Analysis of gate residues in the type 2 secretin PulD

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Without your wisdom, experience, and time, none of this would have been possible.
Insanity would have taken me much sooner…

Abstract

Secretins are gated outer-membrane channels with large internal pore sizes (6-10 nm). They are the outer membrane components of bacterial trans-envelope complexes that assemble/export filamentous bacteriophages as well as pili, complex protein toxins and virulence factors. 12-14 identical subunits form the radially symmetrical channels which share a common architecture - a 3-tiered barrel with middle septum. Secretins are essential components of Gram-negative Type 2/3 secretion systems, spanning the outer membrane and interacting with the inner membrane components of transport machinery. Since secretins have such large pore diameters a simple channel would allow noxious compounds through the normally impermeable outer membrane. The presence of a gate structure allows for the controlled opening and closing of secretin channels, in response to specific cues regulating protein export. Here I have determined gate-structural elements of the *Klebsiella oxytoca* Type 2 Secretin, PulD. Random mutagenesis coupled with selection for open or 'leaky'-gate phenotypes created a library of mutations which were mapped by DNA sequence analysis. Analysis of leaky mutants revealed 12 distinct missense point mutations in *pulD*. Additionally, two deletion mutants were isolated, spanning 5 and 9 amino acids, both conferring a leaky gate phenotype. Comparison of these *pulD* mutations with those previously identified in another secretin gene encoding the *Escherichia coli* filamentous phage f1 secretin pIV, reveals mutations in both are localised in two main clusters that correspond to regions within the secretin homology domain. Named GATE1 and GATE2, these clusters indicate functional gate regions in both secretins.

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1 Introduction

1.1 Secretion Systems of Gram-negative Bacteria

The Gram-negative bacterial outer membrane is an important barrier between the cell and its environment. Various lipoproteins and polysaccharides interweave to form a barrier through which few particles can pass. Despite this impenetrable structure, gram-negative bacteria still manage to secrete and import a wide variety of proteins and bulky molecules in order to modify and survive in their surrounding environment. It is not surprising then, that arrays of large molecular machineries or "secretion systems" have evolved to facilitate passage across the gram-negative inner and outer membranes. What is remarkable about these structures is that despite their complexity and size, their presence does not compromise the integrity of the cell wall.

Spanning both the inner and outer membrane, secretion systems are responsible for supporting the movement of large molecules across these barriers and assembly of filamentous surface-attached structures (pili) or filamentous phages. Four functionally and structurally diverse systems: Type 2 and 3 secretion systems, filamentous phage assembly system and Type 4 pilus system show much diversity in both structure and function. Despite their diversity it has been observed that a highly conserved outer membrane component is present in these systems. Known simply as "secretins", these large, multimeric outer membrane proteins are known to form a channel through which proteins and protein complexes are secreted across the outer membrane. Structural analysis of these secretins has revealed that they share similar attributes: Two rings intersected by an area of density otherwise known as the "gate". Furthermore, a large portion of the primary amino acid sequence has been conserved throughout these proteins. It has been suggested that the conserved nature of these channels may make it a lucrative target for the administration of anti-microbial agents (Mouratou et al., 2007).

1.2 Type 2 Secretion System

Present in a large number of gram-negative bacteria, the Type 2 secretion system (T2SS) has been implicated in many different bacterial survival and pathogenicity strategies. T2SS genes have been found across 15 different genera of γ-proteobacteria and a few α/δ-proteobacteria (Sandkvist, 2001). Mutation studies done on these proteins has implicated the T2SS with the secretion of a wide variety of proteins in different species (Cianciotto, 2005). For many of these species the T2SS is a critical component for survival in their particular environmental niche. Anaerobic denitrifiers (*Azoarcus* sp.) (Rossier et al., 2004), methanotrophic bacteria (*Methylococcus capsulatus*) (Ball et al., 2002), commensal plant bacteria (*Pseudomonas fluorescens*), even the inhabitants of deep-sea vents (*Idiomarina loihiensis*) are known to rely on the T2SS (Campos et al., 2010). In pathogenic bacteria of plants (*Erwinia carotovora, Xanthomonas campestris*) and animals/humans (*Legionella pneumophila, Pseudomonas aeruginosa*); and human pathogens (*Vibrio cholerae* and *Escherichia coli*) survival in the host as well as secretion of virulence factors was found to be dependent on T2SS (Marciano et al., 1999; Ridley, 2006; Schlumberger and Hardt, 2006; Minamino et al., 2010; Saijo-Hamano et al., 2010; Bange et al., 2011; Gray et al., 2011).

1.2.1 T2SS structure and function

The T2SS (general secretory pathway or Gsp) consists of inner membrane (IM) and outer membrane (OM) complexes. The IM complex spans the inner membrane and contains various anchor proteins (GspM, GspF, and GspL), ATPases (GspE), and a "pseudopilin" structure (GspG). The inner membrane complex is tethered to the OM complex through an additional periplasmic protein (GspC). The OM complex contains the secretin multimer (GspD) and an ancillary protein named the "pilotin" (GspS) which is responsible for targeting the secretin monomers to the outer membrane (not included in Fig.1; not all T2SS contain a pilotin). The proteins destined for secretions (secretion substrates) are first translocated across the IM Sec/Tat translocons (Voulhoux et al., 2001). Once they are in the periplasm, they fold and, if they are homo/heteromultimers, associate into mature form (e.g. CtxAB toxin). Folded substrates are specifically recognized by the inner membrane complex of cognate T2SS (Fig. 1). It has been theorized that the substrate protein is positioned at the tip of the GspG pseudopilin. Once this is achieved, the ATPases present at the base of the inner membrane complex

energize assembly of GspG monomers resulting in pseudopilin extension. This acts as a "piston", pushing the substrate towards the outer membrane to interact with the N-terminal domains of the GspD secretin. Interactions between the substrate and these N-domains induces a conformational change in the C-terminal "GATE" region of the secretin, opening the gate and allowing further extension of the pseudopilin where the substrate protein is "pushed" through the opening in the secretin (Johnson et al., 2006; Burkhardt et al., 2011; Jain et al., 2011).

