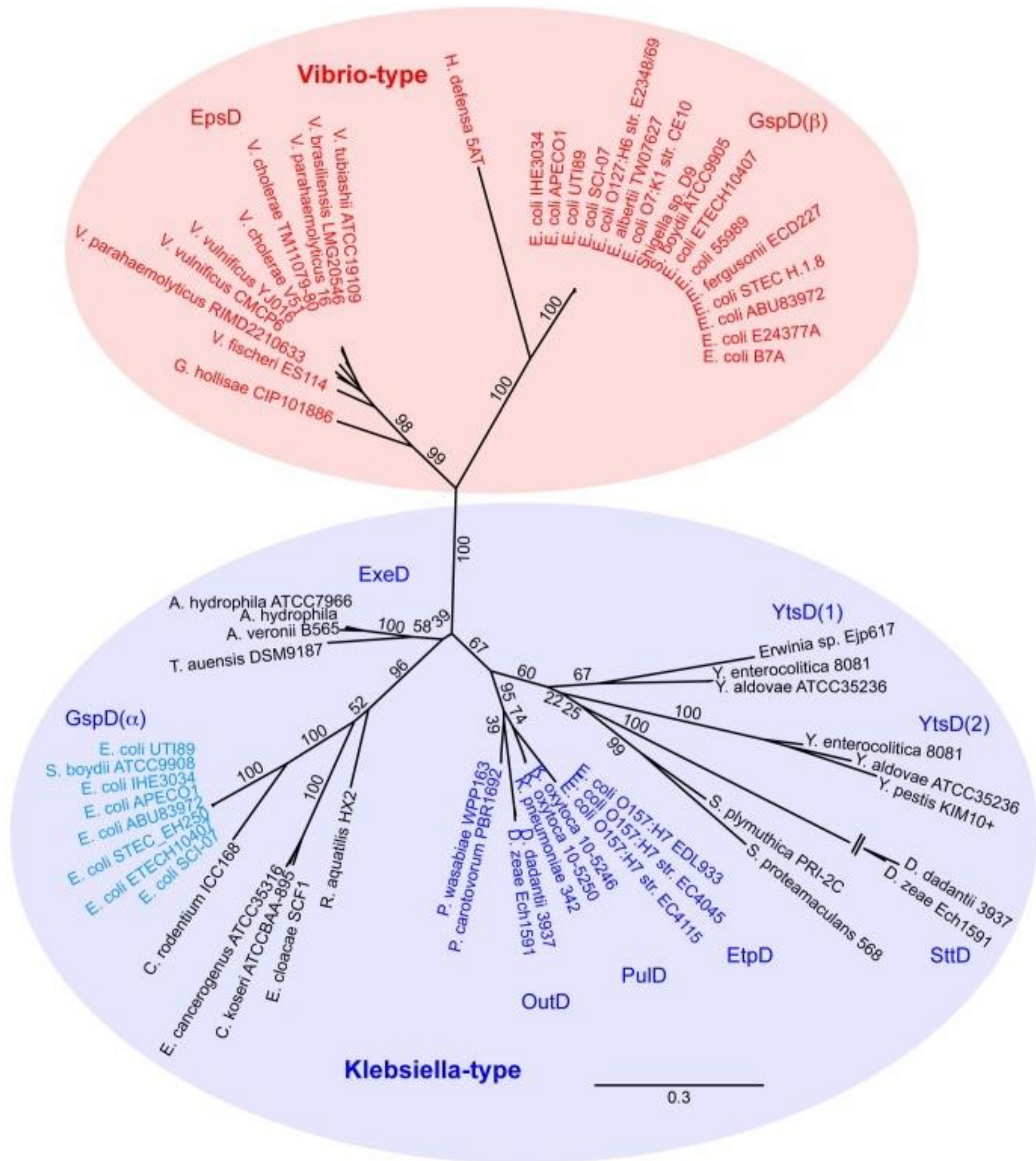


Figure 6: Differentiation of the T2SS in many Gram negative organisms (Dunstan et al., 2013).

1.5.2 The alpha T2SS system

It was found through sequence comparisons that *E. coli* K12 had components that resemble the pullulanase system from *K. oxytoca (*Francetic and Pugsley, 1996). The components were used for complementation assays by being expressed in trans. It was found that GspO could process pre-PulG when *pulO* was inactivated (Francetic and Pugsley, 1996). It was also found that GspG can substitute for PulG in the secretion of pullulinase which can be processed by GspO in a *K. oxytoca* strain that is deficient in *pulO (*Francetic and Pugsley, 1996). These experiments showed that *E. coli* K12 have the components for a working secretion system. Pathogenicity is not associated with the possession of this system as the alpha T2SS system is not normally expressed when grown under standard laboratory conditions as this system is cryptic.

The alpha T2SS secretion system of *E. coli* K12 has two divergent operons (Blattner et al., 1997) consisting of *gspAB* and *gspC-O* (figure 21). The system is known to be cryptic and mechanisms that keep these operons repressed were studied. Fusions were created in which *lacZYA* was fused to *gspD* and *gspA*. These strains could grow on MacConkey lactose agar if the promoters controlling *gspD* and/or *gspA* were induced. Transposon mutants were created and the expression of the fusions were assessed. The promoters were upregulated in *hns* mutants (Francetic et al., 2000) indicating that HNS was the repressor of this system.

Studies were performed in which secretion from this system was shown though immunoblotting using anti-MalE-ChiA antiserum (Francetic et al., 2000). Conditions were used in which the *gsp* promoters were activated and plasmids were induced which carried the *gsp*

proteins, chitinase was also expressed on a plasmid. The studies showed secretion by this system could be observed. Induction of promoters driving expression of the GspAB complex from plasmids was required for secretion to be observed. This was the first study showing secretion by the alpha system. Horstman and Keuhn in 2002 did similar studies. They did experiments in MC4100, a K12 strain, and made observations about the secretion of LT by the T2SS system. Plasmids containing the *gsp* operons were induced in this strain in the absence of *hns*. The secretion of LT was observed and it was found to bind to the bacterial membrane.

1.6 Proteins destined for secretion

1.6.1 AB_5 toxins

E. coli secretes several different proteins for survival and pathogenesis including LT and ChiA. LT is an AB_5 toxin (figure 7) found in *ETEC E. coli* that resembles cholera toxin found in *V. cholera (*Mudrak and Kuehn, 2010; Li et al., 2014). The A subunit indicates that it is the active subunit which performs changes in the eukaryotic cell while the B subunit is used for binding (Tauschek et al., 2002). The B subunit of LT is secreted by the T2SS system (Mudrak and Kuehn, 2010). The B subunit is known to bind to Galβ1,3GalNAcβ1(neuAcα2,3),4Galβ1,4Glc ceramide (G_{m1}) gangliosides that are found on the surface of many eukaryotic cells (Sixma et al., 1991; Mudrak et al., 2009; Becker et al., 2010). The B subunit binds to G_{m1} forming a pore that allows the A subunit to enter the cell (Li et al., 2013). The A subunit ADP-ribosylates the stimulatory GTP-binding protein (G_{sa}) in the adenylate cyclase pathway (Dickinson and Clements, 1995). The activity of the A subunit results in an increase of cyclic AMP (cAMP) within the cell. This increase

in cAMP levels causes the secretion of water and ions into the small intestine. The clinical result of LT attachment to mammalian intestinal cells is diarrhea (for a review of AB_5 toxins see Mudrak and Kuehn, 2010).

1.6.2 Heat-labile Enterotoxin

It is known that LT binds to G_{m1} on the surface of eukaryotic cells, but much further research has been done on this binding interaction. LT is a common AB_5 toxin like CT (Mudrak and Kuehn, 2010). The toxin is delivered to mammalian cells through the release of vesicles (Horstman and Kuehn, 2000; Kesty et al., 2004). The toxin is secreted by the bacterial cell by the T2SS before binding to the surface of the bacterial cell. Outer membrane components are released and the toxin is recognized by eukaryotic cells as vesicles (Horstman and Kuehn, 2002). Studies by Horstman et al in 2003 were performed to see why the toxin binds to the surface of the bacterial cell. *E. coli* contains two core sugars while *V. cholerae* contains only a single core sugar (Horstman et al., 2004). *E. coli* releases vesicles while *V. cholerae* releases soluble toxins. The thought was that the presence of one or two core sugars was the determinate but the experiments proved this to be false as a single or double set of *E. coli* core sugars did not affect the release of vesicles. *V. cholerae* phosphorylates its core sugars while *E. coli* does not. This was found to be the reason for the release of vesicles or the release of soluble toxins (Horstman et al., 2004). The T47 amino acid was found to be important in the binding of the toxin to WT *E. coli* cell surfaces but not its secretion (Mudrak et al., 2009).

1.6.3 Chitinase

Chitin is the second most abundant polymer in nature besides cellulose, being composed of β-1,4-linked N-acetylglucosamine (GlcNAc) residues (Frederiksen et al., 2013). Chitin can be found in crustacean shells and fungal cell walls and the forms the major component of arthropod cytoskeletons (Francetic et al., 2000; Zou et al., 2002; Fredericksen et al., 2013). Chitinases are found throughout nature being present in fungi, yeasts, plants, bacteria, arthropods and humans (Hamid et al., 2013). Chitinases are glycosyl hydrolases that catalyze the degradation of chitin. The degradation product of chitin may or may not be used as a carbon source (Bhattachrya et al., 2007).

The supernatant of wild-type *V. cholerae* cultures has chitinase in the supernatant at detectable levels. Mutational analysis has shown that a deletion in a component of the *eps* system in *V. cholerae* resulted in the absence of chitinase in the supernatant. The results suggested that chitinase is secreted by the *eps* T2SS system in *V. cholerae* (Connel et al., 1998). Research by Francetic et al (2000) in *E. coli* K12 shows that the *chiA* gene is found downstream of the *gspC-O* operon with repression by HNS. The induction of plasmids bearing the *gsp* operons and *ChiA* show that the protein can be secreted by the alpha T2SS upon the examination of supernatant sample immunoblots developed with anti-MalE-ChiA antiserum (Francetic et al., 2000).

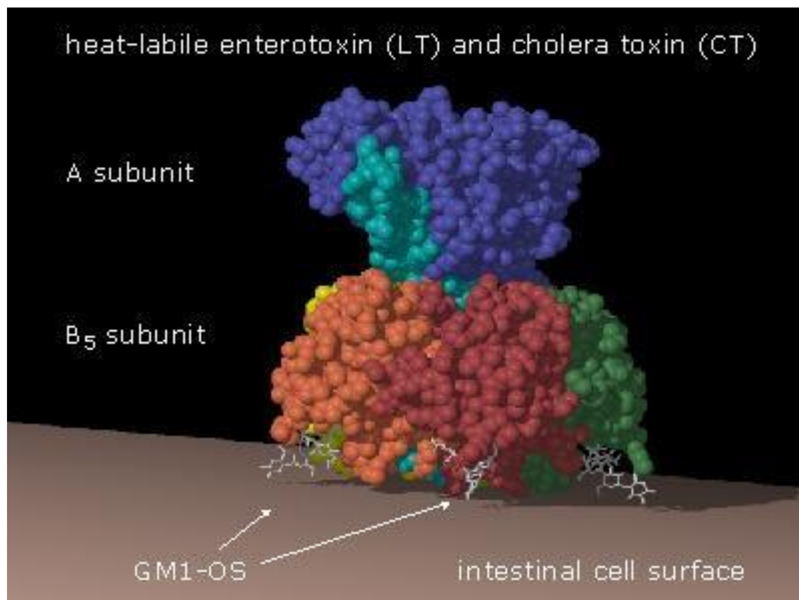


Figure 7: A model of an AB_5 toxin. The toxin consists of five B subunits and one A subunit (http://www.bmsc.washington.edu/WimHol/figures/figs2/WimFigs2.html).

1.7 Bacterial Repressors of the alpha Type 2 Secretion System

HNS is a bacterial transcriptional repressor in the cell which stands for Histone-like Nucleoid Structuring protein. HNS silences genes including the gsp_{α} operons and operons that have a large adenine and thymine content (Atlung et al., 1996; Yang et al., 2007). HNS is known to bind curved segments of DNA as shown by Yamada et al. (1991). The repressor works in several ways including inhibition by trapping RNA polymerase and thus preventing transcription (Brambilla and Sclavi, 2015). HNS has been shown to inhibit the expression of ChiA.

HNS is not the only molecular repressor of the *gsp* operons, another protein known as StpA inhibits the expression of operons when HNS is not present (Free et al., 1998). StpA is another known nucleoid-associated protein functioning to bind DNA segments together (Keatch et al., 2005). A mutant in *Uropathogenic E. coli* in which only StpA was removed from the bacterial cell does not show an effect on transcription rates but a double mutant in both *hns* and *stpA* shows a larger impact than an *hns* mutation on the expression of many genes within the bacterial cell (Müller et al., 2006).

1.8 Inducible promoters used for experiments

The ptac promoter is a synthesis of different promoter elements (de Boer et al., 1983). The DNA upstream of position -20 is from the trypthopan (trp) promoter. The DNA downstream of -20 is derived from the lactose (lac) UV5 promoter (de Boer et al., 1983). The trp promoter has been taken from the *E. coli* tryptophan operon which functions to control the expression of the tryptophan biosynthetic genes (Bass and Yansura, 2000). The *lac* operon consists of *lacZ, lacY* and *lacA* (Hediger et al., 1985). The gene product of *lacZ* is β-galactosidase which cleaves

lactose into glucose and galactose, *lacY* encodes a protein whose function is to bring lactose into the cell, *lacA* produces a protein required for cell detoxification. *lacI* encodes a repressor which silences the *lac* promoter unless lactose is present.

The paraBAD promoter controls the regulation of genes required for the use of arabinose. The promoter is activated in the presence of arabinose and inhibited in the presence of glucose. The promoter is regulated by the AraC protein. This protein binds to AraI and AraQ forming a loop structure in the DNA which prevents transcription. The binding of AraC to AraI in the absence of arabinose prevents the degradation of AraI (Lee et al., 1987). The addition of arabinose activates the promoter by binding to AraC allowing the degradation of AraI and the transcription of genes that are present down-stream of this promoter. Experiments have been done which showed that the paraBAD promoter has a fast induction rate. The promoter was found to be under stronger regulation than the ptac promoters that was used in similar experiments (Guzman et al., 1995).

2. Materials and Methods

2.1 Bacterial Strains and Culture Conditions

In physiological growth experiments, cultures were grown overnight in antibiotic containing 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 1.5-2.0 was reached. An OD_{600} of 2.0 was used initially but changed to an OD_{600} of 1.5 to solve problems such as lysis and double mutant growth control.

The list of strains used can be found in Table 1. All strains were maintained on plates of Luria Broth (LB) medium with 1.6% agar. In liquid culture and plates, antibiotics were used at the following concentrations, unless specified otherwise: kanamycin (kan), 50 µg/ml; chloramphenicol (cam), 30 µg/ml; ampicillin (amp), 100 ug/ml.

2.2 DNA manipulation

2.2.1 P1 Transduction in *E. coli* K12

Transduction was used to move genes from a donor strain of bacteria to a recipient strain using the phage P1. P1 transduction was performed using methods described by Thomason et al (2007) as modified by Timothy Strozen (2012). Donor cells, for example where mutant cells with a resistance cassette inserted into genes of interest were grown overnight before a 1/10 culture dilution was made. The resulting 1/10 dilution had calcium chloride added to 5 mM. 100 µl of P1 phage that was grown on WT *E. coli* strain MG1655 was added. The resulting solution was incubated at 37°C for twenty minutes. 7.5 mls of top agar (2.0g NaCl, 2.0g Tryptone, 1.0g yeast extract and 8.0g Agar topped to 250 ml with ddH_2O) was added to

Table 1: List of Strains

Strains	Relevant Genotype	Resistance Marker	Source or Reference
MG1655	Wild-type K-12 F^{-} λ^{-} *ilvG*$^{-}$ *rfb-50 rph-1*		Hayashi et al., 2006
BW25113	*lacI*$^{+}$ *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR*514 Δ*araBAD*AH33 Δ*rhaBAD*LD78 *rph1* Δ*(araB–D)567* Δ*(rhaD– B)568* Δ*lacZ4787*(::*rrnB3*) *hsdR514 rph-1*		Grenier et al., 2014
BW25113 *hns*	BW25113 *hns*::Kan	Kan^{R}	Baba et al., 2006
BW25113 *stpA*	BW25113 *stpA*::Kan	Kan^{R}	Baba et al., 2006
BW25113 *gspA*	BW25113 *gspA*::Kan	Kan^{R}	Baba et al., 2006
BW25113 *gspD*	BW25113 *gspD*::Kan	Kan^{R}	Baba et al., 2006
NEB5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*		New England Biolabs
XL-1 Blue	*endA1 gyrA96(nal*^{R}*) thi-1 recA1 relA1 lac glnV44 F'[::Tn10 proAB*$^{+}$ *lacI*^{q} *Δ(lacZ)M15] hsdR17(r*$_{K}^{-}$ *m*$_{K}^{+}$*)*		Stratagene
H10407	*wild-type ETEC serotype O78:H11, LT+ ST+*		Evans et al., 1975
BL21(DE3)	*F*$^{-}$ *ompT gal dcm lon hsdS*$_{B}$*(r*$_{B}^{-}$ *m*$_{B}^{-}$*) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])*		Daegelen et al., 2009
TGS60	*H10407 pTac-gspAB*$_{\alpha}$*, pBAD-gspC-O*$_{\alpha}$*, ΔgspD*$_{\beta}$	Kan^{R} Cam^{R}	Strozen, 2012
MBM 1	BW25113 *hns stpA*::Kan	KanR	
MBM 2	BW25113 *stpA hns*::Kan	KanR	
MBM 3	MG1655 *gspA*$_{\alpha}$*-pTac*-FRT-*Kan-Cam*-FRT*pBAD-gspC*$_{\alpha}$	$Kan^{R}Cam^{R}$	
MBM 4	MG1655 *gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD*		
MBM 5	MG1655 *gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD gspB::kan*	KanR	

the solution before 5 mls of the resulting solution was added to 2.5 mM Calcium Chloride and 0.1% glucose LB plates. The non-selective plates were incubated at 37°C overnight.

The top agar was removed from the incubated plates and placed in a centrifuge tube. All of the lysate was removed by adding 5 mls of fresh LB media to the plate, the added media was poured into the same labeled centrifuge tube as the previous step. Approximately 400 µl of chloroform was added to each labeled centrifuge tube. The sample was left to incubate for five minutes at room temperature before being thoroughly mixed. The tubes were centrifuged at > 13 000 x g for ten minutes at 4°C. The supernatants were transferred to sterile falcon tubes and were refrigerated at 4°C.

500 µl of CM buffer (15mM $CaCl_2$ and 30mM $MgCl_2$) with 100 µl of the previously isolated lysate and 100 µl of recipient cells from and overnight culture in LB were added together in a sterile tube. The solution was incubated at 37°C for twenty minutes. The solution was centrifuged for one minute at > 16 000 x g. The resulting supernatant was discarded while the pellet was resuspended in 500 µl of LB + 20mM citrate. The resuspended pellet was incubated at 37°C for two hours. 100 µl of the resulting culture was plated on plates selective for the transferred marker of interest + 20 mM citrate. The plates were incubated at 37°C overnight which allowed growth of cells into colonies that received the selected marker.