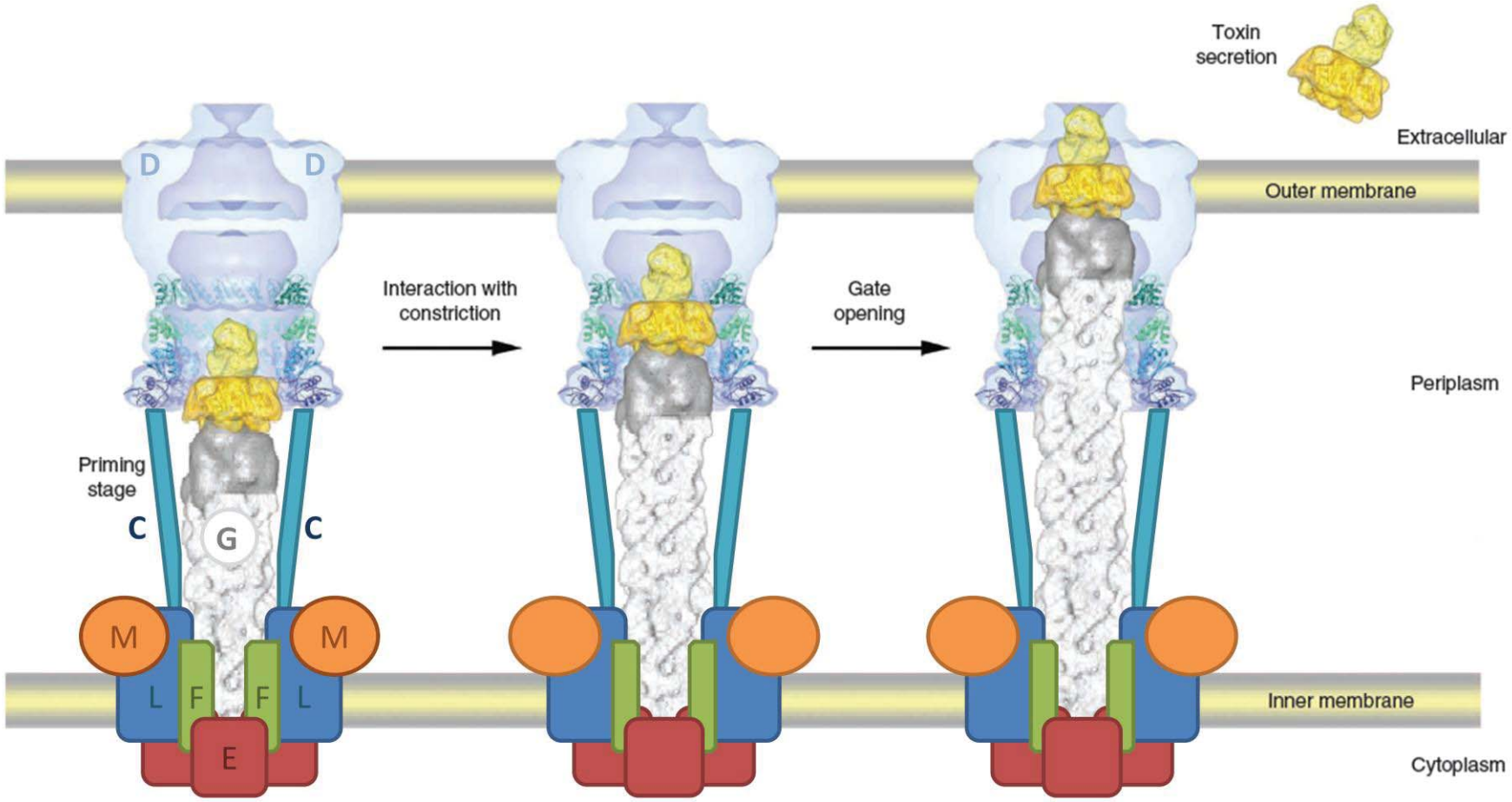

Figure 2. Proposed model of pilus-mediated Type 2 secretion of the cholera toxin AB_5 (yellow) via GspD. The inner membrane component: GspE ATPase (red), GspL (navy), GspF (green), GspG pseudopilin (grey). The periplasmic proteins: GspM (orange), and GspC (blue). The outer membrane component: GspD (teal). GspS pilotin not shown as it is not present in all T2S systems. Once translocated across the inner membrane by the tat system, AB_5 interacts with GspC and the GspG of the T2SS inner membrane component. Here GspC transmits a signal, via conformational change, to GspE. The GspE ATPase then energizes GspG assembly and pseudopilus extention until AB_5 is able to interact with the N-terminal domains of GspD (seen here as a constriction in the channel). At this point a conformational change occurs within GspD causing the GATE region of the protein to enter an opened state, allowing the pseudopilus to extend further pushing AB_5 through the GspD channel. Modified and reproduced with permission from Reichow *et al* (2010).

1.2.2 Type 2 secretin

Cryo-EM structural studies have been carried out on two T2 secretins (also collectively called GspD): EpsD of *Vibro cholerae* (Fig.2) and PulD of *Klebsiella oxytoca.* These proteins appear as multimers of 12 subunits arranged radially around a central axis to form a symmetrical "barrel" structure. The EpsD structure presented by Reichow *et al.* (2010) shows a distinct chamber inside the secretins outer membrane domain. This chamber appears continuous with a large opening at the outer membrane/extracellular end and constricted at the periplasmic end. At the centre of this chamber is the distinct high electron density region called the "gate" or "septum" or "plug" which splits the chamber into two sections. An "extracellular cap" has also been observed in EpsD (but not in PulD) which may contain a secondary extracellular gate (Figure 2a) (Reichow et al., 2010).

At the base of the secretin lay the periplasmic N-domains. Much of the variance between secretins among the homologues from T2SSs from different bacterial species is in the sequence. In different systems: T2SS, T3SS, filamentous phages and T4PS; not only the sequence, but also the number of these periplasmic N-domains differs (Fig. 3). It is also worth noting that the major portion of the secretin channel (~125Å of the total ~200Å in length) lies within the periplasm, much of it composed of the modular N-domains, which appear to be responsible for the many of the system-specific protein-protein interactions of the secretin with the IM complex.

Nouwen *et al.,* (1999) characterised the gating ability of PulD in an electrophysiological study. It was observed that PulD has some unique characteristics among secretins. The maximum conductance of PulD was observed as very low considering the size of the multimer's central channel. PulD exhibited rare and short-lived openings which increased in frequency and length with higher voltages and it was also observed that prolonged exposure to 80mV irreversibly opened the channel. The transition from opened to closed state was much more defined at negative voltages than positive and analysis in asymmetrical media indicated a weak selectivity for cations. These electrophysiological characteristics suggest a very tightly gated central pore and the small fluctuations observed were a result of relatively minor, voltage-induced changes in the structure of the central septum.

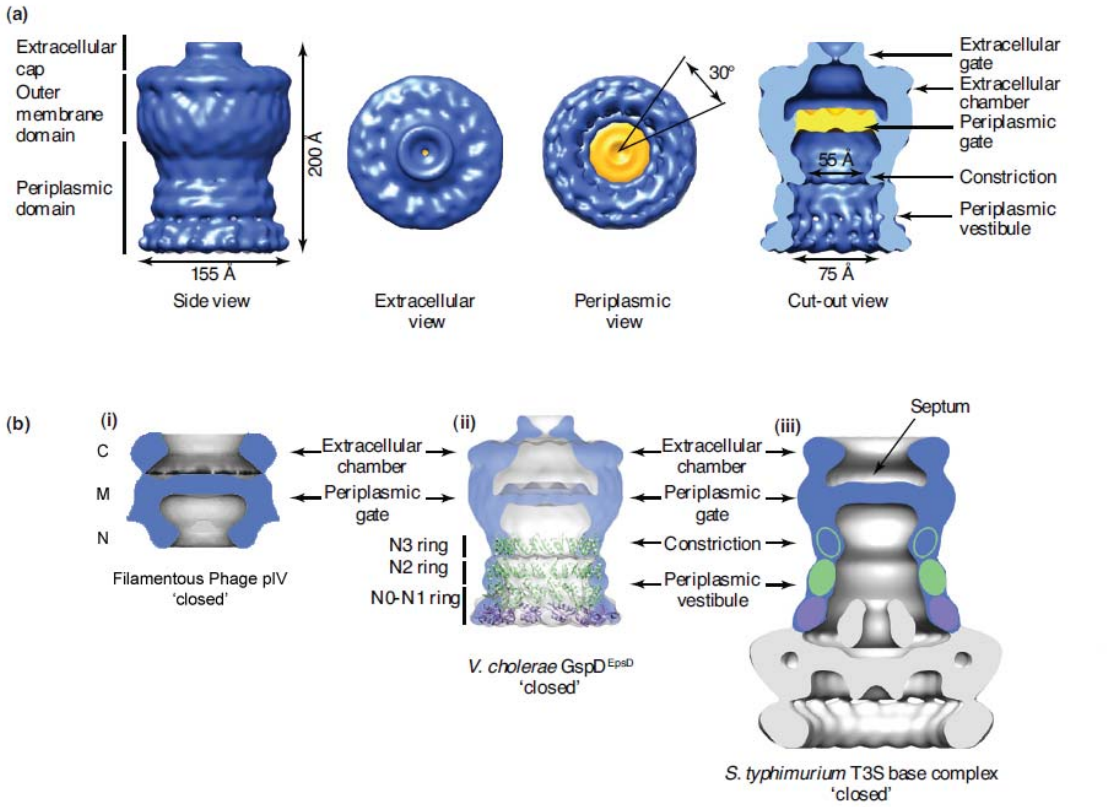

Figure 2. Structures of secretins. (a) Cryo-em reconstruction of *V. cholera* T2SS secretin GspD (or EpsD) **(Reichow et al., 2010)**. Side view, three distinct domains are evident in the EpsD structure: The extracellular cap, the outer membrane domain, and the periplasmic domain. Cross section, the central channel is intersected by a periplasmic gate (yellow) and contains an extracellular chamber and a periplasmic vestibule formed by secretin N-domains. (b) Secretin structure conserved between different secretion systems. (i) Density map of the filamentous phage protein pIV showing the N, M, and C rings. (ii) EpsD density map with fitted N-terminal domains (N0 domain in purple, N1-N3 domains in green). (iii) Reconstruction of the *S. typhimurium* T3SS complex. Secretin in blue containing modeled N-domains (N0, purple ovals; N1-2, green ovals; N3, open green ovals). Modified with permission from Korotkov *et al.* (2011).

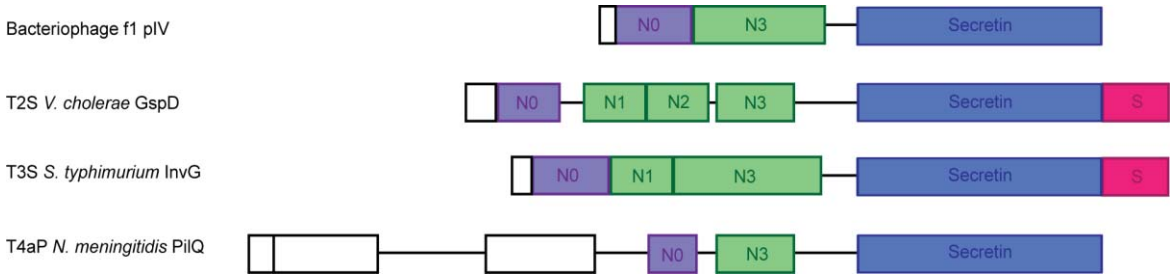

Figure 3. Domain organization of secretins. Modular domain organization of the secretins pIV (f1 bacteriophage), GspD (T2SS), InvG (T3S needle complex), PilQ (T4 pilus). Conserved "secretin homology domain"; blue, variable N domains; green and purple, pilotin signal sequence or "S domain" shown in pink (Note: not all T2 and T3 secretins contain S domains), periphery non-conserved domains shown in white.

1.3 Filamentous phage transport and assembly

Unlike many well-established viral modes of replication, the filamentous phage replication cycle and release of progeny does not compromise the host cell survival. The ability to safely exit host cells is due to the physical structure of the virus particles and a series of phage-encoded proteins which assemble and secrete phage particles rather than being released by inducing cell lysis.

Filamentous bacteriophages form long thin filaments, 6 nm in diameter and 900 nm in length. The phage genomes typically encode three secretion proteins which, when expressed, form a trans-envelope secretion complex. This complex is responsible for the final assembly of the phage coat proteins into the filament at the inner membrane, and extrusion of the phage filament through the outer membrane. This complex is composed of the three proteins. Two of those, pI (ATPase) and pXI, form the inner membrane channel/complex; the third protein, pIV, forms the outer membrane channel.

The outer membrane channel pIV shows much homology, both structurally and in primary sequence with bacterially encoded secretins and appears to operate in much the same manner. Some filamentous bacteriophages (e.g. Ctxφ of *V. cholerae* or MDE of *N. meningitidis*) do not encode a secretin channel, but rather "highjack" a bacterially-encoded secretin that normally works within the bacterial T2SS or Type IV pilus assembly system (Waldor and Mekalanos, 1996; Davis et al., 2000; Bille et al., 2005).

1.3.1 pIV

pIV facilitates the exit of filamentous phage particles without compromising the host's outer membrane integrity. ThepIV channel is a complex of fourteen identical monomers arranged around a central axis, forming a barrel-like structure consisting of three ring domains, 12 nm in length (Fig. 2b). The C-ring, named for its position at the C-terminal domain of each monomer, spans the outer membrane and contains a pore (8.8 nm in diameter). Below this structure lies a region of high density which has been termed the "M-ring" or middle-ring. This region presumably contains a gate which separates the two halves of the secretin central chamber and is thought to be largely responsible for maintaining OM integrity. Resting at the base of pIV, the N-terminal domain or N-ring contains a periplasmic opening, 6 nm in diameter, which interacts with the IM protein complex (pI/pXI)(Opalka et al., 2003).

The gating properties of purified pIV channel have been characterised by Marciano *et al.,* (1999). The author compared *E. coli* expressing pIV and a pIV mutant, S324G, which compromises the barrier function of the gate and gives a "leaky-gate" or "leaky" phenotype, under a number of growth conditions. The leaky pIV^{S324G} mutant rendered *E. coli* sensitive to the antibiotic Vancomycin, in comparison to *E. coli* expressing wild-type pIV, which was resistant. Outer membrane prevents translocation of this antibiotic through the outer membrane, and the wild-type pIV channel has a tightly closed gate which does not breach the outer membrane barrier. The pIV leaky mutant also allowed a strain of *E. coli* otherwise impermeable to a number of maltooligosaccharides (due to deletion of maltoporin LamB) to grow on maltotetraose, maltopentaose, or maltohexaose as the sole carbon source (Marciano et al., 1999).

Wild-type pIV and pIV^{S324G} were further characterised through electrophysiological assays. From these, it was observed that pIV and its mutant shared many characteristics: Both reconstituted into membranes with a common asymmetry, indicated by a larger conductance at positive voltages than negative; both exhibited selectivity for cations, both exhibited a higher probability of opening at higher voltages, and both were more likely to open at negative voltage. The chief difference observed between pIV and its mutant was the voltage at which each secretin was opened: At +80mV the pIV^{S324G} exhibited conductance comparable to wild-type pIV at +200mV. This shift in voltage dependence explained why pIV^{S324G} is permeable to large carbohydrates and confirmed pIV as a tightly gated channel. As an extremely large channel (8.8nm in diameter) an increase in probability of pIV being open would allow any number of large molecules to enter the cell.

As a follow-up to this work, Spagnuolo, *et al.* (2010) developed a method of selecting for pIV mutations which were permeable to maltopentaose. Random mutagenesis followed by this method of selection was used to identify a number of these "leaky" mutations, a majority of which appeared to cluster in two distinct regions. Named "GATE1" and "GATE2" these represent two regions of amino acids responsible for the selective permeability or "gating function" of pIV. More significantly, these two GATE regions lie within the "secretin homology domain", a domain which is shared across all secretin types. This may indicate a possible conserved domain function across the secretin family (Spagnuolo et al., 2010).

1.4 Type 3 secretion system

Known as the "bacterial injectosome", the T3SS is a specialized needle-like secretion system capable of directly depositing (or injecting) effectors into the cytoplasm of a target host cell. This direct method of transportation has allowed many pathogens and symbiotic bacteria to quickly and efficiently deliver proteins to modulate target cell function. Many well-known pathogens utilize this method of virulence, including *Salmonella,* pathogenic *E. coli, Yersinia, Bordetella, Pseudomonas,* and *Shigella* (Hapfelmeier et al., 2005; Carnell et al., 2007; Coburn et al., 2007; Abe et al., 2008). This process shares much in common with the assembly apparatus of the bacterial flagella whose inner membrane ATPase components are homologous to that of the injectosome (Minamino et al., 2010; Saijo-Hamano et al., 2010; Bange et al., 2011).

1.4.1 The Injectosome

The injectosome is a massive nano-machine which forms a direct link between the cytoplasms of bacterial and host cells. Composed of more than 20 proteins, the injectosome bypasses the inner and outer bacterial membranes by forming a channel in much the same manner as other secretion systems. The core of this structure is known as "the basal body" and contains the channel though which proteins are translocated across the bacterial inner and outer membranes (Figure 2b). This basal body has a three-tiered structure where each tier corresponds to different portions of the overall channel (i.e. one tier in the IM, one in the periplasm, and one in the OM). The OM portion of this basal body is a secretin channel (Fig. 2b). A rod-like structure that is assembled within the basal body, called the needle, is constructed by the helical polymerization of several hundred subunits (Marlovits *et al.*, 2004). This needle extends through the central channel of the basal body and out into the extracellular environment. Two proteins which form a pore upon interacting with a eukaryotic membrane are secreted through the needle upon contact with the host cells. It is through this molecular needle that virulence factors can be injected directly inside a eukaryotic cell.

A striking example of the T3SS in action is the invasion and subversion of the host eukaryotic cell cytoskeleton by *Salmonella enterica. Salmonella* encodes two T3SS on two *Salmonella* pathogenicity islands known as SPI1 and SPI2. The SPI1 T3SS is responsible for assisting the invasion of nonphagocytic cells of the intestinal epithelium.

The SPI1 T3SS penetrates the membranes of these cells and secretes a number of effector proteins which activate Cdc42, Rac, and Rho GTPase by either mimicking guanidine nucleotide exchange factors or generation of secondary messengers (Zhou and Galan, 2001; Schlumberger and Hardt, 2006). Activation of a series of signalling pathways ultimately induces the formation of actin-rich membrane ruffles that engulf the *Salmonella* cell (Ridley, 2006). Once safely inside the host, *Salmonella* is sheltered from the host immune system where it may replicate and spread. To facilitate this further, another effector, SptP, is secreted and acts as a GTPase-activated protein to inactivate Rac and Cdc42 so that the host cells cytoskeleton reverts to pre-invasion state (Gruenheid and Finlay, 2003).

Once *Salmonella* is inside the cell, the second SPI2 T3SS is expressed. Effector proteins are then secreted across the vacuole membrane to interfere with phagosome maturation by preventing the delivery of hydrolytic enzymes and inducing the assembly of an actin coat around the membrane-encased bacteria to form a *Salmonella*-containing vacuole. This vacuole is then transported along microtubules towards the perinuclear region by recruitment of the motor protein dynein facilitated by the two effector proteins SseF and SseG (Abrahams et al., 2006). Once correctly positioned, *Salmonella* can begin to manipulate cellular trafficking processes providing access to a supply of nutrients and membrane materials for maintenance of vacuoles and replication.

1.4.2 Type 3 secretin

The secretin is an integral part of the T3SS basal complex, which is an extremely stable structure (in contrast to T2SS and filamentous phage system). Given the stability of the basal body in its intact form, much of the structural characterization has been done on the complete basal complex (Marlovits et al., 2004). Cryo-EM of the *Salmonella typhimurium* basal complex revealed architecture similar to that of the T2S GspD and filamentous phage secretin pIV: a three-ringed structure containing a central pore which has been interrupted by a region of high density much like the septum in GspD and pIV (Figure 2b). Comparison of the basal body structure with that of an assembled needle complex (that includes the assembled needle) highlighted drastic structural changes in the InvG secretin upon needle assembly. The outermost ring appeared to move in an outward motion to accommodate the extending needle structure; furthermore, the inner septum appeared to be dislodged in order to accommodate the needle which traverses

the channel. The structural changes also provide two regions which appear to act as a scaffold for the extending needle, one at the outermost ring of the secretin and another where the central septum was situated.

Electrophysiological studies carried out on another type 3 secretin, YscC, had suggested a weakly gated channel (Hansen and Forest, 2006). It was observed that YscC conductance remained unchanged with variation in both positive and negative voltage (0 mV to 200mV) indicating opening but not closing of the central pore. This characteristic was unique to the YscC secretin as the type 2 PulD and filamentous phage pIV both exhibited nonlinear variation in conductance at different voltages consistent with the opening and closing of a channel. The difference between YscC and other secretins may have been due to the YscC being a component of the T3SS which forms a stable complex and proper gating function requires the presence of additional proteins that belong to this complex.

Although YscC exhibited "leaky" phenotype in electrophysiological experimentation, when YscC was assayed for *in vivo* pore formation, the expression of *yscC* and its pilotin *yscW* did not appear to permeabilize the outer membrane, suggesting that *in vivo* YscC is present in a closed state in the absence of the rest of the T3SS machinery (Burghout et al., 2004).