2.2.2 Lambda Red Recombination

The procedure was performed previously by Datsenko and Wanner (2000) with changes described by Timothy Strozen (Strozen, 2012). The procedure requires more than amplification,

it requires amplification with PCR primers encoding the DNA sequences of the DNA flanking the targeted integration site fused to standard PCR primers to amplify the DNA to be inserted (Figure 8). The lambda RED plasmid (Table 3) prevents single stranded DNA degradation allowing homologous recombination, this plasmid was electroporated into MG1655. 25 mls of LB was inoculated with a 1/100 dilution of an overnight culture of MG1655 cells containing the lambda RED plasmid. The resulting strains were grown to an OD_{600} of 0.1. Once cells reached this OD, arabinose was added to a final concentration of 0.3% to induce the lambda RED plasmid. The cells continued to grow at 30°C until reaching a final OD_{600} of approximately 0.6. The culture was placed at 42°C for fifteen minutes and transferred to an ice water bath for ten minutes to make these cells electrocompetent. The cells were pelleted through centrifugation at 6 000 x g for 5 minutes and washed three times in 20% glycerol + 1 mM MOPS. The final pellet was resuspended in 200 µl of 20% glycerol + 1mM MOPS, 50 µL was used for each electroporation. 1 µl of the PCR amplified product was used for each electroporation. The transformed colonies were recovered in 460 µl of SOC media and placed in a 37°C water bath for four hours. The resulting samples were plated on selective media and allowed to grow at 37°C overnight.

2.2.3 The removal of Kan resistance cassettes using FRT recombination

The method employed was described by Baba et al (2006). Keio collection (Figure 9) mutants were obtained, these mutants have a kanamycin resistance cassette inserted into nonessential genes. The plasmid pCP20 (Dansenko and Wanner, 2000; Table 3) was electroporated into these cells, the gene encoded by the plasmid allows a recombination event

between flanking flippase recognition targets (FRT). 460 µl of SOC media was added to the freshly electroporated cells and the resulting samples were incubated with shaking at 30°C in a water bath for one hour before plating. 100 µl of cells were placed on amp selective plates and placed at 30°C overnight as an increase in temperature causes pCP20 to be cured from the bacterial cell. Colonies were isolated by streaking for isolated colonies on LB plates containing Kan, incubated at 30°C overnight. A single colony was then patch plated onto LB + Kan and LB plates, both plates were placed in a 37°C incubator overnight. Colonies that could grow at 37°C on LB plates and not LB + Kan plates had lost antibiotic resistance and a scar was left where the resistance cassette was excised.

Table 2: Plasmids used in this study

Plasmid	Short Designation	Relevant Genotype	Resistance Phenotype	Source or Reference
pMAL-p4x		M13ori, creation of MalE fusion proteins	Amp^R	New England Biolabs
pBAD322C		*araC, rop, cat*, Pbad promoter upstream of MCSa	Cam^R	Cronan, 2006
pCP20		*FLP⁺, λ cI857⁺, λ pR*	Amp^R Cam^R	Datsenko and Wanner, 2000
pRED/AMP		*Red⁺, Gam⁺, Exo⁺*	Amp^R	Genebriges
pGem-T easy		high copy number vector	Amp^R	Promega
pBad322c(*lt_B*)	pTS76	encodes the B subunit of LT	Cam^R	Strozen T, 2012
pMAL-p4x(*chiAmyc*)	pTS60	*chiA* with a *myc* tag	Amp^R	Strozen T, unpublished
pGem-T easy(pTacFRT-*kan-cat*-FRTpBAD)	pMM1	encodes pTac-FRT*kan-cat*-FRT-pBAD	$Amp^R Cam^R Kan^R$	This Study
pMAL-p4x Δ*lacI* Δ*malE* Ω*tetR/tetA*	pMM2	*tetR and ptetA*	Amp^R	This Study
pMM2(*$lt_β$*)	pMM3	*tetR and ptetA, eLTB*	Amp^R	This Study
pMM2(*chiA-myc*)	pMM4	*tetR and ptetA, chiAmyc*	Amp^R	This Study

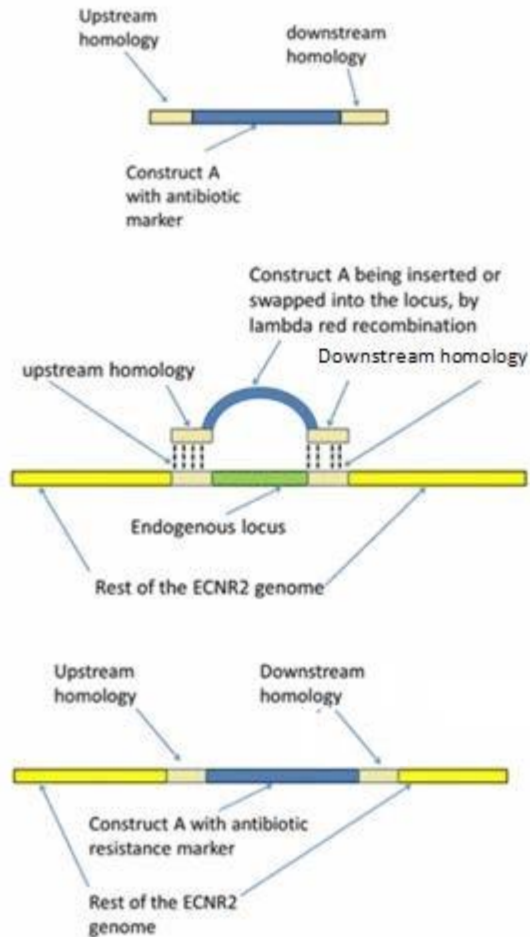


Figure 8: Lambda Red Recombination. The lambda red plasmid consists of *gam*, *exo*, and *beta* genes expressed through the addition of arabinose. Gam prevents the degradation of linear dsDNA; lambda exonuclease (Exo) degrades dsDNA in a 5′ to 3′ manner, leaving single-stranded DNA in the recessed regions; and Beta binds to the single-stranded regions produced by Exo and facilitates recombination by promoting annealing to the homologous genomic target site (http://2011.igem.org/Team:Harvard/Lambda_Red).

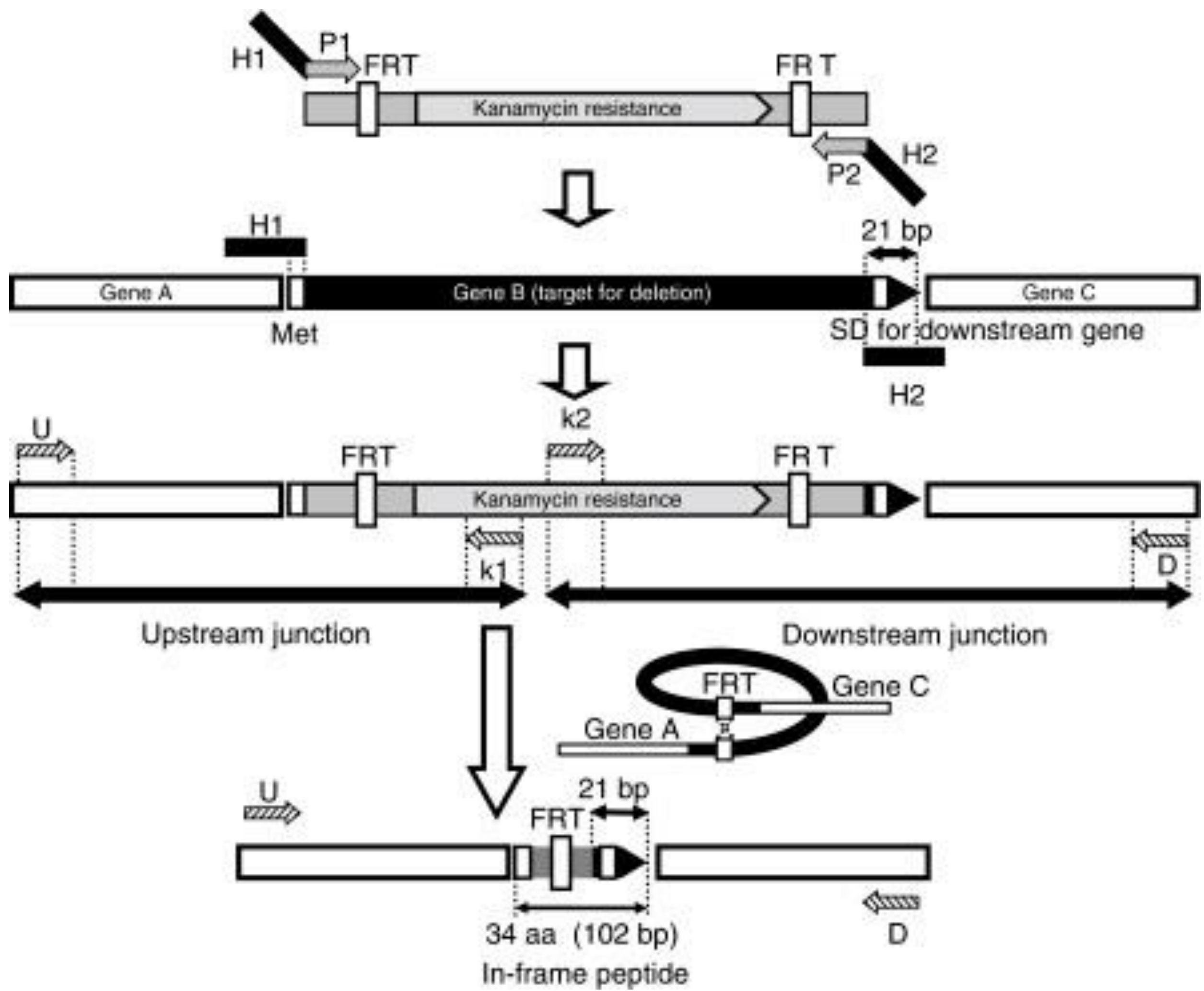


Figure 9: Keio Collection Mutants. Keio collection mutants have a kan resistance cassette inserted into nonessential genes. The figure includes the excision of the kan resistance cassette using the recombination of the flanking FRT sequences. (Baba et al., 2006).

2.3 Ammonium sulfate precipitation

Ammonium sulfate precipitation method as described by Bollag et al (1996) was used to concentrate proteins from a supernatant sample. A given amount of supernatant was removed from samples before concentration. Bovine serum albumin was added to a final concentration of 0.1 mg/ml. Ammonium sulfate was added to give a final 85% saturation (0.58 g of ammonium sulfate was added to 1 ml of sample). The samples were incubated at 4°C overnight after being thoroughly mixed. The samples were centrifuged at > 16 000 x g for 30 minutes. The supernatants were aspirated and the pellet was resuspended in 1x PBS (0.137M NaCl, 3mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4). The amount of PBS added depended on the initial volume of sample that was used. 1ml of sample that was concentrated was resuspended in 40 µl of 1x PBS for a 25x concentrated sample. Supernatants were concentrated 25x for Western Blot analysis and 10x for analysis using ELISAs unless otherwise specified.

2.4 Protein Methods

2.4.1 French Press

The french press was used to release proteins from the cell. All samples for french pressing were kept on ice. The samples were removed from the sample tube and drawn into the machine through negative pressure upon the insertion of the collection tubing with the machine set to the low position, and the valve loosened minimally to let the sample enter the machine. The machine was set to medium after the cells entered through the collection tubing and the valve was firmly tightened, this released air from the sample. The machine was set to the high position and no leakage of the sample occured. The pressure gauge was then set to

1000 psi and the valve loosened until a slow drip of the sample took place. The sample was eluted from the machine and the procedure repeated a second time (https://homogenizers.net/products/french-press-g-m).

2.4.2 Polyacrylamide Gel Electrophoresis (PAGE)

Samples that were run on an acrylamide gel were made up to 50% 2x Sample Buffer (0.125 M Tris pH 6.8, 20% w/v glycerol, 4% SDS, 0.1% w/v Bromophenol blue). The samples were vortexed and heated at 95°C for three minutes. 10 µl of sample were loaded in each lane of the gel. SDS-PAGE gels were created consisting of 10% acrylamide (He, 2011) for medium sized proteins (20-150kDa) or 16% acrylamide (Schägger, 2006) for small proteins (0-20kDa). Very large proteins such as the GspD multimer were run using 3-8% tris-acetate gels (Bio-rad). The gels were run using either running buffer (25mM Tris-Base, 0.192M glycine, and 1%SDS) for 10% gels or Tricine buffer (25mM Tris-Base, 25mM Tricine, 1.734mM SDS) for 14% and 3-8% tris-acetate gels. 10 and 14% gels were run at 100V until the dye band was approximately 1.5 cm from the bottom of the gel while the 3-8% gradient tris-acetate gels were run at 100V until the dye band ran off the gel.

2.4.3 Transferring gels for immunodetection

Acrylamide gels or tris-acetate gels were run as described above before being transferred to PVDF membranes. The transfer procedure utilized a semi-dry transfer apparatus (Bio-Rad). The small proteins (1-20kDa) were transferred using the predefined LowMW program (2.5A, 25V for five minutes). The mid sized proteins (20-150kDa) were transferred using the predefined MixedMW program (2.5A, 25V for seven minutes), while the large proteins (150+

kDa) were transferred with a maximum of 1.4A, 25V for forty-five minutes using Towbin buffer (3.03% Tris, 14.4% Glycine and 20% Methanol; Towbin et al., 1979).

2.4.4 Immunodetection

The transferred membrane was wet in 100% ethanol for a brief period of time before being equilibrated in 1x TN buffer (50 mM Tris-Base, 153 mM NaCl, pH7.4). 2% prime blocking agent (GE Healthcare) in 1x TN to block the membrane was used to prevent the non-specific binding of antibodies. The membrane was incubated in blocking solution at room temperature for one hour before primary antibodies (Table 4) and 0.1% Tween 20 were added to the block solution, following which the membrane was incubated for one hour at 37°C. The PVDF membrane was washed three times with 1x TNT (TN + 0.1% Tween 20) for ten minutes before secondary antibody was added. A new solution of 2% block was placed in a wash container. A concentration of 0.1% Tween 20 was made in the solution before the addition of secondary antibodies. The secondary antibodies have horse radish peroxidase conjugated to them. The membrane was then incubated for one hour in this solution. Development substrates (Lumigen) were added at a concentration of 1:1 (Lumigen). The membrane was then imaged using a chemi-doc XRS+ system (Biorad).

Table 3: Antibody Dilutions

Western Blots

Name	Dilution	Type of Antibody	Source
α-CT	1/1000	Primary Rabbit Polyclonal Antibody	Sigma
α-myc	1/10000	Primary Mouse Monoclonal Antibody	Cell Signalling
α-GspD$_{\alpha}$	1/1000	Primary Rabbit Polyclonal Antibody	Our Lab
Goat anti-rabbit	1/100000	Secondary Antibody	Bio-rad
Goat anti-rabbit	1/200000	Secondary Antibody	Jackson Immunoresearch
Goat anti-mouse	1/100000	Secondary Antibody	Bio-rad

ELISA

Name	Dilution	Type of Antibody	Source
α-CT	1/2500	Primary Rabbit Polyclonal Antibody	Sigma
Goat anti-rabbit	1/1000	Secondary Antibody	Bio-rad
Goat anti-rabbit	1/2000	Secondary Antibody	Jackson Immunoresearch

2.5 DNA Isolation and Analysis

2.5.1 Plasmid DNA Isolation

Plasmid DNA was isolated using the methods described by Sambrook et al (1989). Cells, grown overnight, in 1.5 mls of LB were centrifuged for one minute at > 16 000 x g. The supernatant was removed. 200 µl of buffer P1 (50mM glucose, 25mM Tris pH 8.0, 10mM EDTA, 100ug/ml RNAse) was added and the cells were resuspended, followed by the addition of 200 µl of buffer P2 (0.2N NaOH, 1% SDS) and mixing of tube contents without vortexing. The resulting solution was incubated for five minutes at room temperature. 200 µl of buffer P3 (3M potassium acetate, 5M acetic acid, pH 4.5) was added to the tube and mixed. The resulting sample was then incubated on ice for five minutes. The resulting precipitate that formed was removed by centrifugation for thirty minutes at > 21 000 x g at 4°C. 500 µl of the supernatant was place in a new tube and 250 µl of isopropyl alcohol was added before the tube was centrifuged. The resulting solution was centrifuged at > 16 000 x g for twenty minutes at room temperature. The supernatant was aspirated before the pellet was resuspended with 250 µl of cold 70% ethanol. The sample was centrifuged at > 21 000 x g for ten minutes at 4°C. The supernatant was aspirated and the pellet was dried. The final pellet was resuspended in 50-100 µl of ddH_2O.

2.5.2 Agarose Gel Electrophoresis

The DNA samples generated after PCR amplification using primers listed in Table 5 were added to 6x loading dye (Thermo-Scientific). The resulting samples were loaded on a 0.8% agarose gel [0.40g agarose powder, 50ml 1x TBE (89.15 mM Tris-Base, 88.95 mM Boric Acid, 8.0 ml of 0.5M EDTA pH 8.0]. The samples were separated at 100V for approximately thirty mins. The gel was incubated in a 10ug/ml solution of ethidium bromide for ten minutes to allow staining. The stained gel was then rinsed with water for ten minutes to remove excess ethidium bromide before being monitored for the banding patterns of DNA fragments using a UV transilluminator (UVP).

2.6 Enzyme linked immunosorbant assay (ELISA)

ELISAs were performed as previously described (Ristaino et al., 1983) for the detection of LTB. Bacterial cultures for this assay were either non-concentrated or ten times concentrated. The plate was coated with 0.1 ug/ml GM_1 (Sigma) in 60 mM bicarbonate buffer pH 9.6. The plate was covered and incubated overnight at 4°C. The plate was washed three times in 1x PBST (99.5% PBS, and 0.5% Tween 20) for five minutes each wash. 100 µl of 5% FBS in 1x PBS was added to each well to block the plate. The plate was covered with parafilm before being incubated at 37°C for one hour. The plate was washed in 1x PBS-T. The washing step was repeated three times. The dilutions were calculated for the samples on the plate being done. The examination of supernatants containing LTB was performed in ½ dilutions while the examination of french pressed pellet samples for the presence of LTB was performed at 1/40 dilutions. The plate with samples added was then covered with parafilm and allowed to

incubate for two hours at 37°C. The plate was washed 3x for five minutes per wash with 1x PBST. 100 µl of primary antibody (Table 4), diluted in PBS-T + 1% FBS, was added to each well, the plate was covered and incubated at 37°C for one hour. The plate was washed 3x for five minutes per wash after the incubation in 1x PBS-T and 100 µl of secondary antibody (goat antirabbit HRP) diluted in PBS-T + 1% FBS was added. The plate was covered before being incubated at 37°C for one hour. The plate was washed in 1x PBS-T 3x for five minutes each. ABTS peroxidase substrates (KPL) were mixed at 1:1 concentrations before 100 µl was added to each well. The plate was incubated a room temperature until the appropriate green colour was observed in the wells. 100 µl of 1% SDS was added to each well to stop the reaction. The plate was read at a wavelength of 410 nm.

Table 4: Primers used in this study

Primer Designation	Target	Primer Sequence
US648	Amplify the *hns* region	GAAACGGTGGAAGCCTATCA
US649	Amplify the *hns* region	ATTGGCGGCACAAAATAAAG
US650	Amplify the *stpA* region	GGATTGCCAGTAGCGGTAAA
US651	Amplify the *stpA* region	CAGAAAGCACCAGTGATGGA
US656	Amplify within *gspA/gspC*	AGCCTCACCCAACAAATAGC
US658	Amplify within *gspA/gspC*	TTGGTTGATTAATGATCTGTCCA
US793	Amplify *eltB*	TAATTGAATTCTTCACCAACAAGGACCAT
US794	Amplify *ptetA/tetR*	TAATTCCATCGAATGGAAAGGTTATGCTGCTTTTAA
US795	Amplify *ptetA/tetR*	CGGCCGAATTCCATTTCACTTTTCTCTATCACTGATA
US802	Amplify *gspB*	TACTTAACCACCGAACGCAAGCGT
US803	Amplify *gspB*	GGCAGGGGAGAGTGCCTACTTATATC
US816	Amplify *eltB*	GACTCTGCAGCTAGTTTTCCATACTGATTGCC
US839	Amplify *chiA-myc*	TATGGGATCCAGGAGATATACATATGAAATTAAATATATT
US840	Amplify *chiA-myc*	TATTCTGCAGTGCTTACAGATCCTCTTC

3. Results

3.1 The creation of *E. coli* deletions in *hns* and *stpA*

Previous research by Francetic et al (2000) has shown that the secretion of ChiA by the alpha T2SS system could not occur in the presence of HNS. HNS was disrupted through the insertion of a transposon allowing the characterization of secretion of ChiA by the alpha T2SS system (Francetic et al., 2000). Horstman and Kuehn (2002) showed that secretion of LT by the alpha T2SS system requires HNS to be deleted from the cell. Both of these studies showed secretion by the alpha T2SS system only in the absence of HNS, but levels of secretin assembly were not quantified and the T2SS genes were cloned and expressed on plasmids.