1.5 Type 4 pilus

The type 4 pili are large fibrous structures which extend out from the surface of the bacteria and assist in a variety of bacterial functions, including: adhesion (Virji, 2009), motility (Merz et al., 2000), biofilm formation (Klausen et al., 2003) and DNA uptake (Hamilton and Dillard, 2006). These functions also aid the infection of host cells in many well-known Gram-negative pathogens such as, *Neisseria gonorrhoeae*, *Neisseria meningitides* (Virji, 2009), *Escherichia coli* (Levine et al., 1985), and *Vibrio cholera* (Herrington et al., 1988; Craig et al., 2004). A striking feature of the type 4 pilus is the rapid extentsion and retraction of the filament. A large trans-membrane complex has been revealed as responsible for the assembly and *de*-assembly of this filament: the type 4 pili secretin complex (Craig and Li, 2008). This complex bares much resemblance to those of the type 2 secretion system (Craig et al., 2004; Hansen and Forest, 2006; Johnson et al., 2006).

1.5.1 Type 4 pili assembly complex

While little is known of the inner and outer membrane spanning complex, chemical crosslinking and affinity chromatography have identified a number of proteins involved in this complex including: ATPases, integral inner membrane proteins, an outer membrane secretin, pilin subunits, and a number of other proteins involved in pilus assembly (Hwang et al., 2003).

The inner membrane component spans the IM protruding into the cytoplasm and periplasm. A low resolution EM reconstruction of the *N. meningitidis* inner membrane protein PilG revealed a bullet-shaped structure with four-fold symmetry. The tip of the protein appeared to extend into the cytoplasm where it is thought to interact with the ATPases responsible for pilus assembly (Collins et al., 2007; Craig and Li, 2008). From this IM complex, the pilus is assembled and extended as pilin subunits are diffused through the IM and encounter the assembly complex. The pilin subunit interacts and docks with the base of the growing pilus and conformational changes in the IM complex mediated by cytoplasmic ATPases force the pilus out of the IM opening up a new gap in which a new pilin subunit can dock(Craig and Li, 2008; Campos et al., 2010).

At the OM, the pilus interacts with the OM secretin component. The type 4 pilus secretin provides the gated channel through which the pilus may extend out from the cell curface.

1.5.2 Type 4 pilus secretin

Electron microscopic analysis of the type 4 secretin PilQ from *Neisseria gonorrhoeae* and *Thermus thermophilus* have revealed a surprising variant of the secretin multimer: the *Thermus thermophilus* PilQ secretin exhibited a width consistent with other secretins (15 nm) but a unique length (34 nm). It is thought that this length is to accommodate the thick cell wall layers of *T. thermophilus* which is known to have a diameter of 40 nm. The same multimer treated with SDS however, exhibited an SDS-resistant structure similar to the three-tiered secretins found in other systems: a round barrel structure with a large central 6 nm channel intersected with a region of high density or a septum. It was below this structure that 5 distinct rings are observed in the untreated multimer. These rings extend deep into the periplasm and may interact with the IM components of the type 4 pilus assembly complex (Burkhardt et al., 2011). Further EM imaging of the *Neisseria gonorrhoeae* PilQ have revealed that the secretin complex exhibits a 14-fold symmetry (Jain et al., 2011).

1.5.3 Type 4 pilin subunit

The type 4 pili subunits share three distinct characteristics across all species: an *N*-methylated N-terminus, 25 conserved N-terminal hydrophobic residues, and a C-terminal disulphide bond (Strom and Lory, 1993). The pili are classified into two groups based on differences in primary sequence and length: type IVa and IVb. Type IVa pilins are shorter and more conserved than IVb and are found in many Gram-negative bacteria that have a broad host range (Craig et al., 2004). Whereas type IVb pili are found only in Gram-native bacteria that inhabit the human intestine such as: *V. cholerae* (Shaw and Taylor, 1990), *Salmonella enterica* Typhi (Zhang et al., 2000), and enetropathogenic *E. coli* (Donnenberg et al., 1992).

Each pilin subunits exibits a similar structure: an extended N-terminal α-helical backbone 85 Å in length. The N-terminal half of this helix forms the hydrophobic helical bundle in the core of the filament around which the subunits assemble (Craig et al., 2004). The remaining half is amphipathic and interacts with a globular head. Two

proline residues (at residues 22 and 42) induce subtle kinks in the overall structure of the helix giving it a slight bend. It is thought that this assists in the tight packing of the subunits into the filament by providing flexibility. Above this large helix sits the pilin globular domain which contains a four-stranded anti-parallel β-sheet that creates a hydrophobic core with the N-terminal α-helix forming an αβ-roll. Flanking the β-sheet are two regions which show significant structural and sequence variation (Craig et al., 2004). These regions form the outer edges of the globular domain and appear to facilitate the interactions between pilin subunits and determine the surface chemistry of the filament. The variation seen in this domain can account for the diverse functions of the type 4 pili (Craig et al., 2004).

Similar pilin proteins have also been identified in the *pul* genes of the *K. oxytoca* type 2 secretion system (Filloux, 2004). Five of the 12 genes products (*pulG, pulH, pulL, pulJ, and pulK)* have been described as type 4 pilin-like proteins and have been named pseudopilins. These proteins share extensive conservation in the 30 N-terminal residues which are processed by a type IV prepilin peptidase (Dupuy et al., 1992). Since these proteins share sequence and are processed in the same manner as type 4 pilins it has been proposed that they form a pilus-like structure or pseudopilus. It has been suggested that this pseudopilus acts as a molecular piston to push the substrate protein through the type 2 secretin (Filloux, 2004).

The crystallographic structure of one of these pseudopilin proteins, PulG, shows a characteristic αβ-roll with an antiparallel β-sheet packed against a large α-helix, much like that seen in the type 4 pili. However, PulG contains shorter β-strands, differences in the αβ-region, and appears to lack the larger exposed epitopes the type 4 pili use for substrate specificity (Kohler et al., 2004; Campos et al., 2010). This appears to be due to PulG not having any external receptor or interaction with a host immune response. Overexpression of PulG in conjunction with the *pul* secretion genes has resulted in the production of pilus-like bundles further highlighting the relationship between PulG and type 4 pili (Sauvonnet et al., 2000).

1.6 Aims and significance

The OM secretins of the T2SS, T3SS, and filamentous phage assembly system have shown remarkable homology in the terms of their amino acid sequence and low-resolution structure. The identification of key regions responsible for the gating function of the pIV filamentous phage secretin has posed an intriguing question: Are these regions conserved throughout the secretin families?

The aim of this research has been to answer that question for the T2SS secretin PulD. Since these secretion systems are so widely used in Gram-negative bacteria, functional and structural similarity in gating for both pIV and PulD may have far reaching implications for bacterial processes in which the type II secretion systems are involved. Understanding how T2SS secretins work may lead to a greater understanding of these molecular processes and in doing so we may be able to identify specific weaknesses in the structure or function of secretins which may be a target for antimicrobial agents.

2 Materials and Methods

2.1 Bacterial Strains and Plasmids

Details of bacterial strains and plasmids used in this study are given in Table 1 and Table 2, respectively.

Table 1: List of *E. coli* strains

Strain	Parent	Genotype	Source
XL1-Red		F$^-$ *endA1 gyrA96*(nal^R) *thi-1 relA1 lac glnV44 hsdR17(*rK-mK+) *mutS mutT mutD5* Tn10	Stratagene
XL1-Blue		*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^q ZΔM15 Tn10* (Tet^R)	Stratagene
K1508	MC1400	F- *araD$^-$ lacΔ* U169 *relA- thi-rpsL* (Strep^R) Δ*lamB106*	(Marciano et al., 1999)
K2204	TG-1	TG-1 *ΔlamB106*	P1 transduction from K1508 (J. Spagnuolo; unpublished)

Table 2: List of plasmids used

Name	Expressed protein	Resistance marker	Reference
pGZ119EH	-	Cm	(Lessl et al., 1992)
pCHAP362	PulD	Cm	(d'Enfert et al., 1989)
pAH181	PulS	Km	(Daefler et al., 1997)
pPMR132	pIV	Cm	(Russel, 1994)
pPMR132^{S324G}	pIV ^{S324G}	Cm	(Russel, 1994)

* Cm, Chloramphenicol; Km, Kanamycin

2.2 Media

Minimal medium M63 (Miller, 1972) supplemented with Thiamine (15 mg/mL) and Biotin (2 µg/mL); maltopentaose (0.2%) was as a sole carbon source in the selection experiments. Medium was further supplemented with appropriate antibiotics: Chloramphenicol (25 µg/mL) for growth of cells containing plasmids pCHAP362 pGZ119EH, and Kanamycin (25 µg/mL) for growth of cells containing plasmid pAH181). Expression of PulD was induced with 0.1 mM IPTG. 2xYT rich medium (Sambrook et al., 1989) was purchased from BD and supplemented with the appropriate antibiotics.

2.3 Transformation

Chemically competent cells K1508 and K2204, prepared according to (Maniatis *et al.*, 1982) were used in routine transformation of plasmids listed in Table 2. Mutant libraries (RCA ligations and plasmids isolated from XL1-Red mutator strain) were transformed into K2204 electrocompetent cells (Maniatis et al., 1982). Chemically competent cells of XL1-Red mutator strain were purchased from Stratagene.

2.4 *In vitro* ϕ-29 DNA polymerase-mediated random mutagenesis

Rolling circle amplification (RCA) utilises the phage ϕ-29 DNA polymerase with NNNNNN primers. To achieve error-prone (mutagenic) amplification, the reactions included $MnCl_2$, which promotes the accumulation of errors (Fujii et al., 2004, 2006). The polymerase and hexameric primers were supplied in the TempliPhi kit (GE, Amersham).

To set up a polymerisation reaction, 0.5 µL of 30 pM template DNA (pCHAP362) was added to 5.0 µL of Sample Buffer (TempliPhi kit) containing hexameric primers. The solution was then heated, in a PCR machine, to 95^{o}C for 3 minutes to denature the plasmid and then cooled quickly to 4^{o}C to anneal the hexameric primers. 1 µL of 10 mM $MnCl_2$, 5 µL of Reaction buffer (TempliPhi kit), and 0.2µL of Enzyme mix (TempliPhi kit) was added to the template DNA solution and deionised water to 10 µL. This mixture was then incubated at 30^{o}C for 24 hours. The reaction was then stopped by heat-inactivation at 65^{o}C for 10 minutes.

The replicated DNA was purified using a PCR purification kit (Roche), diluted 5-fold. The product of ϕ-29 DNA polymerase is a large branched dendromer of many products linked together. To separate this product into plasmid monomers, it was digested for 18 hours at 37°C using 5 units of restriction endonuclease *Nco*I to ensure complete linearization of the replicated plasmid DNA. *Nco*I was then heat-inactivated at 65°C for 10 minutes, followed by ligation of 50 ng of linear DNA in a 50 µL ligation reaction containing 2 Units of T4 ligase.

2.4 *In vivo* random mutagenesis

Mutator strain XL1-Red (commercial chemically competent cells; Stratagene) was transformed with both pCHAP362 (expressing *pulD*) and pAH181 (expressing *pulS*) and recovered in 1mL of SOC media for 45 minutes at 37°C. The SOC-recovered culture was then split between two tubes, one containing 2 mL of Chloramphenicol (25 µg/mL) and one containing 2 mL of both Chloramphenicol (25 µg/mL) and Kanamycin (25 µg/mL). 1 mL of these diluted cultures was removed and plated on two 2xYT media plates containing the same antibiotic(s); one was incubated for 18 hours at 37°C and the other at 30°C. The remaining two liquid cultures were incubated overnight at 37°C, then the plasmid DNA was purified from them.

2.5 Selection of maltopentaose-permeable mutants

Ligation mixture from the *in vitro* mutagenesis or two libraries of plasmid DNA from *in vivo* mutagenesis were then electroporated into the Δ*lamB* deletion *E. coli* strain K2204 carrying the pAH181 (*pulS*-expressing) plasmid. The resulting transformant culture was then washed twice in saline solution (0.9% NaCl). Cells were pelleted by centrifugation, supernatant was removed, and cells were re-suspended in an equal volume of saline solution. This washed cell culture was then plated on maltopentaose selection plates (100 µL undiluted) and 2xYT media (100 µL 10^{-3} dilution) and incubated for 36 hours (maltopentaose) or 18 hours (2xYT media) at 37°C. A damp paper towel was included in the incubation container to ensure the maltopentaose media did not dry out during prolonged incubation. The number of colonies grown on each of the plates was counted and frequency of leaky mutations was calculated as a ratio of this number to the number of transformant colonies on the rich medium.

Each of the colonies that had grown on the maltopentaose media were numbered and passaged onto fresh maltopentaose and 2xYT plates to ensure pure cultures remained viable and were the result of leaky mutations, rather than satellite colonies. Each of the colonies was cultured separately and plasmid DNA was isolated using a plasmid preparation kit (Roche). The pulD insert was PCR-amplified and submited for DNA sequencing. The corresponding transformants were subjected to phenotypic characterization (sensitivity to Bac, Van and DOC).

2.6 Sequencing

In order to completely sequence the *pulD* genes present on the pCHAP362 plasmid, four sequencing primers were designed roughly 500 bp apart across the whole gene. Due to the large number of sequencing reactions required to fully sequence every leaky mutant generated (Table 3); sequencing reactions were carried out in 96-well plates using the BigDye™ PCR reaction. The PCR product was then processed by the Alan Wilson Genome Centre and the resulting sequences were analysed using Geneious Pro sequence analysis software (Drummond et al., 2010).

Table 3: List of primers used

Name	Sequence	Position
pCHAP362 1st Forward	5'-GCCTCGGTGAGCATGGCGTT-3'	1,571-1,590
pCHAP362 2nd Forward	5'-AGGCCTGGCGGTACTCGTCG-3'	2,114-2,133
pCHAP362 6th Forward	5'-ACAGCATCGCCCAGTTGCCC-3'	2,642-2,661
pCHAP362 4th Reverse	5'-TCACTCATTAGGCACCCCAGGCT-3'	4,240-4,218

2.7 Antibiotic E-test assays

3mL tubes of 2xYT media were inoculated with each of the strains of interest and grown overnight at 37°C. 100 µL of overnight culture was spread upon 2xYT plates containing selective antibiotics (Chloramphenicol and/or Kanamycin 25µg/mL) and IPTG (0.1mM) and allowed to dry. Vancomycin and Bacitracin (0.17µg to 256µg) E-

test strips (AB Biodisk) were aseptically placed upon the inoculated plates, and incubated at 37^{o}C for 18 hours.

After incubation each of the strains were assessed for antibiotic sensitivity. Often, two distinct measurements were observed from each of the E-test plates: The "halo" or region in which confluent growth is less dense, and the "clearing" or region in which no growth is observed. Due to differences between the two *E. coli* strains K2204 and K1508, the measurements taken from the E-test strips were the "halo" observed with K2204 which corresponds to the "clearing" observed with K1508. The minimal inhibitory concentration (MIC) is measured by the point at which the region of "halo" or "clearing" intersects with the E-test strip scale.

2.8 Plating Efficiency Assays

Tubes containing 3mL of 2xYT media containing the appropriate antibiotics were inoculated with the bacterial strains of interest and incubated overnight. Each of the overnight cultures was then serially diluted to 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}.

2xYT agar was prepared containing Chloramphenicol (25 μg/mL) and/or Kanamycin (25 μg/mL) to allow maintenance of PulD- and PulS-expressing plasmids, IPTG (0.1 mM), and either Bacitracin (50 μg/mL), Vancomycin (50μg/mL), Deoxycholate (0.4%), or no additional antibiotic.

For each of analysed bacterial strains, four dilutions of overnight culture (10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) were spread on these plates. The plates were then incubated for 18 hours at 37^{o}C. Plating efficiency was calculated as number of colonies on each of the Bacitracin, Vancomycin, and Deoxycholate plates divided by the number of colonies present on the plate containing none of these compounds.

3 Results

3.1 Isolation of spontaneous PulD leaky mutants

To ensure PulD was a suitable subject for mutagenesis experimentation, *E. coli* K1508 (MC4100 Δ*lamB*) cells containing plasmids bearing *pulD, pulS,* and previously characterised pIV leaky S324G mutant. Resistance of cells expressing PulD, PulS and both PulD and PulS to the antibiotics Bacitracin (Bac) and Vancomycin (Van) and the detergent sodium deoxycholate (Doc) was tested to confirm that the PulD channel preserves the integrity of outer membrane. Resistance was measured by determining efficiency in the presence of Van, Bac or Doc relative to plating in the absence of these compounds (Section 2.8). Compared to growth without Van, Bac or Doc, cells expressing PulD, PulS, or both proteins, as well as an empty vector pGZ119EEH (instead of pulD-expressing plasmid), showed little to no sensitivity to these antimicrobials (data not shown). Interestingly, cells expressing pIV had a slight decrease in colony count when compared to that of cells expressing PulD and PulS indicating a naturally leaky gate (under these conditions). The cells expressing leaky pIV mutant S324G, however, had a marked reduction in colony count, showing the plating efficiency of 4.3×10^{-5} in the presence of Van, 4.3×10^{-5} in the presence of Bac, and 6.5×10^{-5} in the presence of Doc. Therefore, the expression of wild type PulD and PulS did not sensitize *E. coli* to these antimicrobial agents. It has been reported that PulD is only targeted to the outer membrane in the presence of pilotin PulS. Therefore, only the strains expressing both proteins are expected to contain PulD channels in their outer membranes.

The selection process for this mutagenesis experiment requires that the secretin being expressed is not permeable to the large polysaccharide maltopentaose. To test for this permeability, *E. coli* K1508 (Δ*lamB*) expressing PulD, PulS both PulD andPulS, empty vector pGZ119EH , wild-type pIV, and pIV S324G leaky mutant were plated on M63 minimal medium containing either maltopentaose or maltose as a sole carbon source and incubated for 48 hours. All strains showed confluent growth on maltose monomer, which is expected since the general porins OmpF and OmpC, constitutively produced by *E. coli*, allow entry of this monosaccharide. Growth on maltopentaose,

oligosaccharide which requires a LamB maltoporin to cross the outer membrane, was tested to assess the frequency of spontaneous leaky mutants. Appearance of rare spontaneous maltopentaose-permeable mutants would confirm that PulD has been integrated correctly in the outer membrane and therefore could be mutated to acquire a leaky phenotype. Cultures expressing PulD or PulS alone, or the vector control did not show growth on maltopentaose. The leaky pIV S324G exhibited significant growth as a confluent lawn on the plate indicating permeability to maltopentaose. Wild type pIV did not grow as a lawn; instead a small number of maltopentaose- colonies appeared. The only other strain that allowed appearance of these spontaneous maltopentaose-permeable mutations is the strain that produces both PulD secretin and PulS pilotin, required to target PulD to the outer membrane. In the case of pIV, no accessory proteins are required for its targeting to the OM, hence expression of this secretin alone is sufficient to form a channel in the outer membrane. Consistently, spontaneous maltopentaose-permeable mutants were viable (Marciano, 1999).