3.1.1 The creation of double mutants in *hns* and *stpA*

Mutant collections have been made with a kanamycin resistance cassette inserted into non-essential genes of *E. coli* K12, the mutants are known as the Keio collection (Baba et al., 2006). Mutations in *hns* and *stpA* were created in which should allow the expression of the cryptic alpha T2SS system *(*Francetic et al., 2000). The kanamycin cassette was flanked by flippase recognition targets (FRT), the presence of flanking FRT cassettes allows the excision of the resistance genes through recombination using the *flippase recombinase* genes found on plasmid pCP20 (see materials and methods). The unmarked strain was used as a recipient strain for P1 transduction. The donor strains used contain the kanamycin resistance cassette inserted into *hns* or *stpA*. P1 transduction was used to place a marked deletion in *hns* or *stpA* into a strain with an unmarked deletion created in the other gene. The resulting strains had a deletion in both *hns* and *stpA* preventing the expression of either of these proteins. The created strains

thus have either a kan cassette inserted into *hns* or *stpA* and a deletion in the other gene. The strains are resistant to kan and were grown on LB + kan selective plates.

The plasmid pTS76 was transformed into the mutants. The transformed plasmid encodes LTB which is secreted by the beta T2SS system. This gene was not expressed unless isopropyl β-D-1-thiogalactopyranoside (IPTG) was added since the plasmid was derived from pMAL-p4x. In addition, plasmid TS60 was inserted as well. pTS60 encodes *chiA* with a myc tag which is version of a protein shown to be secreted by the alpha T2SS (Francetic et al., 2000). This gene was not expressed unless arabinose was added since the plasmid was derived from pBAD322c.

Following the construction of these strains, their *hns* and *stpA* genotypes were confirmed by PCR. Databases (Ecocyc) show that the *hns* gene amplification has a WT fragment of approximately 835 base pairs while *stpA* was slightly larger being approximately composed of 986 base pairs. Strains used as controls have a deletion scar of *hns* or *stpA,* these strains have a banding pattern consisting of approximately 526 base pairs for an *hns* deletion scar and 686 base pairs for a *stpA* deletion scar. Virtual clones showed that an amplification of a gene with a kan resistance inserted into *hns* causes the gene to become larger, the gene became 1748 base pairs from 835 base pairs while the same occurrence happened in *stpA* which went from approximately 986 base pairs to 1908 base pairs. The *hns* region of the cell was amplified with primers US648 and US649 while the *stpA* region was amplified using primers US650 and US651. The amplification of the *hns* and *stpA* regions of strains with plasmid pTS76 is shown in Figure 10 while the *hns* and *stpA* regions amplified from strains with plasmid pTS60 are found in Figure 11. The figures indicate that double mutants were created in which *hns* and *stpA* were deleted in the bacterial cell.

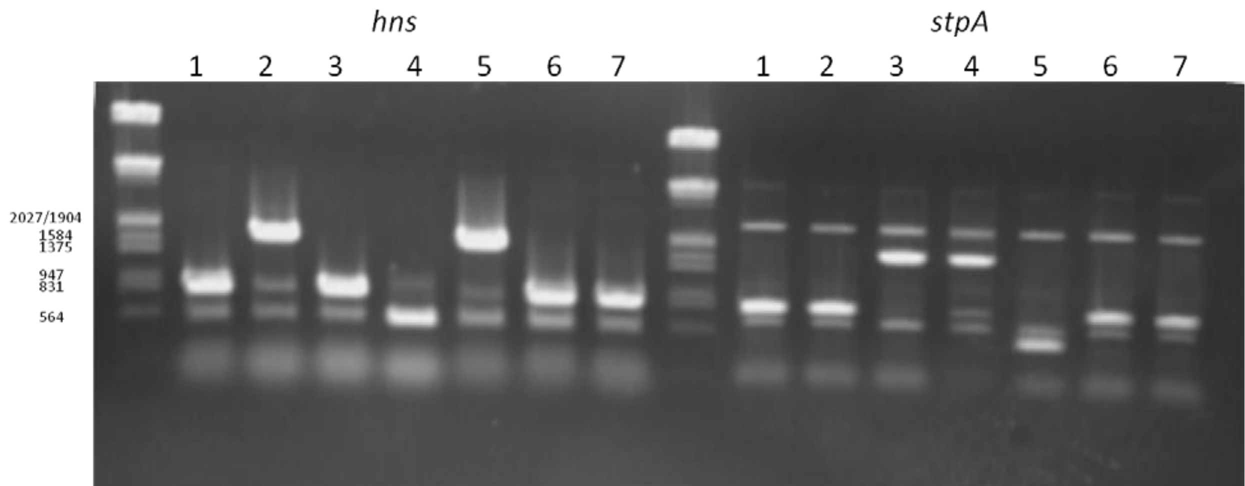


Figure 10: An agarose gel showing an amplicon from single and double mutants of *E. coli* K12 MG1655 in *hns* and *stpA* expressing pTS76. The left side shows the amplification of *hns* while the right side shows the amplification of *stpA.* Lane 1, MG1655 (pTS76); Lane 2, BW25113 *(Δhns:kan*, pTS76); Lane 3, BW25113 *(stpA:kan,* pTS76)*;* Lane 4*,* MBM 1 *(Δhns, ΔstpA::kan,* pTS76)*;* Lane 5, MBM 2 *(ΔstpA, Δhns::kan,* pTS76)*;* Lane 6*,* BW25113 *(ΔgspA:kan,* pTS76)*;* Lane 7, BW25113 *(ΔgspD:kan,* pTS76). The *hns* region was amplified by the use of primers US648 and US649 while the amplification of *stpA* was through the use of primers US650 and US651. *hns* amplification: approximately 835 bp for WT, 526 bp for the excision scar and 1748 bp for a kan insert. *stpA* amplification: approximately 986 bp for WT, 686 bp for an excision scar and 1908 bp for a kan insert.

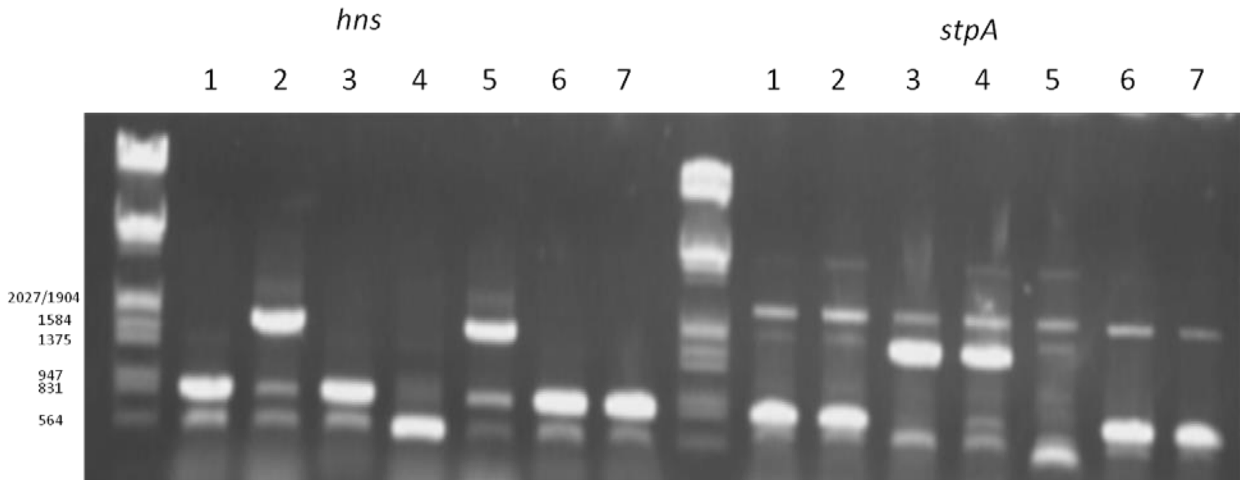


Figure 11: An agarose gel showing an amplicon from single and double mutants of *E. coli* K12 MG1655 in *hns* and *stpA* expressing pTS60. The left side shows the amplification of *hns* while the right side shows the amplification of *stpA.* Lane 1, MG1655 (pTS60); Lane 2, BW25113 *(Δhns:kan*, pTS60); Lane 3, BW25113 *(ΔstpA:kan,* pTS60)*;* Lane 4*,* MBM 1 *(Δhns, ΔstpA::kan,* pTS60)*;* Lane 5, MBM 2 *(ΔstpA, Δhns::kan,* pTS60)*;* Lane 6*,* BW25113 *(ΔgspA:kan,* pTS60)*;* Lane 7, BW25113 *(ΔgspA:kan,* pTS60). The *hns* region was amplified by the use of primers US648 and US649 while the amplification of *stpA* was through the use of primers US650 and US651. *hns* amplification: approximately 835 bp for WT, 526 bp for the excision scar and 1748 bp for a kan insert. *stpA* amplification: approximately 986 bp for WT, 686 bp for an excision scar and 1908 bp for a kan insert.

3.1.2 Secretion by the *hns* and *stpA* mutants

The mutants (Δ*hns* and Δ*stpA)* and the wild type, each with pTS76 and pTS60 were grown to an OD_{600} of 2.0 in LB media containing antibiotics, strains with pTS76 were grown in the presence of amp while strains with pTS60 were grown in the presence of cam. The supernatants and the pellets were separated through centrifugation. The supernatants were passed through a 0.2um filter removing whole cells from contaminating the supernatant. The samples were prepared for electrophoresis as described in Chapter 2. The detection of LTB required the use of 14% Tricine gels while the detection of ChiA-myc required the use of 10% SDS-Acrylamide gels. Aliquotes from supernatants and pellets were separated by SDS PAGE containing plasmids pTS76 (*LTB)* or pTS60 (*chiA-myc*). Proteins in the gels were transferred to a PVDF membrane using a LowMW program for the detection of LTB or the MixedMW program for the detection of ChiA-myc. The membrane was stained with primary antibodies against CT which bind to the protein of interest, either LT or anti-myc for the detection of ChiA-myc. The CT antibody is a polyclonal antibody in which goat anti-rabbit secondary antibody was used whereas the anti-myc antibody is a monoclonal antibody in which goat anti-mouse secondary antibody was used. The secondary antibody binds to the primary antibody, the secondary antibodies allows detection as a biomarker is attached to the antibody to allow detection.

All strains with induction should allow the expression of LTB as all strains contain the inducible plasmid. The western blot (Figure 12) showed the amount of LTB within the cell. The strains in the first two lanes were used as a control as they both contain MG1655 (pTS76), these strains should not show secretion upon examination of the supernatant as the alpha T2SS system is not

activated. The *hns*, *stpA*, and double mutant strains are the strains of interest when examining the supernatant.

A western blot was run of the supernatants (Figure 13) which indicated that there maybe other mechanisms of secretion by *E. coli* other than the T2SS system. There is no evidence of natural secretion by *E. coli* K12 strain MG1655. MG1655 appeared to show secretion of LTB but so did strains with a deletion in *gspA* or even *gspD*. The apparent very low levels of secretion being observed in any of these strains cannot be due to the presence or absence of the alpha T2SS, and may have occurred through low levels of cell lysis. LTB was not secreted by the alpha T2SS system upon the deletion of *hns* or *stpA*.

A western blot of pellets was run for pTS60 but the results were similar of that of pTS76. A western blot was run of supernatant samples grown with the expression of pTS60 (Figure 14). The blot at first appearance showed that *E. coli* has a different mechanism of secretion besides the alpha T2SS system. The MG1655 strain containing pTS60 that was induced seems to have a band in it corresponding to the ChiA-myc band observed in lane 1. Mutating either *hns* or *stpA* seemed to result in more secretion but the negative controls with a mutation in *gspA* or *gspD* still showed the band corresponding to secretion indicating that the appearance of a band with the same size as the secreted protein indicating that this phenomenon cannot be secretion via the alpha T2SS system.

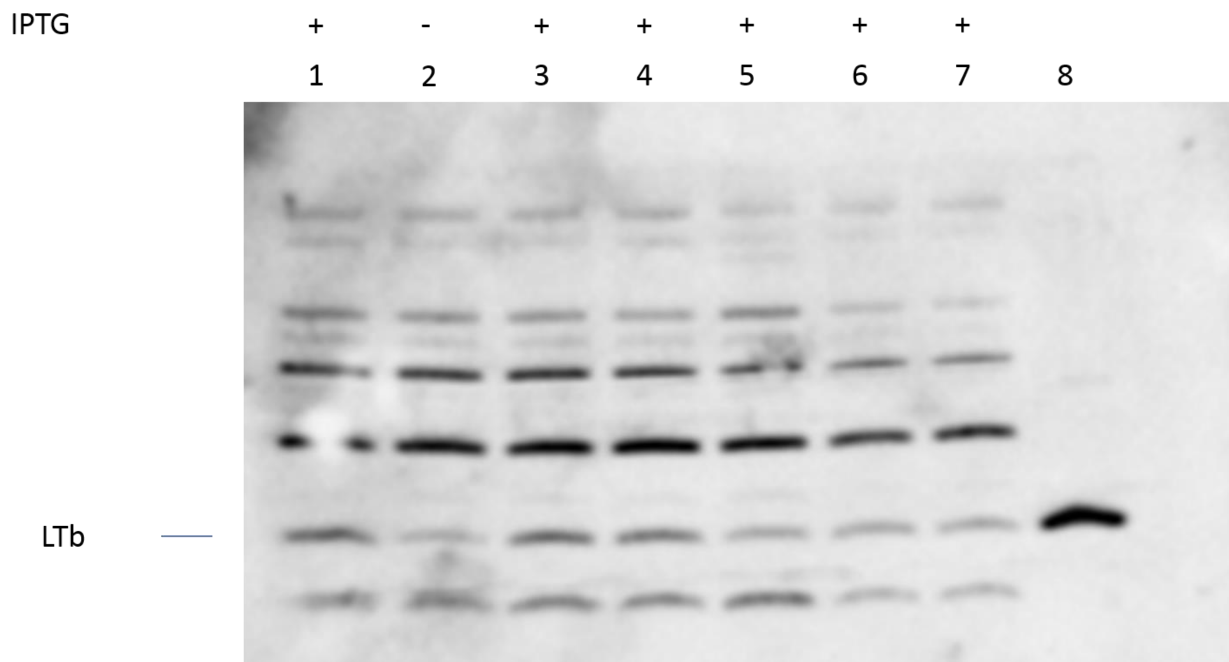


Figure 12: An immunoblot detecting LTB in the pellets of WT, *hns* and *stpA E. coli* K12 strain BW25113 mutants expressing pTS76. The western blot was developed against LTB which used anti-CT to show the amount of LTB in the pellets of *hns* and *stpA E.* coli mutants, mutants contain pTS76 expressing LTB. Lane 1, MG1655 (pTS76) with induction; Lane 2, MG1655 (WT) with no induction; Lane 3, BW25113 *(Δhns:kan*, pTS76) without induction; Lane 4, BW25113 *(ΔstpA:kan,* pTS76) with induction*;* Lane 5*,* MBM 1 *(Δhns, Δ stpA::kan,* pTS76) with induction*;* Lane 6*,* BW25113 *(ΔgspA:kan,* pTS76) with induction*;* Lane 7, BW25113 *(ΔgspD:kan,* pTS76) with induction*;* Lane 8, 10ug/mL of LTB. + or – signifies the induction levels.

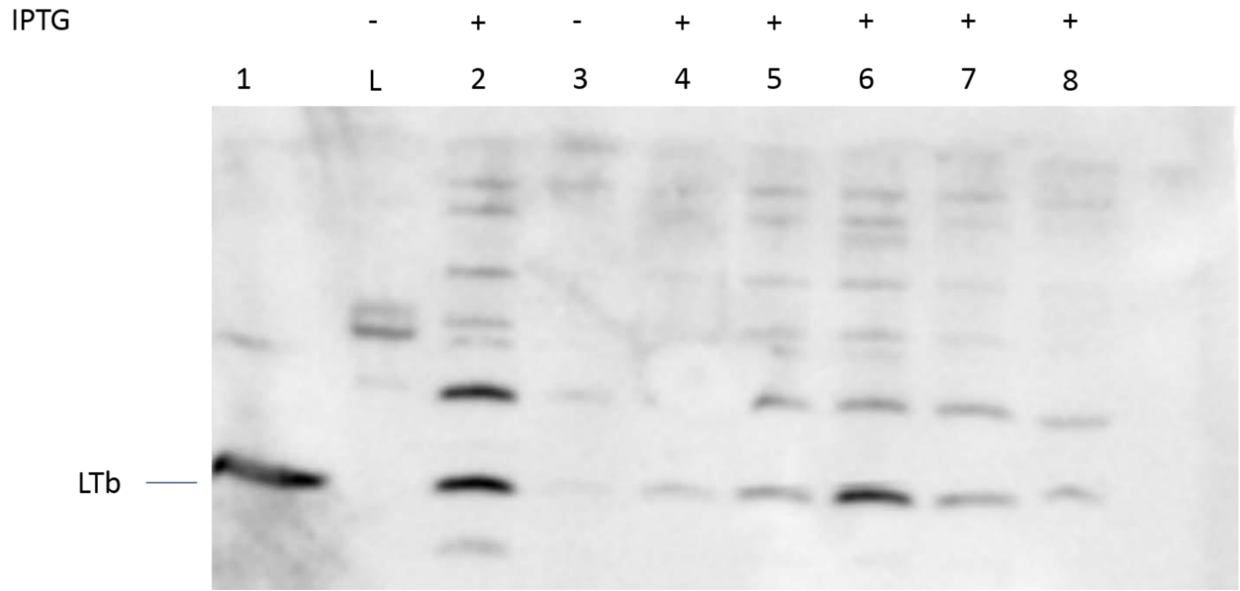


Figure 13: An immunoblot detecting LTB in the supernatant of WT, *hns* and *stpA E. coli* K12 strain BW25113 mutants expressing pTS76. The western blot was developed against LTB using anti-CT, the development showed the amount of LTB in the supernatants of *hns* and *stpA E. coli* mutants, mutants had the plasmid pTS76 expressing LTB. Lane 1, 10ug/mL of LTB; Lane 2, molecular weight ladder; Lane 3, MG1655 (WT, pTS76) with induction; Lane 4, MG1655 (WT, pTS76) without induction; Lane 5, BW25113 (*Δhns:ksn,* pTS76*)* with induction; Lane 6, BW25113 (*ΔstpA:kan*, pTS76) with induction; Lane 7, MBM 1 *(Δhns, Δ stpA::kan,* pTS76) with induction*;* Lane 8 BW25113 (*ΔgspA:kan*, pTS76) with induction*;* Lane 9, BW25113 (*ΔgspD:kan,* pTS76) with induction*.* + or – signifies the induction levels.