Thirteen spontaneous PulD mutants were clonally purified by passaging onto a fresh maltopentaose plate. DNA sequencing of *pulD* from thirteen spontaneous PulD leaky mutants revealed four distinct mutations (Table 4). Of those, three were missense mutations: G458A, G466C, and I475T; and one was an in-frame deletion mutant: Δ477-480 (Figure 5b). All mutations were located within the region corresponding to the C-terminal secretin homology domain. Mapping PulD mutations to a PulD-pIV alignment showed that the spontaneous maltopentaose-permeable PulD mutations were all located in a region of PulD that corresponds to that of the pIV GATE1 (Figure 6).

It had been previously established that expression of the leaky pIV S324G mutant in *E. coli* K1508 sensitized the organism to Bacitracin, Vancomycin, and Deoxycholate (Spagnuolo et al., 2010). To determine if the spontaneous PulD mutants exhibited a similar phenotype, *E. coli* K1508 expressing these mutants were tested for susceptibility to these compounds (Table 4). Mutant G466C showed sensitivity to Deoxycholate, Vancomycin, and Bacitracin. I475T showed significant sensitivity to Vancomycin, a slight sensitivity to Bacitracin and complete resistance to Deoxycholate. G458A exhibited sensitivity to Vancomycin, slight sensitivity to Deoxycholate, and Bacitracin. The Δ477-480 deletion mutant displayed the most striking phenotype; highly sensitive to both Vancomycin and Deoxycholate with moderate sensitivity to Bacitracin (Table 4).

The resistance of wild-type PulD expressing *E. coli* to these antimicrobial agents and the spontaneous generation of leaky PulD mutants on maltopentaose selective medium confirmed the suitability of PulD for further mutagenesis and characterization. The resistance profiles of spontaneous mutants to Vancomycin, Bacitracin, and Deoxycholate also indicated that maltopentaose-permeable mutants are leaky-gate mutants who allow passage of several large molecules which cannot cross the outer membrane or wild-type secretin. To provide a more detailed map of gating regions in PulD, libraries of random mutants of *pulD* gene were generated and selected for growth on maltopentaose as a sole carbon source.

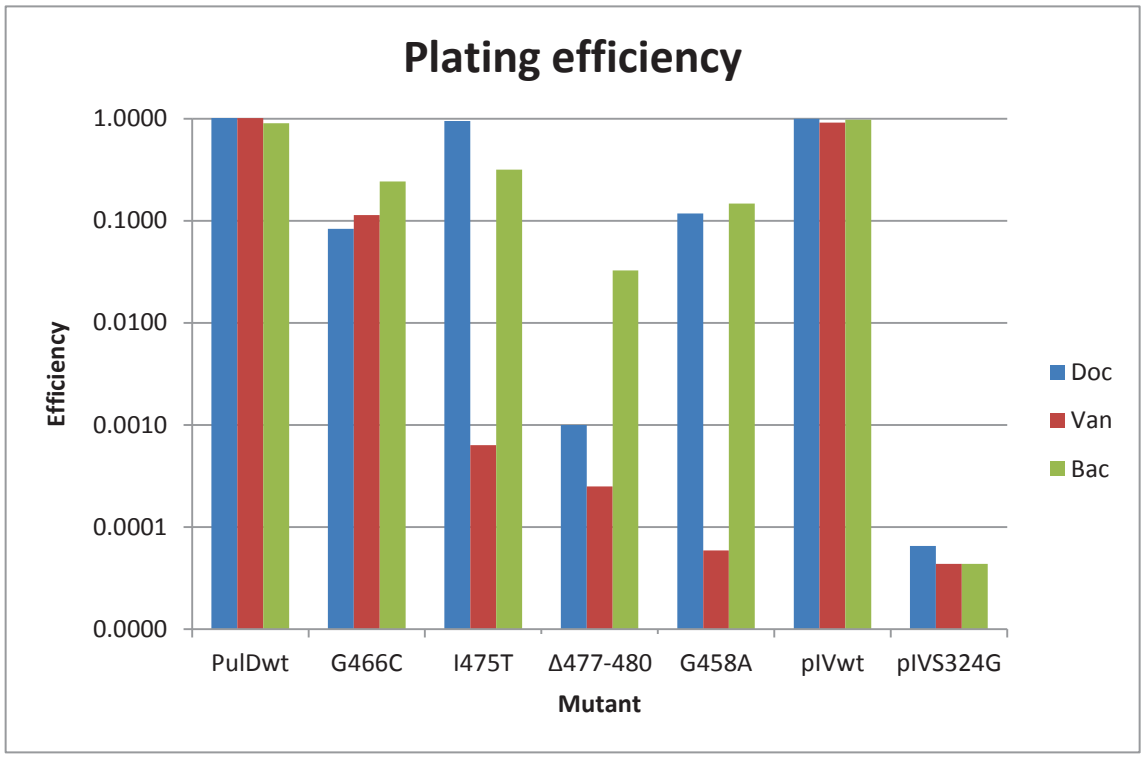

Figure 4: Plating efficiency of spontaneous mutants. Efficiency of plating was calculated as a ratio of the number of colony forming units in the presence relative to the number obtained in the absence of each of the compounds.

3.2 *In vitro* mutagenesis

One way to generate libraries of random mutations in a gene is *in vitro* replication under error-prone conditions. Reportedly, phage polymerase ϕ-29 generates equal numbers of transitions and transversions in the presence of $MnCl_2$. In a preliminary experiment using lacZ of plasmid pUC18 as a target, the $MnCl_2$ concentration (10 mM) that gave the same frequency of $lacZ^-$ mutants as *in vivo* mutagenesis was determined (J. Spagnuolo, unpublished) (Fujii et al., 2004, 2006). The *in vitro* mutagenesis had a perceived advantage over *in vivo* mutagenesis because of observed lethality of some pIV mutants in the mutator strain XL1-Red (Spagnuolo et al., 2010). *In vitro* mutagenesis, which bypasses transformation of a mutator strain, was expected to allow isolation of more severely leaky mutants than *in vivo* mutagenesis.

To generate mutant libraries, 10 amplification reactions were carried out (Section 2.3). The resulting amplification product was processed (Section 2.3) and used to transform *E. coli* strain K2204 (a Δ*LamB* strain), which is incapable of importing large maltodextrins. The resulting culture was washed with saline to remove all remaining nutrients and plated on M63 minimal media containing maltopentaose to select for leaky mutants.

Little growth was observed after 3 days of incubation. Any colonies that were present remained very small and did not appear to grow any further after a fourth day of incubation. 30 of these colonies were isolated and plasmid DNA was isolated and then sequenced.

12 of the 21 maltopentaose-permeable mutants had a wild type *pulD* and 9 contained one or more point mutations. Of the 9 sequences containing mutations, 6 contained multiple nucleotide changes. Of those, 4 mutants contained multiple amino acid changes, while 2 had only one amino acid change combined with a silent nucleotide changes (Table 4). When mapped on the PulD sequence, the few missense mutations appeared spread across the whole PulD sequence with no apparent clustering.

However, due to the rate at which these mutants grew on M63 minimal media containing maltopentaose and the frequency of multiple mutations it became evident that this particular method of mutagenesis was not producing true leaky mutants, rather it is likely that a separate event within the K2204 strain occurred which allowed very

slow growth on maltopentaose and the mutations present in the *pulD* plasmids were found due to a high frequency of mutations. This set of mutants was not pursued further; instead in vivo mutagenesis, which was successfully used in pIV gate mapping, was undertaken.

3.3 *In vivo* mutagenesis and mapping of the PulD gate

XL-1 Red Mutagenesis employs the error-prone replication of the *E. coli* strain XL-1 Red. This particular strain lacks a variety of DNA repair pathways and is prone to accumulating errors at a predictable rate. Here a mutant library can be easily generated by transforming XL-1 with the desired plasmid, incubating in liquid culture for a select period of time, and isolating the plasmid DNA.

XL-1 Red was transformed with both plasmids encoding PulD and PulS, to mitigate potentially lethal effect of the PulD protein inserting into the IM of the strain (Guilvout *et al.*, 2006) (the inclusion of PulS ensures correct translocation into the OM). The resulting transformants were then inoculated into two tubes containing 2xYT liquid media: one containing only Chloramphenicol required for *pulD* containing plasmid and one containing both Chloramphenicol and Kanamycin for both *pulD* and *pulS* containing plasmids. Single antibiotic culture aimed to capture pCHAP362 transformants only, in case this is more beneficial to cell survival. Two plasmid preparations from these two transformed cultures represent two libraries of PulD mutants: one obtained in the presence and one in the absence of PulS. After transformation of the a Δ*LamB* strain K2204, the maltopentaose selective plates contained much larger and more numerous colonies than those observed after *in vitro* mutagenesis.