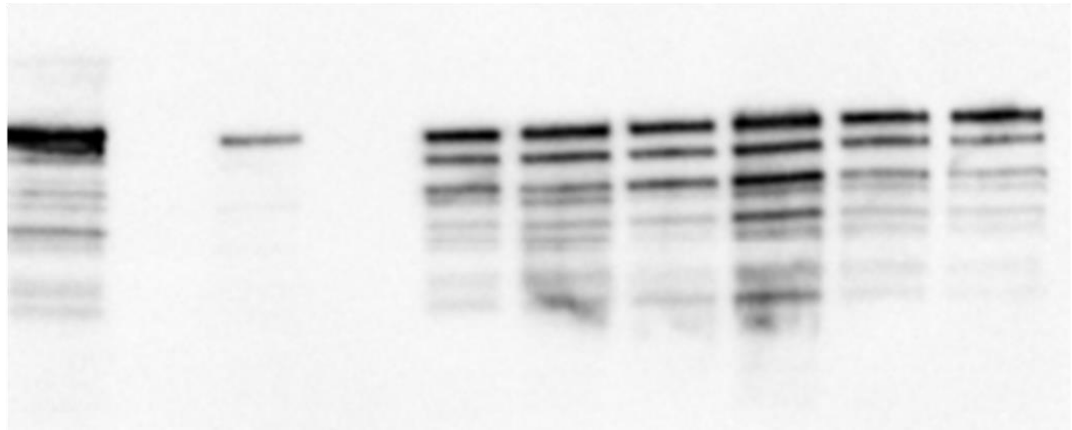


Figure 14: An immunoblot detecting ChiA in the supernatants of WT, *hns* and *stpA E. coli* K12 strain BW25113 mutants expressing pTS60. The Western blot show the detection of ChiA-myc in the supernatants of *hns* and *stpA* mutant *E. coli,* antibodies that were used were targeted against the myc tag. The mutants all had pTS60 expressing ChiA-myc, pTS60 was expressed through the addition of arabinose. Lane 1, BW25113 (pellet control, *hns* pTS60); Lane 2, molecular weight ladder; Lane 3, MG1655 (WT, pTS60) with induction; Lane 4, MG1655 (WT, pTS60) without induction; Lane 5, BW25113 *(hns*, pTS60) with induction*;* Lane 6, BW25113 *(stpA*, pTS60) with induction*;* Lane 7, MBM 1 *(hns, stpA::kan,* pTS60) with induction*;* Lane 8, MBM 2 (*stpA, hns::kan,* pTS60) with induction; Lane 9, BW25113 (gspA, pTS60) with induction; Lane 10, BW25113 (*gspD,* pTS60) with induction. Induction (+) means that 0.5% arabinose was added to the culture before growth. A dash (-) indicates that arabinose was not added.

3.1.3 Expression of the alpha T2SS system in the *hns* and *stpA* mutants

The T2SS system contains many different proteins encoded within the gsp_{α} operon as described in the introduction. The $GspD_{\alpha}$ protein must first assemble before the alpha T2SS becomes active. GspD must go from a monomeric state to a multimeric state before secretion can occur. The wild type and mutant strains were therefore analyzed for the expression and assembly of $GspD_{\alpha}$ as a measure of assembly of this T2SS.

Multimeric $GspD_{\alpha}$ is a large complex and requires a low percentage gel and a long running time to separate. The monomer consists of one 70.698 kDa protein while the multimer consists of 12 copies of the monomeric protein assembled together. 3-8% tris-acetate gels were required for the separation of this large protein. The gel was run for approximately four hours before being transferred and the immunoblot was developed with antibodies directed against $GspD_{\alpha}$.

The results (Figure 15) showed that the alpha T2SS system was not activated by mutations in *hns* or *stpA*. None of the samples tested have $GspD_{\alpha}$ in the multimeric form. Small amounts of monomer are detected in the *hns* and double mutant samples meaning that the repressors may have an impact on the expression of $GspD_{\alpha}$. The experiment does not have a positive control but the $GspD_{\alpha}$ multimer can be observed in other Figures such as Figure 24. Using single and double mutants in *hns* and *stpA* did not result in significant expression of GspD or multimerization of the small amount that was synthesized.

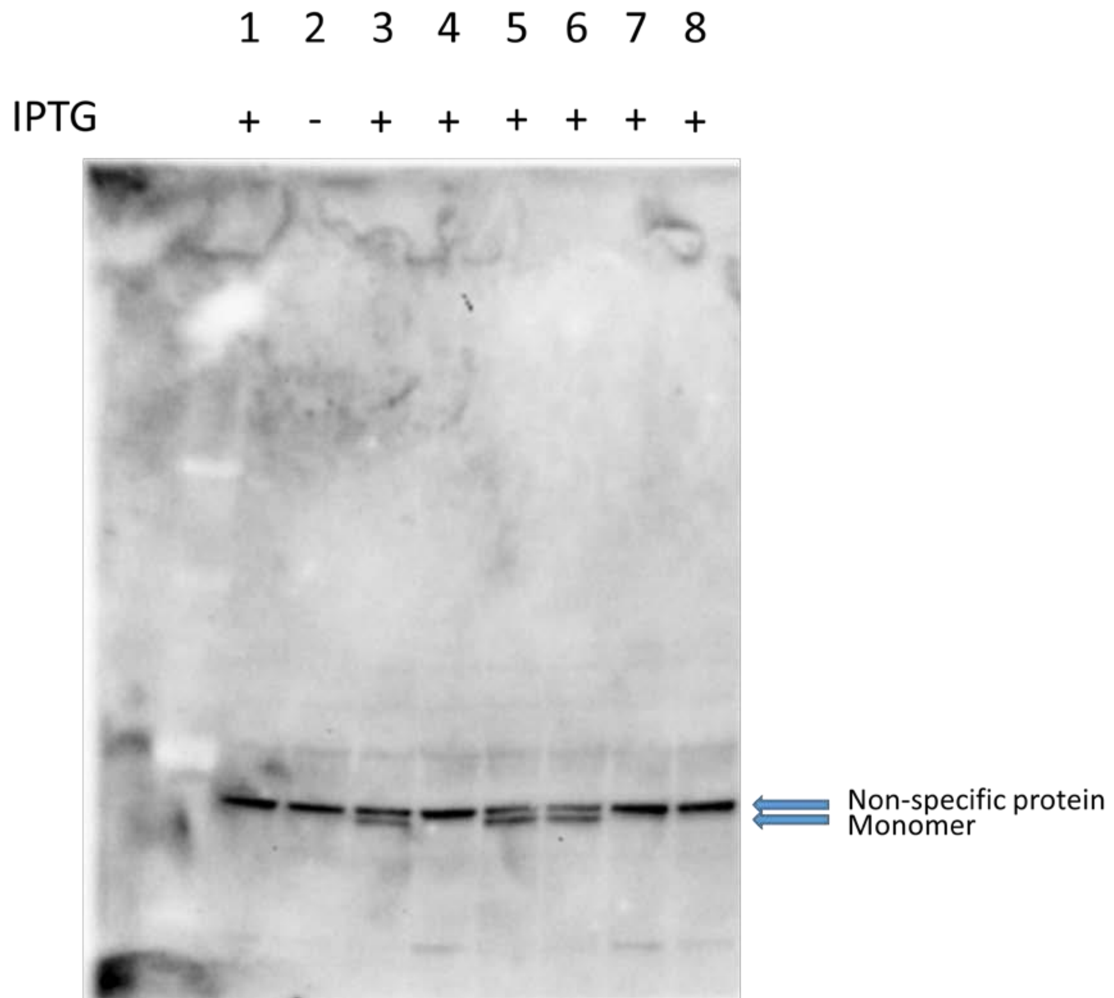


Figure 15: An immunoblot detecting GspD$_\alpha$ found in the pellet of *hns* and *stpA E. coli* K12 strain MG1655 cells. Antibodies that were used were targeted against *gspD$_\alpha$*. The higher band of the bands present corresponds to a non-specific protein while the lower band corresponds to the GspD$_\alpha$ monomer. Samples were concentrated 5x before 10µl of sample was run on the gel. Lane 1, MG1655 (WT,pTS76) with induction; Lane 2, MG1655 (WT, pTS76) without induction; Lane 3, BW25113 *(Δhns:kan*, pTS76) with induction; Lane 4, BW25113 *(ΔstpA:kan,* pTS76) with induction; Lane 5, MBM 1 *(Δhns ΔstpA::kan*, pTS76) with induction; Lane 6, MBM 2 *(ΔstpA, Δhns::kan,* pTS76) with induction; Lane 7, BW25113 *(ΔgspA:kan,* pTS76) with induction; Lane 8, BW25113 *(ΔgspD:kan,* pTS76) with induction. + or – signifies induction levels.

3.2 Studies of secretion and GspD$_\alpha$ assembly with inducible promoters controlling the alpha *gsp* operons

It has previously been reported that the alpha T2SS system can secrete proteins. Francetic et al (2000) showed that chitinase could be secreted by the alpha T2SS system. The results were gathered by the induction of plasmids controlling the secreted protein and the *gsp*$_\alpha$ operons in the absence of HNS. The paper did not include controls such as a control over the addition of inducer. It has been shown in *Erwinia Crysanthemi* that over expression of GspD can restore the function of a secretion deficient mutant (Condemine and Shevchik, 2000). A paper written by Horstman and Kuehn (2002) reported that the alpha T2SS system can be activated through the expression of *gsp*$_\alpha$ plasmids which has been shown to secrete LT. These papers showed that secretion by the alpha T2SS system required inducible plasmids housing the *gsp*$_\alpha$ operons.

3.2.1 The creation of *E. coli* strains with inducible promoters controlling the *gsp*$_\alpha$ *operons*

E. coli K12 strain MG1655 mutants were created in which natural promoters controlling the expression of the *gsp*$_\alpha$ operons were replaced with promoters from pMAL-p4x and pBAD322c. The inducible bi-directional promoter fragment (Figure 16) to be inserted between *gspA* and *gspC* was amplified from strain TGS49, the resulting amplicon included the flippase recognition target flanked resistance cassette. The fragment was ligated into pGem-T easy for easy amplification, the resulting plasmid is known as pMM1. The plasmid was isolated and used as a template for amplification with primers US656 and US658. The pure template and the primers

mentioned allowed amplification of the resistance cassette and two promoters between the *gspAB*$_{\alpha}$ and *gspC-O*$_{\alpha}$ inducible promoters.

The p*tac* promoter from pMAL-p4x was used to control *gspAB*$_{\alpha}$, while the *paraBAD* promoter from pBAD322c was used to control the expression of *gspC-O*$_{\alpha}$. The promoters are thus controlled through the addition of IPTG and arabinose, respectively. The inducible promoters used were separated with a FRT-kan-cam-FRT resistance cassette.

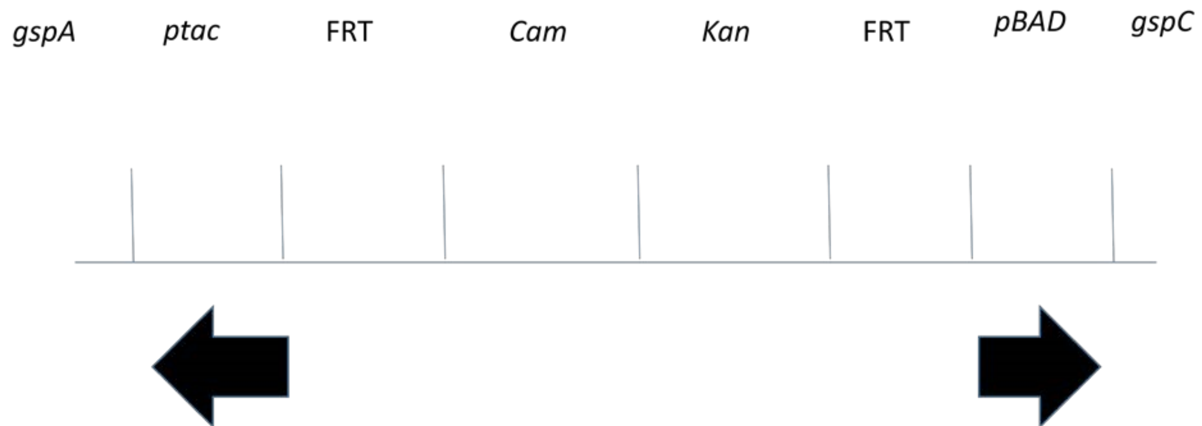


Figure 16: The inducible promoter fragment used for the replacement of natural *gsp*$_{\alpha}$ promoters in *E. coli* K12 strain MG1655. The strain that will be discussed contains gspAB$_{\alpha}$ controlled through the addition of IPTG under the control of the pTAC promoter and arabinose controlling the expression of the gspC-O$_{\alpha}$ operon.

The inducible promoters were used for the replacement of the natural *gsp*$_{\alpha}$ promoters. The amplified fragment was electroporated into MG1655 cells containing the lambda red plasmid which allowed a recombination event (Mosberg et al., 2010).

The cells that grew on the selective plates were isolated and made competent and the pCP20 plasmid expressing flippase recombinase was electroporated into them. The resulting culture was grown on and plated on LB plates containing no antibiotic, and colonies tested on LB plates containing kan (30 µg/ml) and cam (2.5 µg/ml) and incubated at 37°C overnight. The cells that grew on LB media but not LB media containing selective antibiotics were used in further experiments after verifying that the plasmid and the resistance cassette were cured from the cell. The promoters were confirmed to be present by sequencing, while the resistance cassette was not

3.2.2 Assembly of GspD$_{\alpha}$ in *gspAB*$_{\alpha}$ *- Tac* and *gspC-O*$_{\alpha}$ *– Bad* MG1655 mutants

IPTG and arabinose were added during the growth of the MG1655 *gspAB*$_{\alpha}$ - *Tac* and *gspC-O*$_{\alpha}$ - *Bad* mutant strain. The cells were grown to an OD_{600} of approximately 2.0 , and then samples taken, electrophoresed on a 3-8% tris-acetate gel, and transferred to a PVDF membrane. The membrane was developed using primary antibodies against GspD$_{\alpha}$ with secondary antibodies against the rabbit polyclonal antibody (Figure 17).

The results show that assembly of GspD$_{\alpha}$ can occur when the *gspC-O*$_{\alpha}$ operon was induced. The gel shows the banding pattern that was observed using different concentrations of the inducers. The darkest band of assembled secretin was found upon the maximal induction of *gspC-O*$_{\alpha}$ through the use of arabinose and the minimal concentration of IPTG added controlling

the *gspAB*$_{\alpha}$ operon. The results suggest that GspAB$_{\alpha}$ was not required for assembly of this system in contrast to *Aeromonas hydrophila (*Strozen et al., 2011). The alpha T2SS system showed GspD$_{\alpha}$ only when the *gspC-O*$_{\alpha}$ operon was expressed through the addition of arabinose during growth.

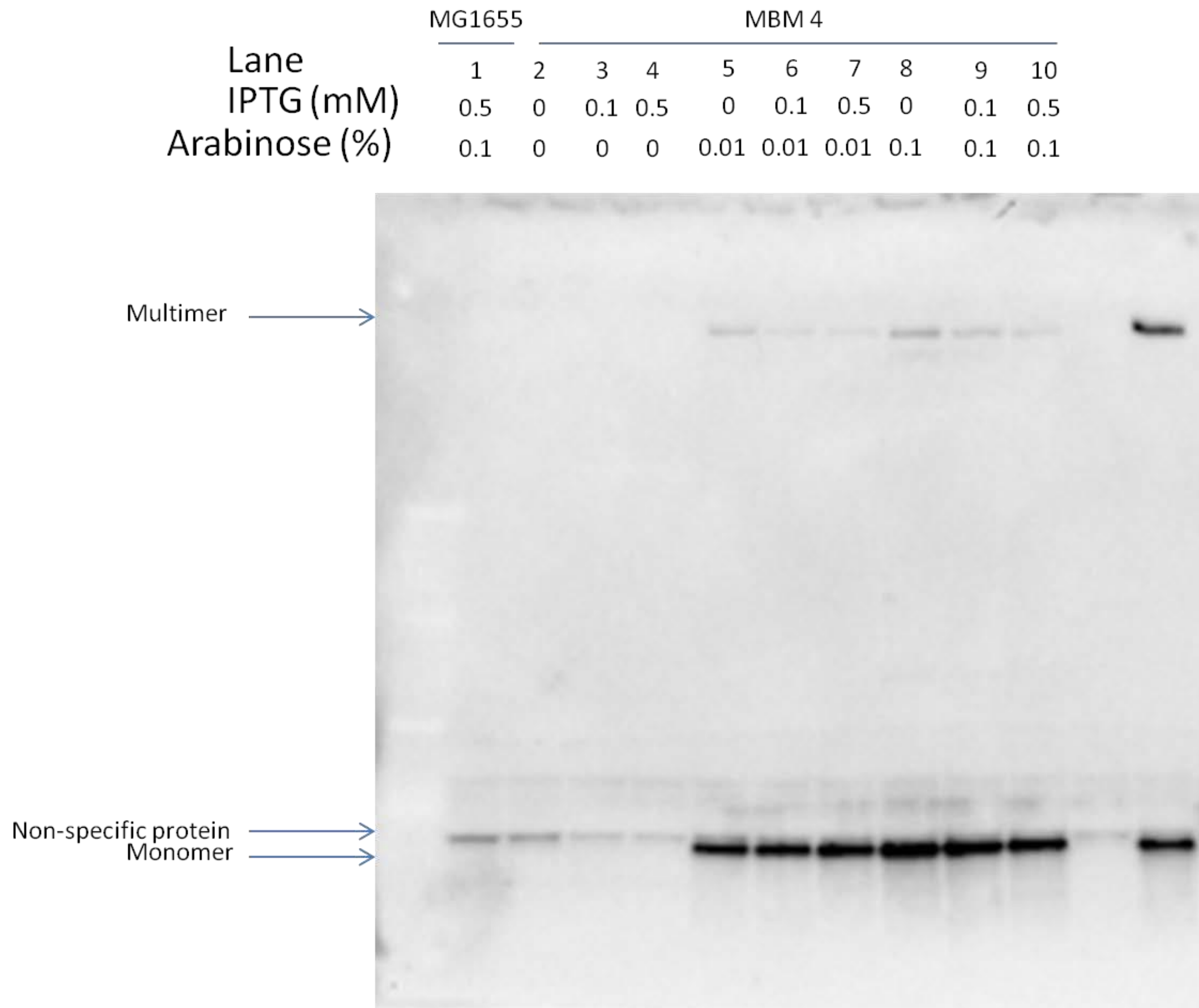


Figure 17: A western blot detecting GspD$_{\alpha}$ in WT and MBM4 *E. coli* K12 strain MG1655 cells. Antibodies were used that were targeted against GspD$_{\alpha}$. The samples do not contain a plasmid that can be induced. The samples were concentrated 5x before being run on the gel. Lane 1, MG1655 (WT); Lanes 2-10, MBM 4 (*gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD)*; Lane 11, MG1655 (WT, negative control); Lane 12, MBM 4 (positive control, *gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD*). The concentration of the inducers added is indicated at the top of the figure.

3.2.3 Activation of the alpha T2SS in *$gspAB_{\alpha}$ - Tac* and *$gspC\text{-}O_{\alpha}$ – Bad* MG1655 mutants containing pTS76 or pTS60

Mutant cells with the inducible *gsp* promoters containing either pTS76 or pTS60 were grown as described above, with different concentrations of arabinose and IPTG added. Culture samples were electrophoresed and transferred and the immunoblot was developed with antiGspD for the detection of the $GspD_{\alpha}$ multimer in the cells that contained pTS76 (Figure 18) or pTS60 (Figure 19).

The immunoblots (Figures 18 and 19) show that $GspD_{\alpha}$ formed a multimer when the *$gspC\text{-}O_{\alpha}$* operon was expressed. Both figures show that multimerization of GspD was increased in the absence of IPTG controlling the *$gspAB_{\alpha}$* operon. The results further suggest that $GspAB_{\alpha}$ was not required for the assembly of the $GspD_{\alpha}$ multimer. The multimer was only found upon expression of the $GspD_{\alpha}$ protein when arabinose was added to the growing culture. As shown in Figure 19, a low level of arabinose and a high level of IPTG resulted in no multimer appearing in cells containing pTS60.

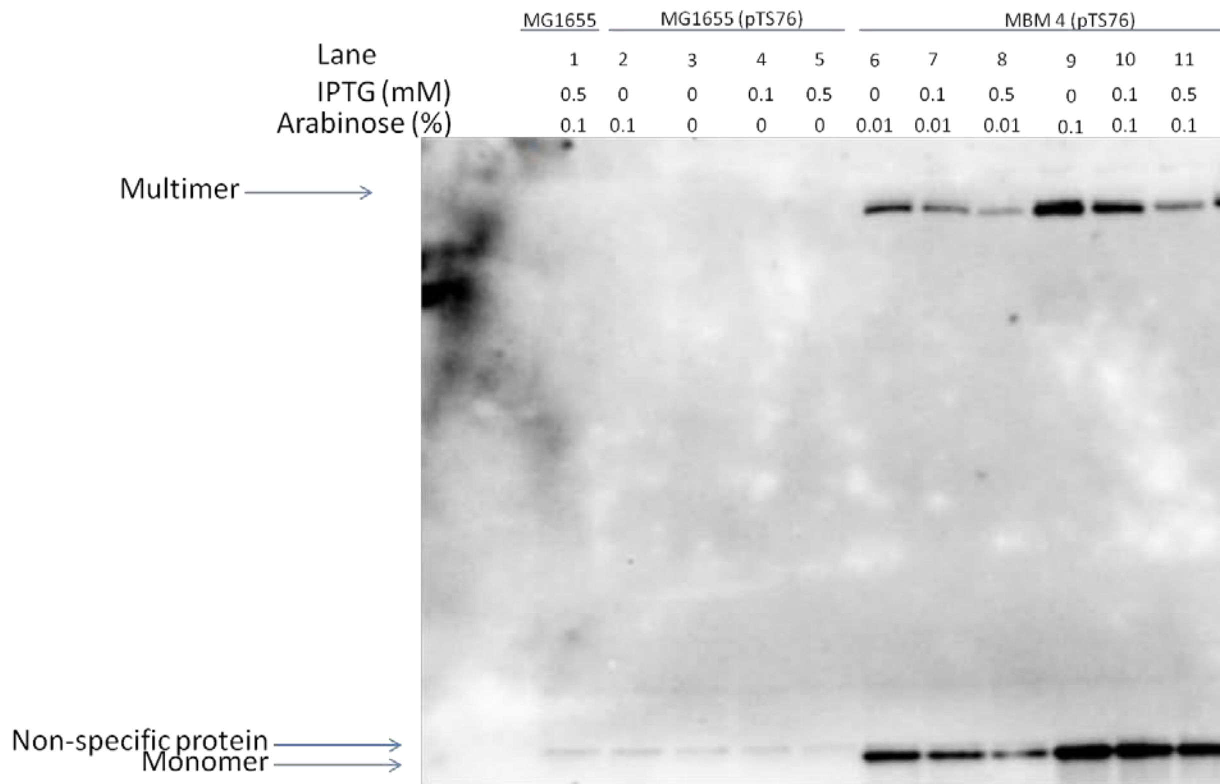


Figure 18: An immunoblot detecting GspD$_\alpha$ in WT and MBM4 *E. coli* K12 strain MG1655 cells expressing pTS76. Antibodies were used which were targeted against GspD$_\alpha$. The cell pellets were concentrated 5x before being run on this gel. Lane 1, MG1655 (WT); lanes 2-4, MG1655 (WT, pTS76); Lanes 5-11, MBM 4 (*gspA$_\alpha$-pTac gspC$_\alpha$-pBAD,* pTS76*)*. The addition of IPTG controlled the expression of *gspAB$_\alpha$* and the expression of pTS76 encoding LTB while the addition of arabinose controlled the expression of the *gspC-O$_\alpha$* operon.

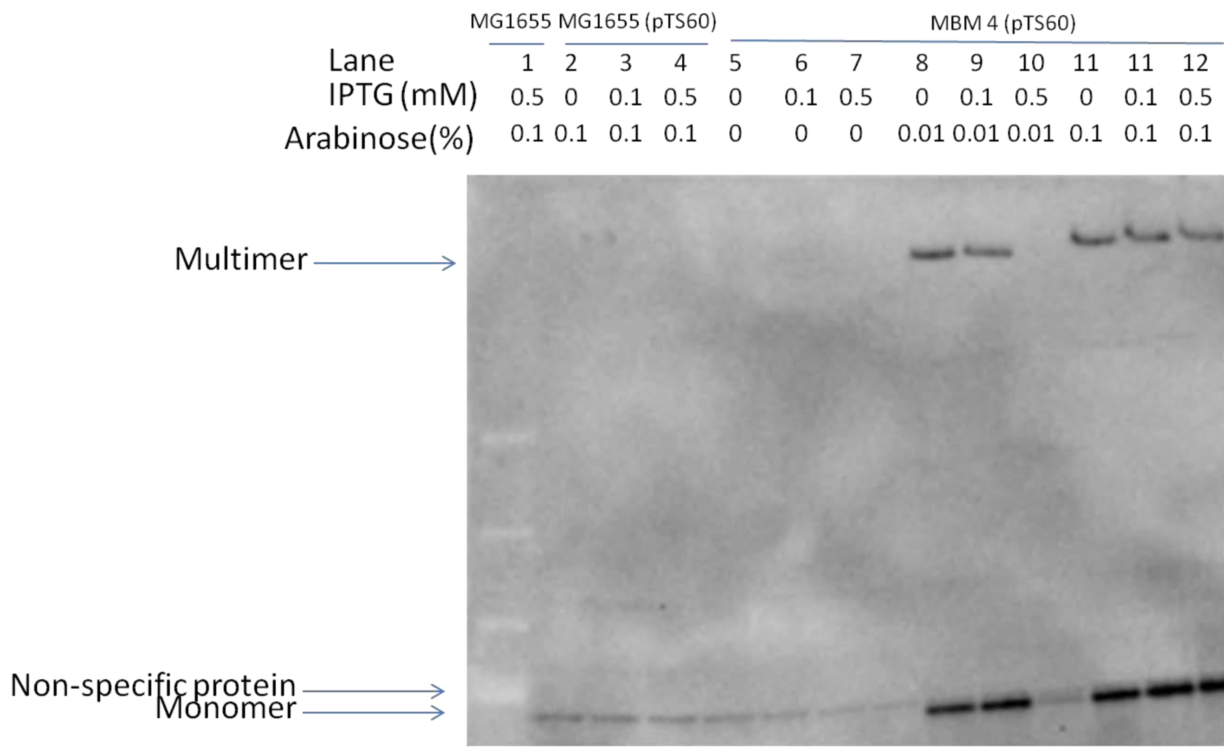


Figure 19: An immunoblot detecting $GspD_\alpha$ in WT and MBM4 cells expressing pTS60. Antibodies that were used are against $GspD_\alpha$. Samples were concentrated 5x before being run on the gel. Lane 1, MG1655 (WT); Lane 2-4, MG1655 (WT, pTS60); Lanes 5-13; MBM 4 (*$gspA_\alpha$-pTac $gspC_\alpha$-pBAD*, pTS60*)*. pTS60 (expressing ChiA-myc) was induced through the addition of arabinose as well as the *$gspC$-O_α* operon, the addition of IPTG controlled the expression of *$gspAB_\alpha$.*

3.2.4 Secretion of ChiA-myc in *gspAB*$_{\alpha}$ *- Tac* and *gspC-O*$_{\alpha}$ *– Bad* MG1655 mutants

The *gspAB*$_{\alpha}$ - *Tac* and *gspC-O*$_{\alpha}$ – *Bad* MG1655 mutants containing pTS60 were grown as described above. The supernatants were filtered using a 0.2 µm filter. Samples were electrophoresed on 10% SDS Acrylamide gels and after transfer, the immunoblots were developed with anti-myc antibodies (Figure 20).

As the figure shows, the immunoblot was essentially blank showing only the band in the control lane corresponding to ChiA-myc. The gel results thus suggest that even with the assembly of GspD$_{\alpha}$, secretion of ChiA-myc did not occur.

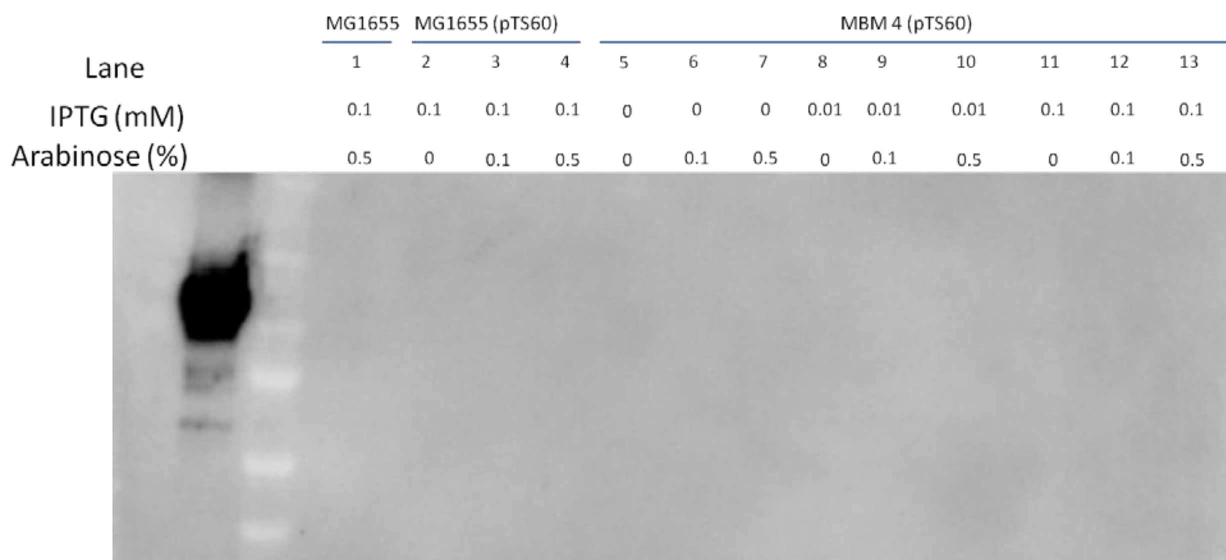


Figure 20: An immunoblot detecting ChiA-myc in supernatants of WT and MBM4 *E. coli* K12 strain MG1655 cells expressing pTS60. Lane 1, MBM 4 (pellet control, *gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD,* pTS60); Lane 2; molecular weight ladder. Lane 3, MG1655 (WT); Lane 4-6, MG1655 (pTS60); Lanes 7-15, MBM 4 (*gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD,* pTS60*)*. A dash (-) indicates that none of the listed inducer was added. Arabinose controlled the expression of pTS60 and *gspC-O*$_{\alpha}$ while IPTG controlled the expression of *gspAB*$_{\alpha}$.

3.3 Secretion and $GspD_{\alpha}$ assembly studies using inducible plasmids controlling the expression of LTB and ChiA-myc

3.3.1 The creation of tetracycline inducible plasmids controlling the expression of LTB and *chiA-myc*

Initial studies showed that a single inducer controlling multiple promoters (ie. *gspAB* and LTB) made it difficult to examine the effects of *gsp* induction level on secretion. Plasmids were made from pMAL-p4x where the *lac* promoter within the plasmid was therefore replaced with a tetracycline inducible component, *tetR* and *ptetA (*Bina et al., 2014). Since tetracycline is a potent antibiotic, the much less active derivative anhydrotetracycline was used for induction. The *tetR* gene encodes a repressor that binds to the operator found within *ptetA*. Tetracycline or its derivitives bind the repressor and allow expression from the promoter (*ptetA*). The tet inducible plasmid was created through the use of primers US794 and US795. The primers made use of the restriction sites PflMI and EcoRI. The *tetR/ptetA* gene was cloned from TN10 transposon. The amplified gene and promoter was ligated into plasmid pMAL-p4x. The resulting plasmid was named pMM2 (Figure 21).

The plasmid, once created, was used to construct two different expression plasmids. The plasmid pMM3 expresses LTB (Figure 22) while plasmid pMM4 expresses *chiA-myc (*Figure 23*)*. Plasmid pMM3 was created by using primers US793 and US816.

The primers US839 and US840 were used for the amplification of *chiA-myc* from plasmid pTS60 to form pMM4 (Figure 23). Creation required the BamHI and PstI sites found on the

primers for its insertion into pMM2. The resulting plasmids allowed the expression of LTB or ChiA-myc through the addition of anhydrotetracycline.

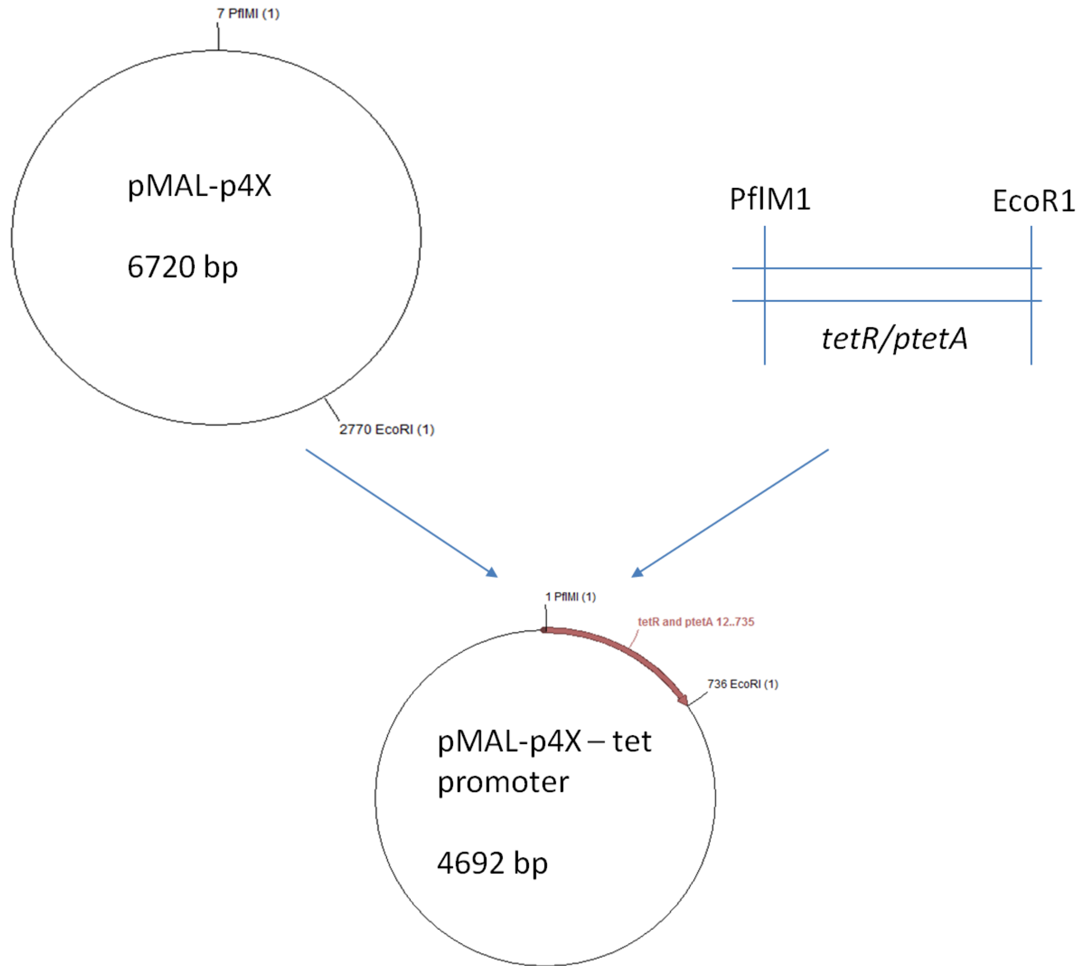


Figure 21: A diagram showing the creation of pMM2. The resulting plasmid contained a promoter (ptetA) which was induced through the addition of anhydrotetracycline.

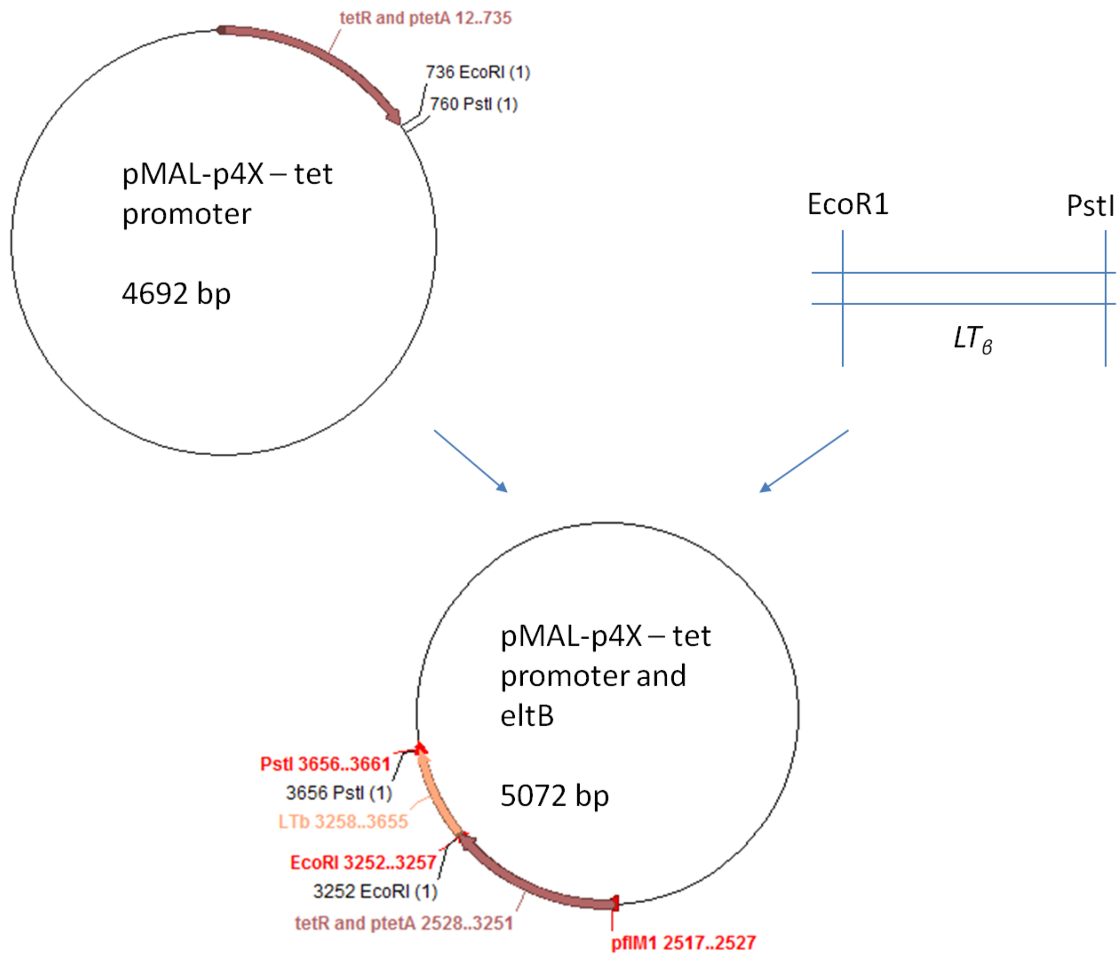


Figure 22: A diagram showing the creation of pMM3. The gene, LTB, was expressed through the induction of *ptetA* through the addition of anhydrotetracycline.