64 colonies were clonally purified, 32 from each transformation, by passaging on fresh maltopentaose selective plate, taking care to choose representatives of every shape and size. Plasmid DNA was isolated from these clones and *pulD* was sequenced in each of them. Of the 64 sequenced mutants, 11 distinct mutations were discovered. 8 new missense mutations: E334K, A359V, L431F, G458S, S467L, T469A, R529L, and G547S; one double missense mutation: V511M + D521N; and one deletion Δ513-522 (Figure 5b). G458S was the most frequently isolated mutant occurring in 9 maltopentaose-positive transformants (Table 4).

Mutant	Nucleotide change	Van MIC[1]	Bac MIC[1]	Number of Isolates	Mutagenesis method
E334K	G1000A	1	24	1	XL1R[4]
A359V	C1076T	1.5	16	1	XL1R[4]
L431F	C1291T	1	32	1	XL1R[4]
G458S	G1372A	1	8	9	XL1R[4]
G458A	G1373C	1.5	12	4	Spont[3]+XL1R[4]
G466C	G1396C	128	R	1	Spont[3]
S467L	C1400T	1	12	1	XL1R[4]
T469A	A1405G	12	R	8	XL1R[4]
I475T	T1424C	6	R	1	Spont[3]
Δ477-480	Δ1429-1443	1	8	6	Spont[3]
V511M+D521N	G1531A+G1561A	6	R	1	XL1R[4]
Δ513-522	Δ1537-1566	1.5	12	1	XL1R[4]
R529L	G1586A	1	R	2	XL1R[4]
G547S	G1639A	128	R	1	XL1R[4]
P246S+Q258*	C736T+C772T			1	RCA[5]
Q263R+A338P+A390T	A788G+G1012C +G1168A			1	RCA[5]
D328V	A983T+[G1326A][2] +[C1620T][2]			1	RCA[5]
A354T+G382D	G1060A+G1145A			1	RCA[5]
G365C	G1093T			1	RCA[5]
A369T	G1105A			1	RCA[5]
A264V+A388V +A417V+P492S	C791T+C1163T+ C1250T+C1474T			1	RCA[5]
I445N	[C1083T][2]+T1334A+[C1641T][2]			1	RCA[5]
A534T	G1600A			1	RCA[5]

Table 4: Summary of mutants and phenotypes.

[1] Minimal inhibitory concentration of Vancomycin and Bacitracin as measured by concentration gradient strips in μg/mL (Section 3.4). R, completely resistant at the highest concentration tested (256μg/mL).

[2] Nucleotide changes which do not result in an amino acid substitution.

[3] Spontaneous mutants which form colonies on maltopentaose media plated with strain K1508 expressing PulS and wild-type PulD (Section 3.1).

[4] Mutants isolated from XL1-Red *in vivo* mutagenesis (Section 3.3).

[5] Mutants isolated from Rolling Circle Amplification (RCA) *in vitro* mutagenesis (Section 3.2)

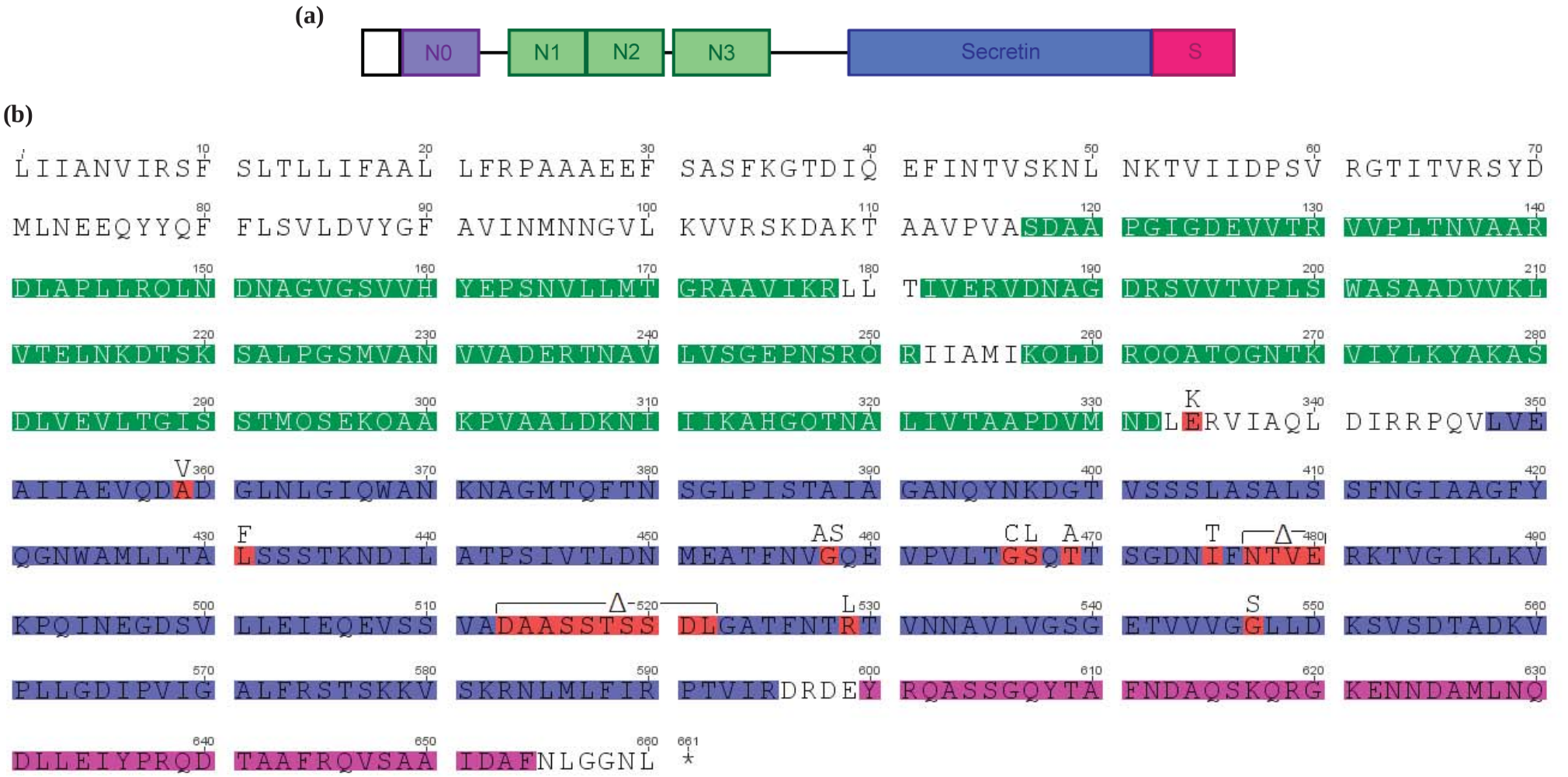

Figure 5: (a) Schematic of the Type 2 secretin domain organization (GspD). **(b)** Complete amino acid sequence of PulD highlighting N-domains (Green), Secretion homology domain (Blue), S-domain (PulS-binding domain; pink). The 11 substitution and 2 deletion mutations are highlighted (Red) with mutant substitutions or deletions indicated above the sequence.

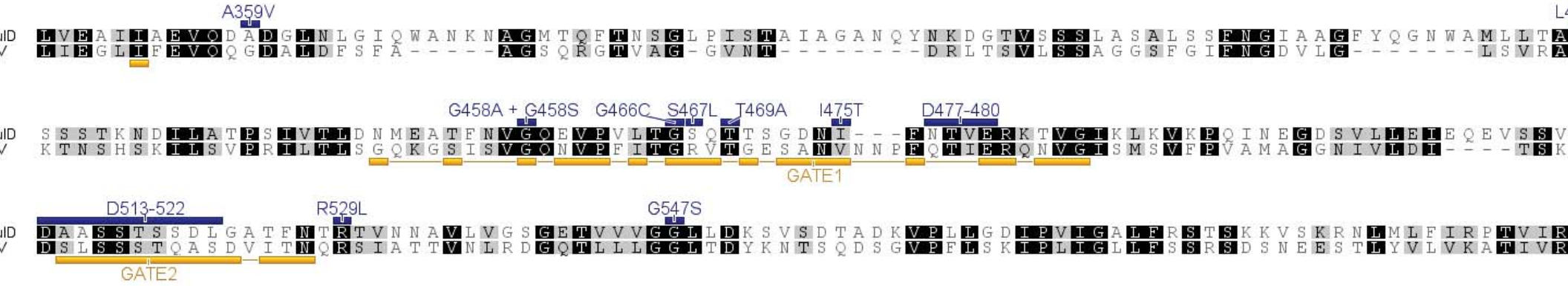

Figure 6: Alignment of secretin homology domains of PulD (top) and pIV (bottom) with leaky mutations of PulD (blue) and pIV GATE regions (yellow) highlighted. Image indicates conserved residues (black), similar residues (grey) and dissimilar residues (white).