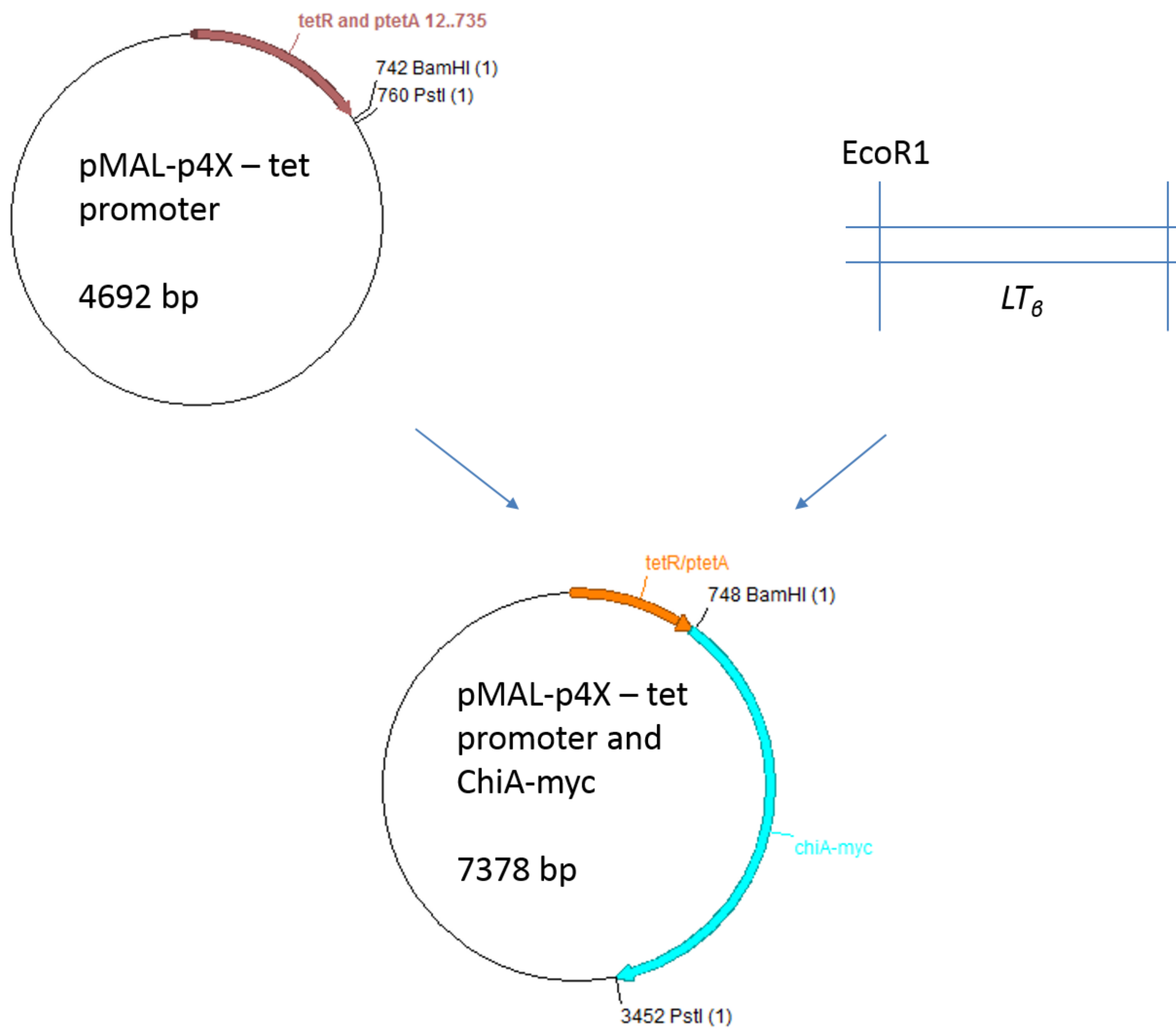


Figure 23: A diagram showing the creation of pMM4. The gene, *chiA-myc,* was expressed by *ptetA* through the addition of anhydrotetracycline.

3.3.2 Assembly of GspD$_\alpha$ *gspAB$_\alpha$ - Tac* and *gspC-O$_\alpha$ - Bad* MG1655 mutants expressing the tetracycline inducible LTB plasmid

Strains of *E. coli* MG1655 with *gspAB$_\alpha$ - Tac* and *gspC-O$_\alpha$ – Bad* containing pMM3 were grown to an OD_{600} of 1.5 in LB media containing amp. A further growth experiment included samples in which the FRT cassette was left inserted between the *gsp$_\alpha$* inducible promoters. The resistance cassettes may prevent the interference of added inducers that control the inducible promoters. The samples were electrophoresed on a 3-8% tris-acetate gel and transferred to a PVDF membrane. The immunoblot was developed with antibodies against GspD$_\alpha$.

Strains with inducible promoters controlling the *gsp$_\alpha$* operons without the resistance cassette (Figure 24) show a similar pattern as seen before, as only those strains grown with the inducer arabinose controlling the *gspC-O$_\alpha$* operon showed assembly of the GspD$_\alpha$ multimer. The second lane showed that induction of the *gspC-O$_\alpha$* operon was required for assembly of the GspD$_\alpha$ multimer. The blot showed that induction of the *gspAB$_\alpha$* operon was not required for assembly, since the same amount of multimerization was described with or without the addition of IPTG.

Figure 25 shows the blot from cells which still contain the FCKF cassette between the inducible promoters. The first five lanes in this experiment were controls, in which the first two lanes are inducible promoter mutants with arabinose added and the next three lanes have no arabinose. The results showed that there does not seem to be interference between inducers.

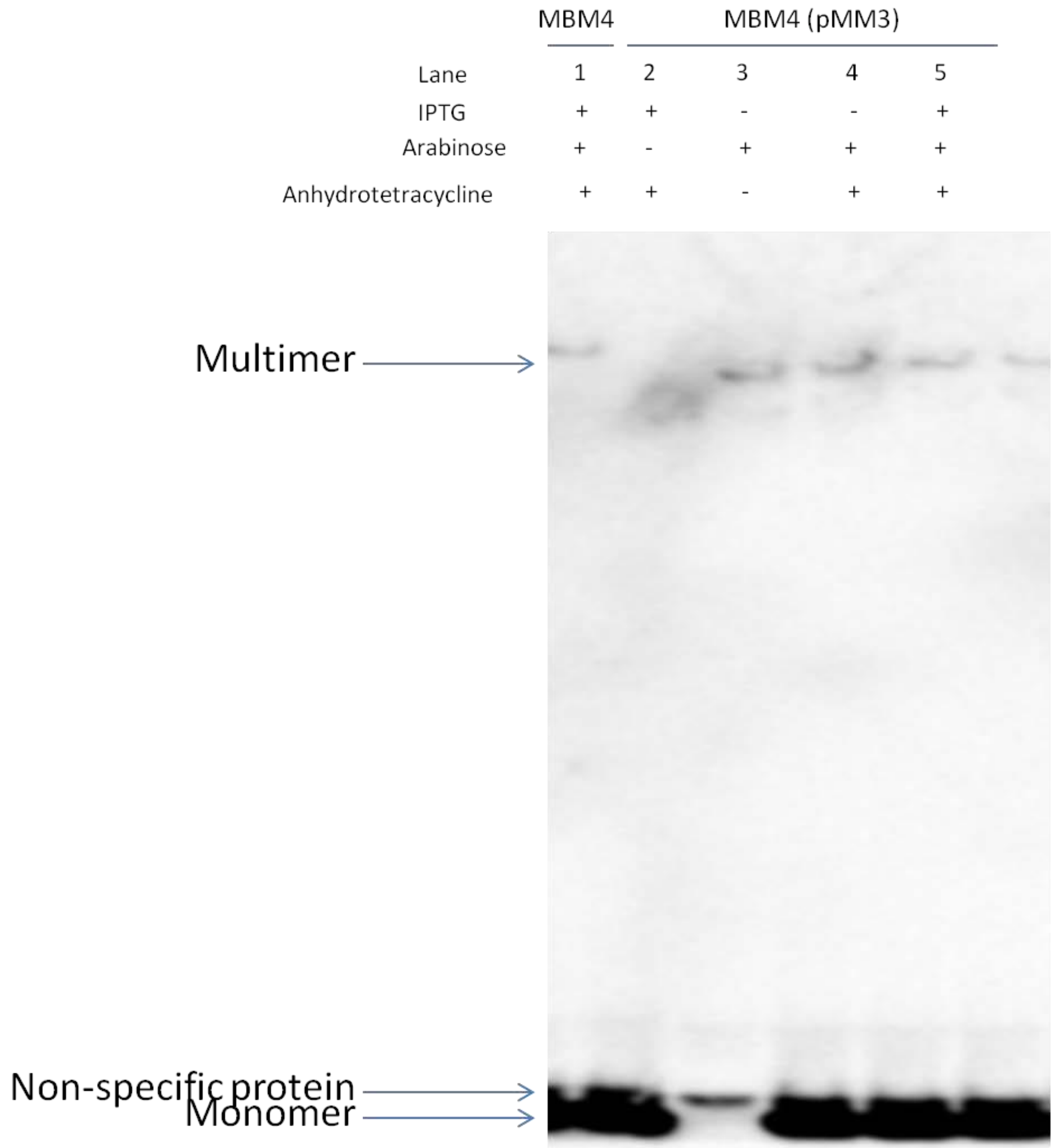


Figure 24: An immunoblot detecting GspD$_\alpha$ in WT and MBM4 *E. coli* K12 strain MG1655 cells expressing pMM3. Lane 1, MBM 4 (*gspA$_\alpha$-pTac gspC$_\alpha$-pBAD)*; Lane 2-5, MBM 4 (*gspA$_\alpha$-pTac gspC$_\alpha$-pBAD,* pMM3). Arabinose was used (+) at 0.01%, IPTG was used (+) at 0.1mM and anhydrotetracycline was used (+) at 40ng/ml. A minus symbol (-) indicates that the inducer was not added.

Figure 25: An immunoblot detecting GspD$_{\alpha}$ in WT and MBM3 *E. coli* K12 strain MG1655 cells expressing pMM3. Lane 1, MBM 4 (*gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD)*; Lane 2-8, MBM 3 (*gspA*$_{\alpha}$*-pTac*-FRT-*Kan-Cam*-FRT-*pBAD-gspC*$_{\alpha}$*,* pMM3). Arabinose was used (+) at 0.01%, IPTG was used (+) at 0.1mM and anhydrotetracycline was used (+) at 40ng/ml. A minus symbol (-) indicates that none of the inducer was added.

3.3.3 Secretion of LTB in *$gspAB_\alpha$ - Tac* and *$gspC\text{-}O_\alpha$ - Bad* MG1655 mutants using a tetracycline inducible promoter controlling the expression of LTB

The *$gspAB_\alpha$ - Tac* and *$gspC\text{-}O_\alpha$ - Bad* MG1655 mutants with and without the FCKF resistance cassette containing pMM3 were grown and centrifuged as described above. The supernatants were 10x concentrated using ammonium sulfate (materials and methods), while the pellet samples were lysed using a French press. The pellet and supernatant samples were analyzed with a LT ELISA. The pellet (Figures 26 and 28) and the 10x supernatant samples (Figures 27 and 29) were developed with antibodies against CT.

The results were used to compare the amount of secretion in strains with and without the resistance cassette between inducible promoters. The results showed that strains with inducible promoters controlling the expression of the *gsp_α* operons expressed the LTB gene only when anhydrotetracycline was added during growth.

The supernatants of cells with or without the resistance cassette between inducible promoters showed that no secretion takes place. Supernatants from cells grown without anhydrotetracycline showed the presence very small amounts of LTB compared to those of induced cells. Thus the ELISA showed that secretion does not take place in these cells. The induction of these strains resulted in very small amounts of LTB being detected in the media. The values are so small compared to the pellet samples that the results were evaluated as zero.

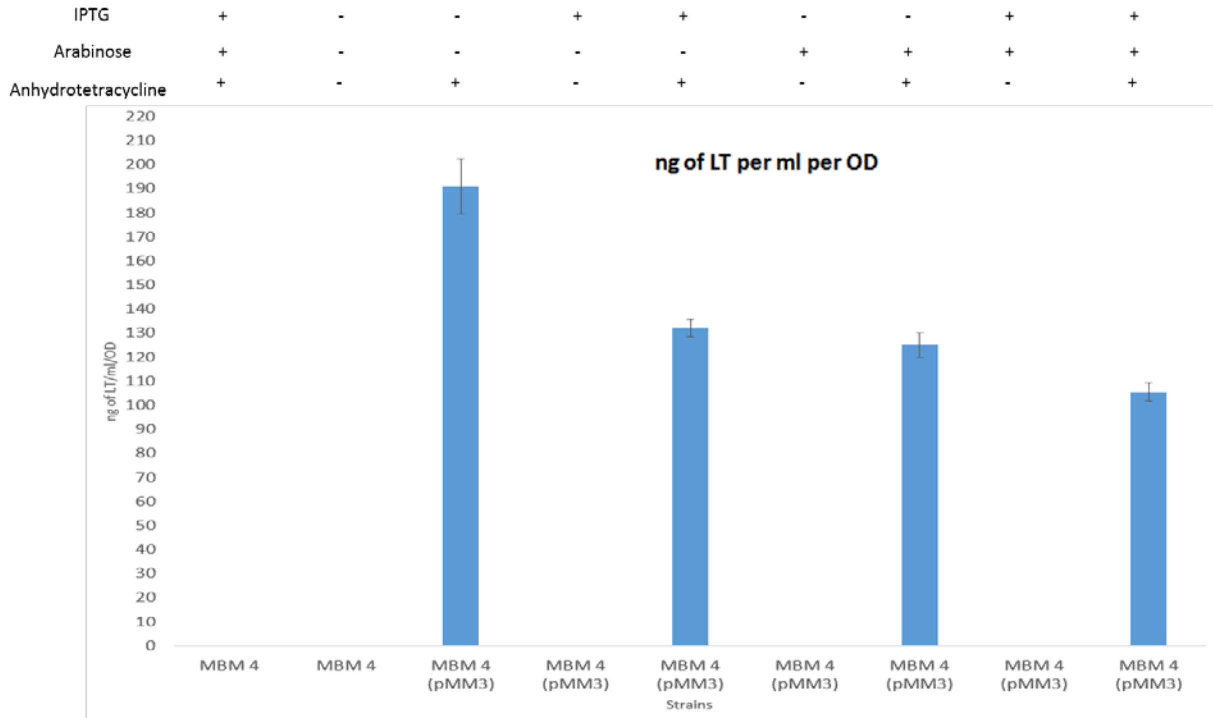


Figure 26: An ELISA detecting LTB within MBM4 *E. coli* K12 strain MG1655 cells expressing pMM3. The cell pellets were french pressed before analysis. Column 1 and 2, MBM 4 (*gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD)*; Columns 3-9, MBM 4 (*gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD,* pMM3). Arabinose was used at 0.01 %, IPTG was used at 0.1mM and anhydrotetracycline was used at a concentration of 40 ng/ml. The scale goes to 210 in increments of 10.

IPTG	+	+	-	-	+	+
Arabinose	+	-	+	+	+	+
Anhydrotetracycline	+	+	-	+	-	+

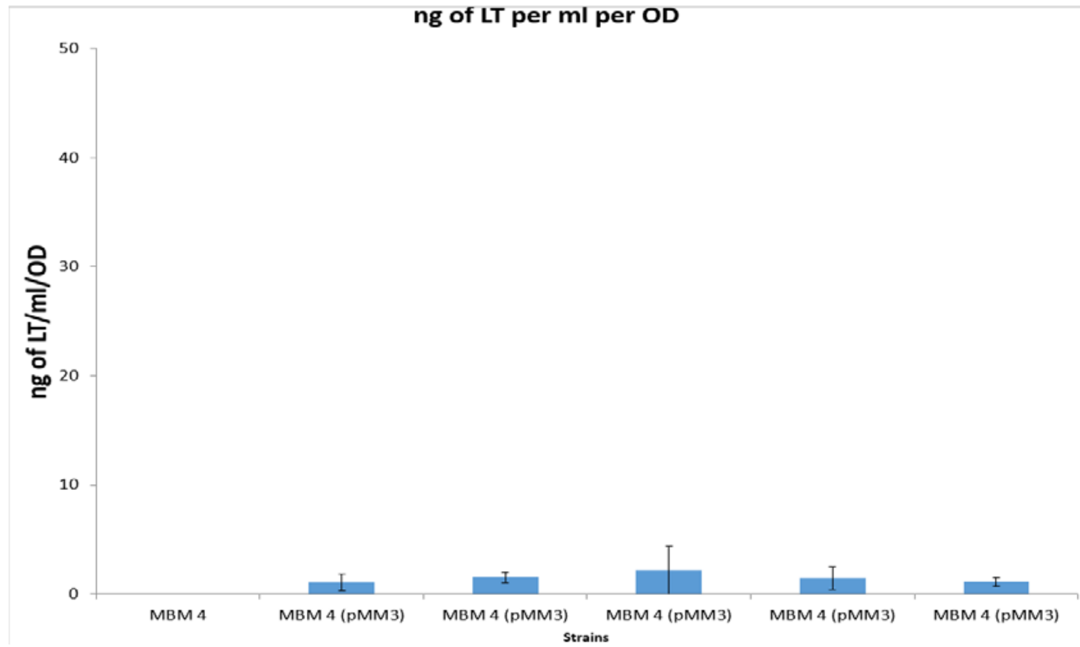


Figure 27: An ELISA detecting LTB in the supernatants of MBM4 *E. coli* K12 strain MG1655 cells expressing pMM3. Supernatants are concentrated 10x before the detection of LT_{β}. Column 1, MBM 4 (*$gspA_{\alpha}$-pTac $gspC_{\alpha}$-pBAD*); Columns 2-6, MBM 4 (*$gspA_{\alpha}$-pTac $gspC_{\alpha}$-pBAD*, pMM3). Arabinose was used at 0.01%, IPTG was used at 0.1mM and anhydrotetracycline was used at 40 ng/ml. The scale goes to 50 in increments of 10.

Arabinose	+	-	-	-	-	+	+	+	+
IPTG	+	-	+	-	-	+	+	-	+
Anhydrotetracycline	+	+	+	-	+	-	+	+	+

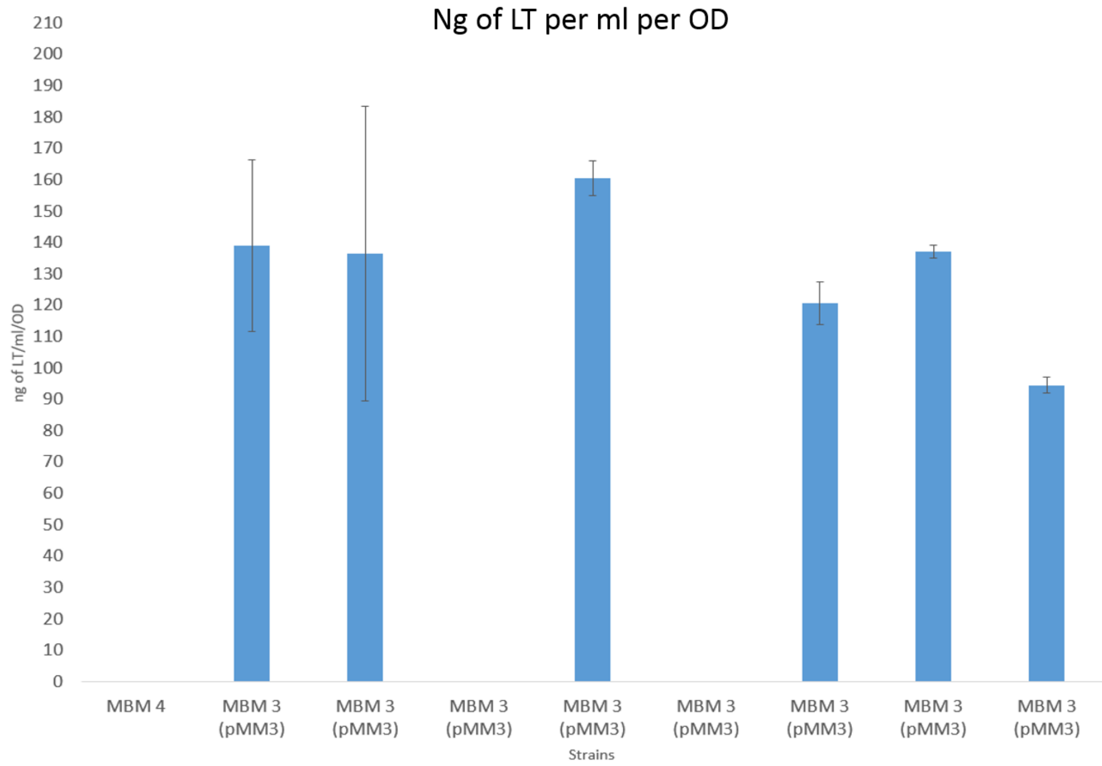


Figure 28: An ELISA detecting LTB in MBM3 and MBM4 *E. coli* K12 strain MG1655 cells expressing pMM3. Cell samples were french pressed before they are used for analysis. Column 1, MBM 4 (*gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD*); Columns 2-9, MBM 3 (*gspA*$_{\alpha}$*-pTac* FCKF *gspC*$_{\alpha}$*-pBAD,* pMM3). Arabinose was used at 0.01 %, IPTG was used at 0.1mM and anhydrotetracycline was used at a concentration of 40 ng/ml. The scale goes to 210 in increments of 10.

Arabinose	+	-	-	+	+	+
IPTG	+	+	-	+	-	+
Anhydrotetracycline	+	+	+	+	+	+

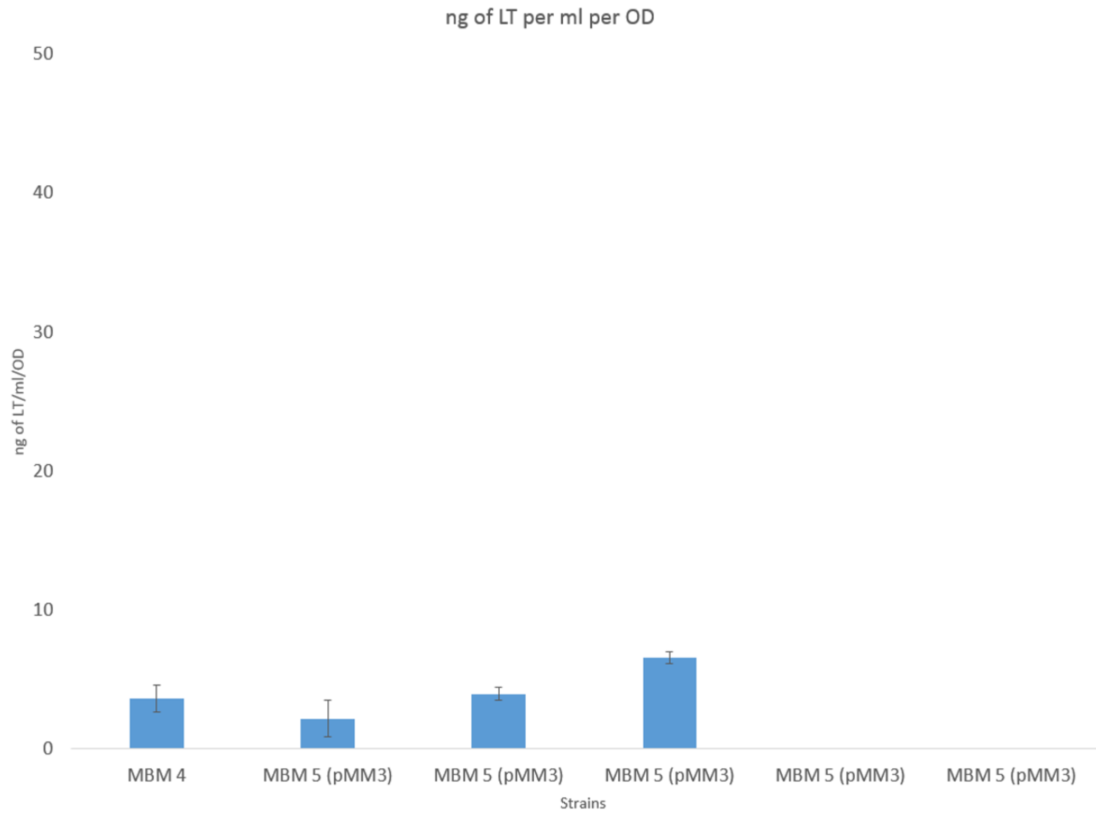


Figure 29: An ELISA detecting LTB in the supernatants of MBM3 and MBM4 *E. coli* K12 strain MG1655 cells expressing pMM3. Supernatants are concentrated 10x before the detection of LTB. Column 1, MBM 4 (*gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD)*; Columns 2-6, MBM 3 (*gspA*$_{\alpha}$*-pTac* FCKF *gspC*$_{\alpha}$*-pBAD,* pMM3). Arabinose was used at 0.01%, IPTG was used at 0.1mM and anhydrotetracycline was used at 40 ng/ml. The scale maximizes at 45 in increments of 10.

3.3.4 Secretion of *chiA-myc gspAB*$_{\alpha}$ *- Tac* and *gspC-O*$_{\alpha}$ *- Bad* MG1655 mutants for the using a tetracycline inducible plasmid controlling the expression of *chiA-myc*

The *gspAB*$_{\alpha}$ *- Tac* and *gspC-O*$_{\alpha}$ *- Bad* MG1655 mutants containing pMM4 were grown in LB media containing amp to an OD_{600} of 1.5. The supernatants were concentrated 25x. The pellet and concentrated supernatant samples were electrophoresed on a 10% acrylamide gel before being transferred to a PVDF membrane. The immunoblots were developed with antibodies against the myc tag.

The cell sample immunoblot (Fig. 30) showed a similar pattern to what was previously observed, samples grown in the presence of anhydrotetracycline express the chiA-myc gene while those without did not.

The results of the detection of ChiA-myc in the supernatants is shown in Fig. 31. The left most lane shows a positive control in which ChiA-myc has been previously detected. The lower band in each sample corresponds to BSA which was used as a carrier in ammonium sulfate precipitation. The immunoblot shows that the samples did not give prominent results as many strains show very small amounts of secretion. Some of the samples that show secretion did not contain arabinose meaning that the presence of the band corresponding to ChiA-myc is likely an artifact. The cells do not seem to secrete appreciable amounts of ChiA-myc.

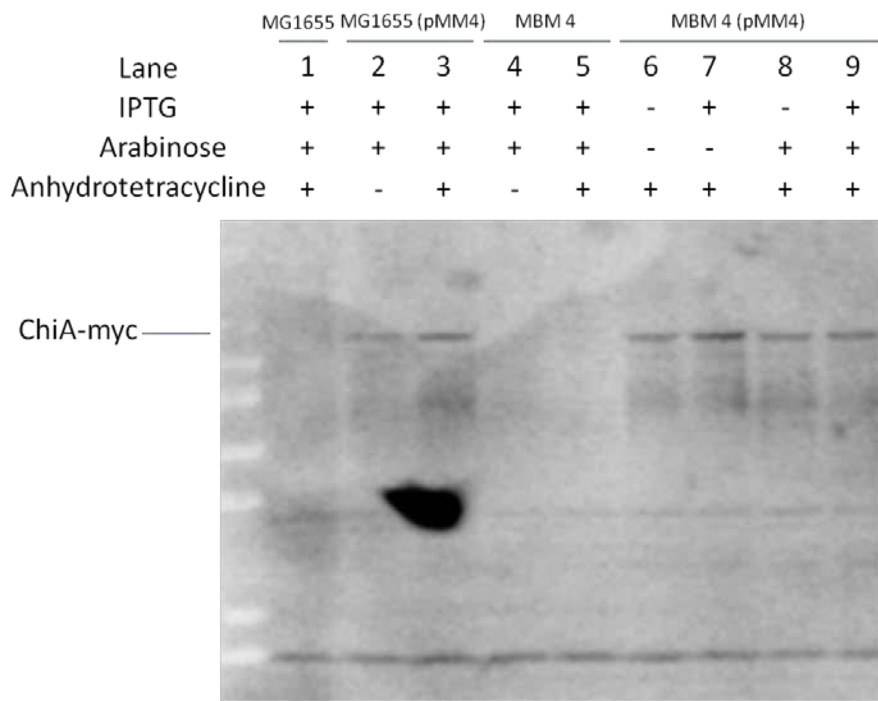


Figure 30: An immunoblot detecting ChiA-myc within WT and MBM4 *E. coli* K12 strain MG1655 cells expressing pMM4. The samples were not concentrated prior to loading. Lane 1, MG1655 (WT); Lanes 2+3, MG1655 (WT, pMM4); Lanes 4+5, MBM 4 (*gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD*); Lanes 6-9, MBM 4 (*gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD,* pMM4). Arabinose was used (+) at 0.01%, IPTG was used (+) at 0.1% while anhydrotetracycline was used (+) at 2 ng/ml. A minus sign (-) indicates that the inducer given was not added.

	MG1655		MG1655 (pMM4)		MBM 4		MBM 4 (pMM4)			
Lane	1	2	3	4	5	6	7	8	9	10
IPTG	+	+	+	+	+	-	+	-	+	-
Arabinose	+	+	+	+	+	-	-	+	+	-
Anhydrotetracycline	-	+	+	-	+	+	+	+	+	+

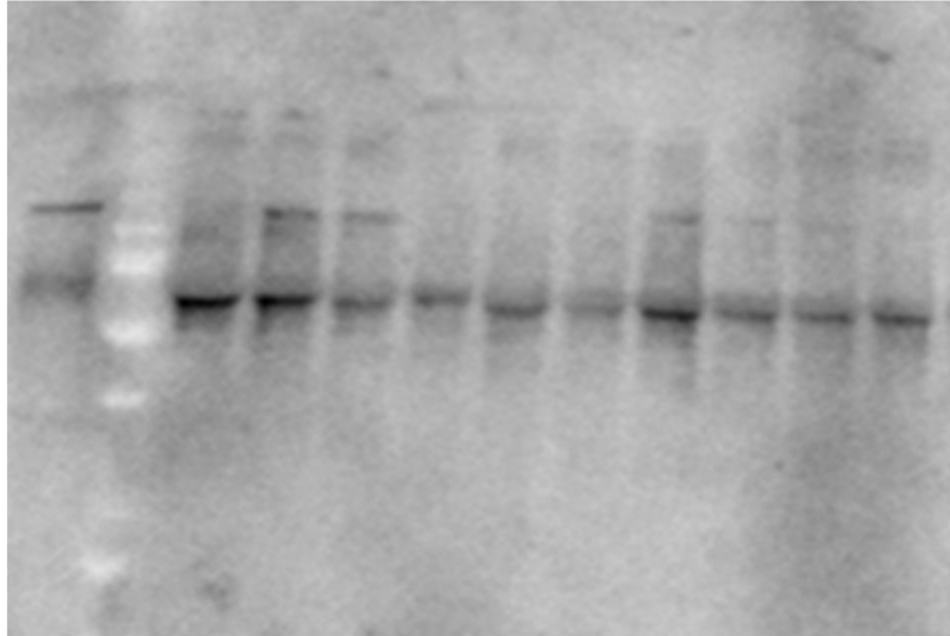


Figure 31: An immunoblot detecting ChiA-myc within the supernatants of WT and MBM4 *E. coli* K12 strain MG1655 cells expressing pMM4. The samples were concentrated 25x prior to loading. Lane 1, Positive control; Lane 2, molecular weight ladder; Lane 3, MG1655 (WT); Lanes 4+5, MG1655 (WT, pMM4); Lanes 6+7, MBM 4 (*gspA$_\alpha$-pTac gspC$_\alpha$-pBAD*); Lanes 8-11, MBM 4 (*gspA$_\alpha$-pTac gspC$_\alpha$-pBAD,* pMM4). Arabinose was used at a concentration of 0.01%, IPTG was used at a concentration of 0.1% while anhydrotetracycline was used at a concentration of 2 ng/mL. A minus sign (-) indicates that none of the given inducer was added.

3.4 Assembly of $GspD_{\alpha}$ in *$gspAB_{\alpha}$ - Tac* and *$gspC\text{-}O_{\alpha}$ - Bad* MG1655 mutants with a deletion of GspB

It has been shown in different bacteria that the GspAB T2SS proteins are involved in secretin assembly. *Aeromonas hydrophila* requires these proteins for secretion while *Vibrio cholerae* does not require the proteins for secretion but requires the proteins for full levels of psecretin assembly. In *Aeromonas hydrophila* the GspA protein interacts with peptidoglycan while the GspB protein interacts with the secretin. The two accessory proteins have not been well studied for the secretion of proteins in *E. coli*. A paper that was written by Francetic et al (2000) showed that GspAB increased the apparent secretion of ChiA, but assembly of the secretin multimer was not studied.

A keio collection strain with a kan resistance cassette inserted into *gspB* was attained and used as a template for amplification with US802 and US803. The amplified product was used to replace the existing *gspB* gene in the strains which had inducible promoters controlling expression of the alpha T2SS system operons. The resulting strain, MBM5, contained the inducible promoters controlling the expression of the alpha *gsp* system operons with a kan resistance cassette inserted into *gspB.*

The creation of MBM 5 allowed an examination of the assembly of GspD. The results shown in Fig. 32 indicated that the absence of the GspB protein did not decrease the amount of multimer detected compared to that observed in the experiment shown in Fig. 25. Furthermore in both experiments the addition of IPTG controlling the expression of the *$gspAB_{\alpha}$* operon had no effect

on secretin assembly. The results thus suggest that the accessory proteins GspA and GspB are not required for secretin assembly in this T2SS system.

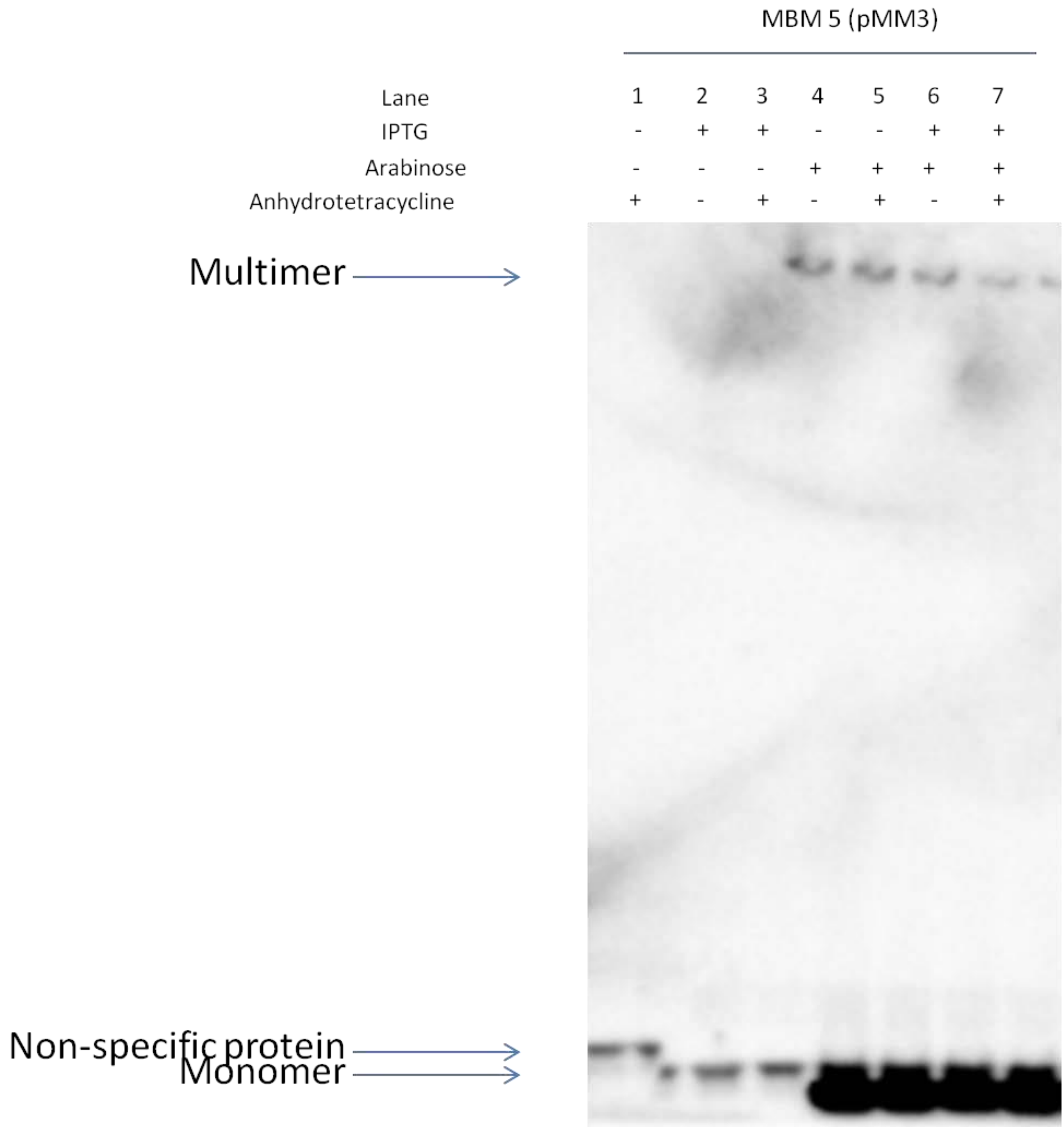


Figure 32: An immunoblot detecting GspD$_{\alpha}$ in MBM5 *E. coli* K12 strain MG1655 cells expressing pMM3. Lane 1-7, MBM 5 (*gspA*$_{\alpha}$*-pTac gspC*$_{\alpha}$*-pBAD gspB::kan,* pMM3). Arabinose was used (+) at 0.01%, IPTG was used (+) at 0.1mM and anhydrotetracycline was used (+) at 40 ng/ml. A minus sign (-) indicates that the given inducer was not added.

3.5 The assembly of $GspD_{\alpha}$ and the secretion of proteins in Enterotoxigenic *Escherichia coli*

3.5.1 Assembly of $GspD_{\alpha}$ in *$gspAB_{\alpha}$ - Tac* and *$gspC\text{-}O_{\alpha}$ - Bad $\Delta gspD_{\beta}$ E. coli* H10407 mutants

There are many different strains of *E. coli* with the alpha or the beta T2SS. Some strains have both the alpha and the beta T2SS system, such as *ETEC E. coli* H10407. This strain has been shown to secrete LTB using the beta T2SS but the alpha system, as in strain MG1655, appears to be cryptic. It was thus of interest to examine the multimerization of $GspD_{\alpha}$ and secretion of LTB in an H10407 strain in which the alpha T2SS system could be activated.

An H10407 mutant, TGS60, was created by Timothy Strozen (2011) with inducible promoters controlling the *gsp_{α}* operons. The pBAD promoter was used to control the *$gspC\text{-}O_{\alpha}$* operon which was induced through the addition of arabinose while *$gspAB_{\alpha}$* was induced through the addition of IPTG. In addition a kan resistance cassette was inserted into *$gspD_{\beta}$* to inactivate secretion from the beta T2SS system. The cells were grown as before and samples were electrophoresed on a 3-8% tris acetate gel and transferred to a PVDF membrane. The immunoblot was developed with $GspD_{\alpha}$ antibodies, and is shown in Fig. 33.

The first three lanes run on the gel were used as controls as none of the strains have an inducible promoter controlling the alpha T2SS system. The WT or H10407 ETEC *E. coli* strains with a deletion in *$gspD_{\beta}$* did not show expression or assembly of the $GspD_{\alpha}$ monomer. Lanes 4-8 show that TGS60 assembled small amounts of multimeric $GspD_{\alpha}$ protein when the strain was grown in the presence of arabinose. Lane 8 was used as a positive control in which the multimer of $GspD_{\alpha}$ had been previously detected. The results showed that arabinose controlling the *gspC-*

O_α operon was necessary during growth for the expression of $GspD_\alpha$ and multimerization of the protein.

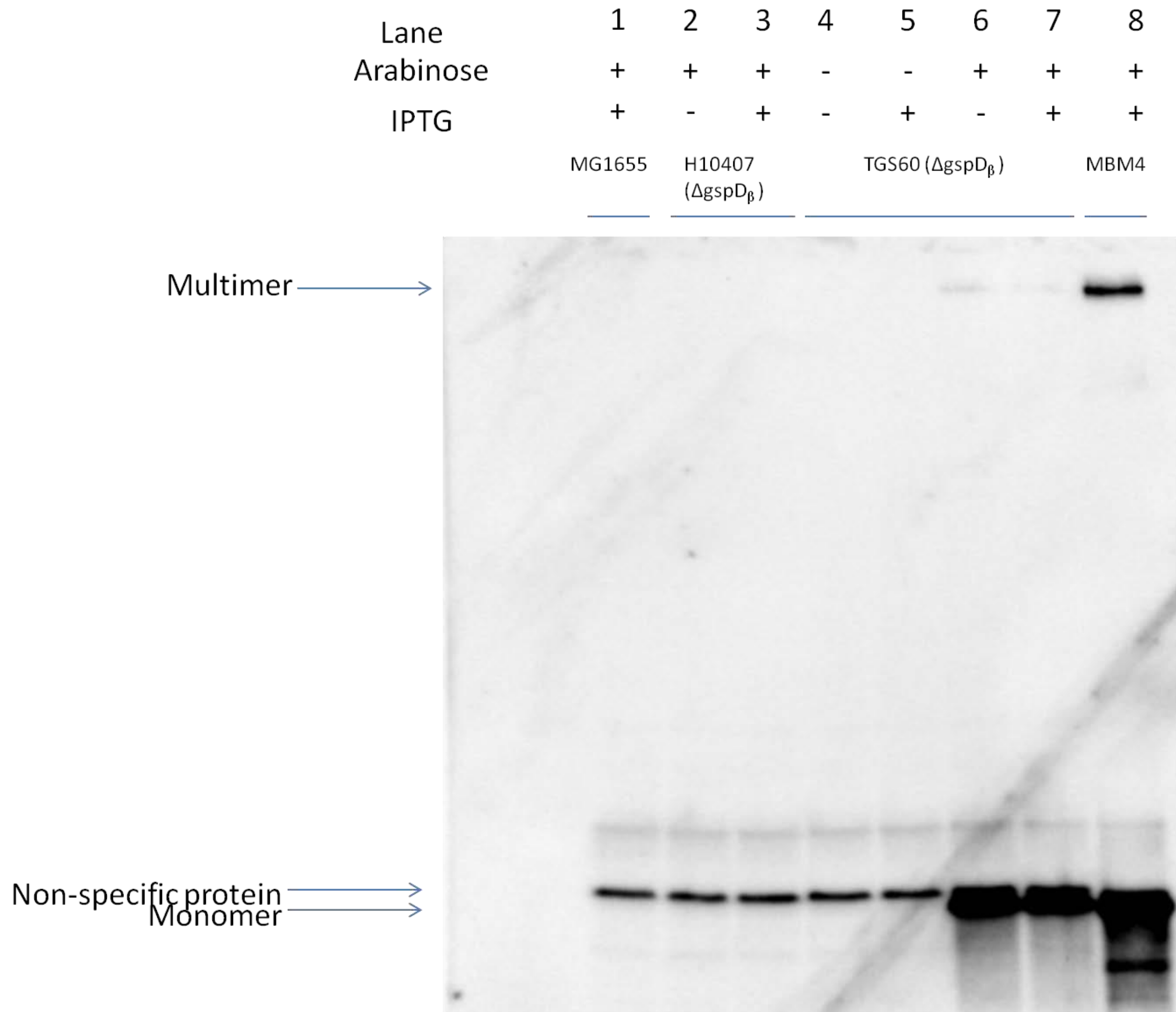
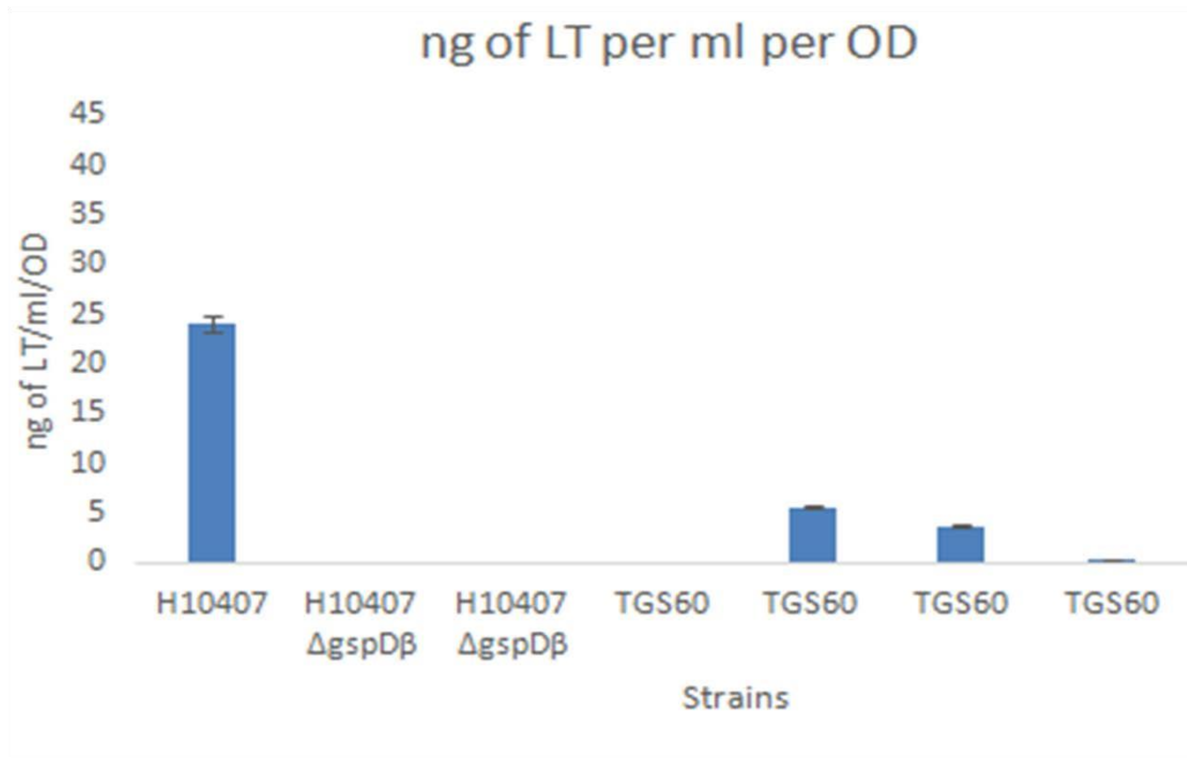


Figure 33: An immunoblot detecting $GspD_\alpha$ in WT, H10407, TGS60 and MBM4 *E. coli* cells. The cell pellets were concentrated 5x before being run. Lane 1, MG1655 (WT); Lane 2+3, H10407 ($\Delta gspD_\beta$); Lane 4-7, TGS60 ($pTacgspAB_\alpha$, pBAD-$gspC$-O_α, $\Delta gspD_\beta$); Lane 8, MBM 4 (positive control, $gspA_\alpha$-$pTac$ $gspC_\alpha$-$pBAD$). Arabinose was used (+) at 0.01% while IPTG was used (+) at 0.1mM. A minus sign (-) indicates that the inducer was not added.

3.5.2 Secretion of LTB in *gspAB*$_{\alpha}$ *- Tac* and *gspC-O*$_{\alpha}$ *- Bad* Δ*gspD*$_{\beta}$ *E. coli* H10407 mutants

WT H10407, H10407 Δ*gspD*$_{\beta}$ *and* TGS60 were grown to an OD_{600} of approximately 1.5, supernatant and cell samples were obtained through growth and centrifugation. An ELISA was used to measure of LTB in the supernatants to see if secretion occurred.

The ELISA (Figure 34) showed the amount of LTB in the supernatant of *ETEC E. coli* cells. The wild-type H10407 was used as a positive control. The sample demonstrates normal levels of secretion occurred from the active beta T2SS. The second and third samples are negative controls in which secretion from the beta T2SS system was inactivated through the deletion of *gspD*$_{\beta}$. The third to seventh samples contain the samples of interest. These samples contain cells with inducible promoters controlling the expression of the *gsp*$_{\alpha}$ operons. The amount of secretion from these strains was very minimal and did not show a consistent pattern since secretion when either operon was induced appeared greater than when both operons were induced.



	1	2	3	4	5	6	7
Arabinose	+	+	+	-	-	+	+
IPTG	+	-	+	-	+	-	+

Figure 34: An ELISA detecting LTB found in the supernatants of H10407, H10407 $\Delta gspD_{\beta}$ and TGS60 ETEC *E. coli* cells. The supernatants are not concentrated for the use in this ELISA. Column 1, H10407; Columns 2+3, H10407 Δgsp_{β}; Columns 4-7, TGS60. Arabinose was used (+) at 0.01% while IPTG was used (+) at 0.1mM. The scale on the y axis goes to 45.

4. Discussion

This is the first study done in which expression of the chromosomal copies of the alpha *gsp* T2SS system genes have been manipulated to study the function of the T2SS in secretion. The results have demonstrated that induction of the alpha T2SS system leads to multimerization of the $GspD_{\alpha}$ protein but this multimerization does not lead to the secretion of the proteins examined.

Experiments used a *lacZYA* fusion in the new alpha T2SS secretion system (Francetic et al., 2000) to indicate conditions in which these fusions were expressed. Transposon mutagenesis revealed that transcription was increased when mutations in the *hns* gene occurred. Further experiments revealed that the alpha T2SS system can secrete both LT (Horstman and Kuehn, 2002) and ChiA (Francetic et al., 2000) when the *hns* gene was deleted. The chromosomal copies of the system were not induced in these experiments, the alpha T2SS system was induced through the presence of the *gsp* genes on inducible plasmids. Secretion was only observed when the repressor protein HNS was removed from the bacterial cell.

In this work, studies were performed in which *hns* and *stpA*, were deleted, either independently or together. The strains tested could not express *hns* or *stpA* through the insertion of a kan resistence cassette (Baba et al., 2006) the levels of secretion from the T2SS system and the assembly of $GspD_{\alpha}$ into the multimeric form were examined. The secretion results showed that the plasmids used were present and expressing either LTB or ChiA as only the pellet samples contained the induced protein upon induction. Analysis of the supernatant samples indicated however that secretion did not occur. Although LTB or ChiA appeared to be

present in the supernatants of the *hns* and *stpA* mutant cells, this also occurred in WT cells and in cells in which components of the alpha T2SS were removed through deletion. Thus the results obtained were an artifact, perhaps caused by small amounts of cell lysis. The strains were tested for the assembly of $GspD_{\alpha}$ into the multimeric form from the monomer. In all strains tested, the multimer was not present. Strains with a mutation in *hns* showed small amounts of the monomer which indicated that expression of the alpha *gsp* operon was increased in this mutant. However, the deletion of both *hns* and *stpA* did not result in the assembly of the $GspD_{\alpha}$ multimer or result in the secretion of proteins that were tested including LTB or ChiA.

In an effort to further increase expression, strains of *E. coli* K12 were created in which promoters controlling the expression of the *gsp* operons were replaced with inducible promoters which are not repressed by cellular repressors. The *ptac* promoter from pMAL-p4x was used to control the expression of *gspAB (*de Boer et al., 1983) while the *paraBAD* promoter from pBAD322c was used to control the expression of *gspC-O (*Lee e al., 1987)*.* The promoters were induced with IPTG and arabinose, respectively. The created *E. coli* K12 strains, through lambda red recombination (Strozen, 2012), were used for experiments assaying the assembly of $GspD_{\alpha}$ and the secretion of LT and ChiA. The strains assembled $GspD_{\alpha}$ into the multimeric form only when arabinose was added to the culture during growth. Expression of the operon containing *gspD*$_{\alpha}$ resulted in the expression of the monomer and assembly of the secretin multimer. However, multimerization of the alpha T2SS secretin may be a self-assembled complex as can be observed upon the over-expression of OutD in *Erwinia crysanthemi*

*(*Condemine and Shevchik, 2000). The overexpression of *outD* in a strain that carried a deletion in *outB* in which secretion was not observed showed secretion upon the overexpression of *outD.* The $GspD_{\alpha}$ protein may be over-expressed in which the secretin multimer could selfassemble providing false results.

However, assembly of the $GspD_{\alpha}$ multimer did not result in secretion by the system. Initial experiments used pTS76 and pTS60 (Strozen, 2012) for the secretion of LTB and ChiA. LTB and ChiA genes on these plasmids are induced through the addition of IPTG and arabinose respectively which may have caused artifacts since the induction of the alpha *gsp* operons was also controlled with these inducers. The analysis of cell samples showed that the proteins were being expressed. In any case however, secretion was not observed in any of the strains used. Plasmids were created in which the promoter from pMAL-p4x (*ptac*) was replaced with a tetracycline inducible promoter from TN10 (Bina et al., 2014). The tetracycline promoter was induced through the addition of anhydrotetracycline. This allowed three different inducible promoters to be used to control the expression of the alpha *gsp* operons and the expression of LTB or ChiA. Results from experiments in which these plasmids were used showed similar negative secretion results. Secretion was not evident in any of the strains even when the supernatants were concentrated. Any results that showed secretion could be attributed to an artifact such as lysis since the apparent secretion could be witnessed in WT cells.

In *Aeromonas hydrophila* and *Vibrio cholera*, GspA and GspB form part of the T2SS (Ast et al., 2004). Studies on these proteins have shown that the ExeAB complex is required for secretion in *Aeromonas hydrophila*. ExeA has a peptidoglycan binding domain while ExeB interacts with

ExeD (Vanderlinde et al., 2014). In *Vibrio cholerae,* secretion is not affected but assembly of the secretic system is decreased when *epsA* is deleted (Strozen et al., 2012). These studies show that the A and B proteins are involved in secretin assembly and thus in secretion. Before this work, studies had not been performed on the role of *gspA*$_\alpha$ and *gspB*$_\alpha$ with regards to the formation of the GspD$_\alpha$ secretin in *E. coli*.

In these studies with the *E.* coli K12 mutants containing inducible *gsp*$_\alpha$ promoters, it was repeatedly observed that secretin assembly did not depend on the expression of *gspAB*$_\alpha$. To confirm this result, a Keio collection mutant with a kan resistance cassette inserted into *gspB*$_\alpha$ (Baba et al., 2006) was used to create a strain in which *gspB*$_\alpha$ was deleted from the *gspAB*$_\alpha$ operon under the control of the tac promoter. This strain still showed assembly of the GspD$_\alpha$. Thus multimerization of the GspD$_\alpha$ secretin was not affected upon deletion of *gspB*$_\alpha$ or in the absence of IPTG when *ptac* controlled *gspAB*$_\alpha$ expression showing that GspB was not required for assembly of the T2SS. This suggested that GspB$_\alpha$ was not required for assembly of the multimer but was shown to be required in secretion through immunoblot analysis (Francetic et al., 2000).

Experiments were also performed on an ETEC *E. coli* strain with an inducible chromosomal copy of the alpha *gsp* operons (Strozen et al., 2012). The promoters that had been used in *E. coli* K12 were used in this strain which consisted of *ptac* controlling *gspAB*$_\alpha$ and *paraBAD* controlling the expression of *gspC-O*$_\alpha$. *ETEC E. coli* is known to secrete LT though the beta T2SS system and thus the strain used also contained a deletion of *gspD*$_\beta$*.* Although this strain could

be shown to multimerize the seretin upon induction with arabinose, this did not result in the secretion of LTB.

In summary, the results of these studies indicate that the alpha T2SS system does not function in the secretion of LT or ChiA. Previous studies done on the alpha T2SS (Francetic et al., 2002) did not include lysis controls on quantification of the proportion of these proteins secreted. Furthermore, mutations created in known repressors of the alpha *gsp* system, including *hns* and *stpA*, did not result in assembly of the $GspD_{\alpha}$ multimer. The deletion of these repressors also did not result in the secretion of LT or ChiA from the bacterial cell, above background levels also observed in WT or $gspD_{\alpha}$ mutant cells. Thus the small amounts of apparent secretion observed are attributed to artifacts such as lysis. The creation of K12 strains with inducible promoters controlling the expression of the alpha *gsp* operons did show assembly of $GspD_{\alpha}$ upon induction of the operons but did not result in secretion. Not all of the components of the secretion system have been tested, it may be that the secretion system under the conditions employed is not fully assembled and thus conclusions about the natural substrates for the system cannot be drawn. The substrates used, chiA and LT, were not secreted under the conditions employed.

5. Future Directions

It has been shown by Francetic et al. (2000) that *hns* represses promoters controlling expression of the gsp_{α} operons, however the amount of repression or expression was never quantified in these experiments. qPCR of the *gsp* operons in such would allow a more precise determination of the role in the repression of these operons.

Dunstan et al. (2013) have identified many different strains of *E. coli* carrying an alpha or beta T2SS or both. Some organisms may have only an alpha T2SS system and the absence of a beta T2SS system but still secrete proteins. An organism such as this would allow the study of secretion by the alpha T2SS system. This would allow for the detection of currently unknown proteins that are substrates for secretion by the alpha T2SS system. Gathering and studying such strains would thus allow the characterization of secretion by the alpha T2SS system.

It has been reported by Horstman and Kuehn (2002) that LT secreted by the beta T2SS attaches to the bacterial surface before being released to attach to mammalian cells. Supernatants of these bacteria should be checked for the leakage of periplasmic proteins as the presence of such proteins would suggest partial rupture of the outer membrane under the conditions used. Vesicles from the outer membrane that are used to deliver the toxin to bacterial cells could represent such ruptures containing release of proteins associated with the bacterial outer membrane.

Studies by Strozen et al. (2012) showed that ETEC *E. coli* have a second T2SS system classified as the alpha system and used in these studies. Sequence comparisons done in *E. coli*

K12 show that K12 encodes both the alpha T2SS system and a truncated beta T2SS system. The beta system of *E. coli* K12 strain MG1655 only has *yghJ, pppA, yghG, gspC, gspL* and *gspM*, the replacement of the entire system with the beta T2SS system of *ETEC E. coli* would allow the characterization of secretion by the beta T2SS system by *E. coli* K12 strain MG1655, and thus a detailed study of the role of vesicles in secretion by the beta T2SS system.

It has previously been shown in our lab that little of the LT synthesized by ETEC *E. coli* is secreted from the bacteria (Rinesland et al., unpublished). Promoter mutants in which the beta T2SS system is made inducible need to be created. This would allow a determination of whether limited expression of this T2SS is responsible for the low efficiency of secretion as well as more detailed studies of the secretion mechanism.

Horstman and Kuehn (2000) have shown that the delivery of LT to mammalian cells is done through the delivery of vesicles. The release of vesicles has been shown through the induction of plasmids bearing the *gsp* operons in MC4100, a K12 strain that does not contain a functioning secretion system (Horstman and Kuehn, 2002). Vesicles can be an artifact of lysis as they are composed of outer membrane blebs. The blebs could have formed from the outer membrane being lysed in which periplasmic contents could be released including LT. Detailed studies on the secretion of vesicles between the alpha and beta T2SS would allow a characterization of secretion between these two systems.

Ast et al. (2002) showed that the ExeD secretin of *Aeromonas hydrophila* resides in the inner membrane if the ExeAB complex is not present, but also that this could be at least partially multimerized, especially if ExeD was overexpressed. Assembly of GspD in the inner

membrane is not associated with the secretion of proteins. The studies done here have shown that you can get multimerization of the $GspD_{\alpha}$ protein but the assembly of this protein has not been shown to take place in either the inner or the outer membrane. Studies should be done in which the membrane of these cells are fractionated. The separated inner and outer membranes when run on a gel could be used to detect if GspD is assembling in the inner membrane or if this secretion system component is being properly assembled in the outer membrane. If assembly was found to be in the inner membrane, it could lead to the identification of as of yet unknown factors involved in secretin assembly into the outer membrane.

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