3.4 Characterization of leaky mutants

In order to characterize the severity of each of the 14 leaky mutants discovered through mutagenesis, a series of antibiotic assays were carried out using Bacitracin, Vancomycin, and the bile salt component Deoxycholate, none of which can cross the outer membrane of *E. coli* expressing the wild-type PulD in combination with PulS pilotin. The number of colony-forming units of E. coli strain K2204 (TG-1 *ΔlamB*) expressing each PulD mutant, wild type PulD, pIV, pIV mutant S324G, and control containing empty vector pGZ119EH in the presence of Vancomycin (50 μg/mL), Bacitracin (50 μg/mL), or Deoxycholate (0.4%) was determined as described in material and methods (Section 2.8). Efficiency of plating was calculated as a ratio of the number of colony forming units in the presence relative to the number obtained in the absence of each of the compounds.

Of all strains tested, only the leaky pIV mutant S324G mutant showed a decrease in colony count between media with no agent and the three test plates. As for the other strains, there was no discernable difference in colony count between the strains and across the different media, however the colonies obtained in the presence of Van, Bac and DOC were very small and transparent, whereas the colony size was equal to that of empty vector-containing control strain in the absence of these compounds. Given the difference in colony size, signifying poor growth in the presence of Van and Bac, commercially available gradient *E*-test strips (AB Biodisk) were used to measure minimal inhibitory concentration (MIC) (Section 2.7).

Again the pIV S324G mutant showed the most prominent sensitivity to the antibiotics with a MIC of 1 μg/mL for Vancomycin and 3 μg/mL for Bacitracin. Strains expressing wild-type pIV and PulD exhibited resistance to both antibiotics (MIC >256μg/mL). The PulD mutant strains were variably sensitive with 12 showing sensitivity to Van (MIC between 1 and 96 μg/mL) 8 showing sensitivity to Bac (MIC between 3 and 64 μg/mL) (Table 4). The lawns of cells expressing most of the PulD mutants exhibited a decrease in growth density around the E-test strips (forming a "halo") indicating the cells could still grow at a low rate. This behaviour indicated that there were two populations of

cells in the lawn, one that was more sensitive to Van and Bac than the other and that the less sensitive population was able to slowly overcome the effects of the antibiotic.

The two *ΔlamB* strains used for selection of maltopentaose-permeable mutants, K1580 (used in spontaneous PulD mutant selection, Section 3.1) and K2204 (used in RCA and XL1-Red mutant selection, Sections 3.1 and 3.3), have a difference in parentage; the latter is derived from TG-1, and the former from MC4100 (Table 1). TG-1 and its derivative K2204 are more resistant to toxicity of some leaky secretins (Spagnuolo, unpublished) than MC4100 and its derivative K1508. Strain K2204 (and TG-1) appear to have ability to tune down expression of genes that cause envelope stress in a sub-population of cells. This has been reported in the literature for *E. coli* and *Salmonella enterica*, but the mechanism of control is poorly understood (Perez-Rodriguez et al., 2011; Sturm et al., 2011).

To determine the extent of increased sensitivity of strain K1508 to Van and Bac, plasmids expressing *pulD* mutants were transformed into K1508 (together with the *pulS* encoding plasmid) and the *E*-test was repeated on this strain. It was observed that K1508 gave clear zones of inhibition which matched the areas of decreased growth density ("halo") seen with K2204, the most sensitive being G458S and Δ477-480 which had an inhibitory concentration of 1 μg/mL Van and 8 μg/mL Bac respectively (Table 4).

4 Discussion

4.1 Leaky mutations

To identify gating regions of a T2SS secretin and make a comparison between the filamentous phage secretin pIV and T2SS secretin PulD, 14 leaky mutants were obtained both spontaneously and through XL-1 Red mutagenesis. XL-1 Red mutants G458S, S467L, and T469A clustered with the spontaneous G458A, G466C, I475T, and Δ477-480 mutations (Figure 5). When compared to leaky mutations in pIV, this cluster appears to be located in the GATE1 region outlined by the Spagnuolo *et al.* (2010). L431F appears upstream (Figure 6). The large 10 amino acid Δ513-522 deletion occurred within the GATE2 region providing an almost complete GATE2 deletion (Figure 6). R529L appears near the end of GATE2 with G547S present further downstream. The final two mutations occur at the very N-terminal end of the secretin homology domain, with E334K just outside and A359V only 12 amino acids into the domain (Figure 5b).

The alignment of both pIV and PulD leaky mutations revealed conserved residues in both proteins that, when mutated, result in a leaky phenotype: L431, G458, G466, E480, S516, S517, G547. Residues of a similar structure or polarity are also conserved between PulD and pIV: I475, V482, A514, T518, and S525, leaving only 6 of the 21 altered residues not conserved between the two proteins (Figure 6). While the sites of mutation hint at a conserved function between the two proteins, the new residues which have been substituted do not match between the pIV and the PulD mutants. The phenotypic change seen in these mutants is likely to be due to both the particular substituted residue being important for function, folding or assembly of the multimer in the gate region and the newly incorporated residue interacting with other parts of the protein.

The two mutants G458A and G458S gave an insight into this phenotypic change as the residue has been conserved between both PulD and pIV; The first change from Glycine to Alanine, the second to Serine, and the pIV counterpart being changed to Aspartic acid. G458S exhibited a much higher sensitivity to Bacitracin than G458A, but both exhibited a similar sensitivity to Vancomycin, while the pIV counterpart was observed

to have no sensitivity to either compound (Spagnuolo et al., 2010). It is not surprising that G458S exhibited a higher sensitivity as the change from a Glycine residue to a much larger hydrophilic Serine is much more drastic than the addition of the Alanine CH_3 side-chain. What is surprising, however, is these substitutions have a much greater effect in PulD than what was observed in pIV despite the change to aspartic acid (in pIV) being a much more drastic change than those seen in PulD. This would suggest that, while the position of this Glycine and the surrounding residues appears conserved to alignment software, it is functionally dissimilar between the two proteins. A comparison of a number of type 2 secretins quickly reveals a larger region of conservation than was seen between PulD and pIV, in which case, this residue may only be important for the type 2 secretin (Figure 7). It is possible that Glycine458 is an important residue in the GATE1 loop structure by providing flexibility. If this was indeed the case, the more bulky and less flexible Serine residue may alter the GATE1 loop structure interfering with multimerization or channel closure.

Indeed, the G458S mutant appears is one of the multimerization-defective mutants of PulD reported by Gulivout *et al.* (2011), where the multimerization kinetics of this particular mutation was assessed. Gulivout *et al.* observed that after *in vitro* synthesis the majority of PulD G458S monomers were unable to form multimers in liposomes after 45 minutes; 90% of the PulD G458S protein was observed as monomeric (Guilvout et al., 2011).

While it is clear that the incorporation of a Serine at this site has an immediate effect on the ability of this protein to multimerize correctly in an *in vitro* setting, however, the ability of a G458S mutant to grow on maltopentaose suggests formation of multimers in the outer membrane *in vivo*. Interestingly, this particular mutation was one of the most prominent having been isolated on 9 separate occasions during XL-1 Red mutagenesis. There are two possible explanations for this phenomenon: It may be that the conditions within the outer membrane and the addition of the PulS pilotin assist the assembly of this mutant and that the conditions present in the experiments presented in this study favour G458S in a monomeric state. Alternatively unstable multimers with high turnover may be responsible. Should G458S be able to form temporary multimers it would provide ample opportunity for compounds to enter the cell through the temporary channel formed by this multimerization event. Once this had occurred, the multimer

would then break up due to insufficient forces to sustain its structure. The majority of the protein would then appear on an SDS-page immunoblot as a monomer with the small amount of multimer being the proportion of proteins attempting multimerzation at the time of sampling. In order to establish whether multimers were forming correctly, a secretion assay would have to be carried out utilizing a construct expressing the full complement of *pul* genes and the G458S PulD mutant. This would assess the efficiency at which the mutant was able to secrete the substrate PulA and further establish whether PulD G458S was assembling properly.

G458S is not the only Glycine to Serine substitution to be found in a region of high conservation. G547S is present in a region of very high conservation (Figure 7). A Pfam consensus of all secretin homology domains shows this region (VVVGGLL) is one of the best conserved. Furthermore, the G547 residue itself is present in almost all secretins. A pIV leaky mutant isolated in the corresponding highly conserved residues affecting the homologous residue (Russel, 1994). The sequence VVVGGLL contains five hydrophobic residues intersected by two Glycines which, in conjunction with the high conservation, indicate the presence of a transmembrane region. Indeed, when put through the TMBETA algorithm to predict β-strands, the G547S mutant appears in the centre of a β-strand predicted C-terminal of the GATE-2 region. It is possible that the presence of a Serine residue in this conserved region may have altered the assembly of the PulD multimer in the same manner as G458S; however this mutation was not identified in the published screen for de-stabilizing PulD mutations (Guilvout et al., 2011). Another explanation for this mutant may be that the two Glycine residues in the centre of this region may provide important flexibility which a Serine would interfere with. This loss of flexibility would restrict the movement of the GATE2 loop and possibly restrict the channel to a semi-closed state. Consistently, G547S exhibits almost complete resistance to Bacitracin and only moderate sensitivity to Vancomycin while still allowing passage of maltopentaose.

4.1.1 GATE deletions

The GATE 1 deletion mutant Δ477-480 exhibited the highest sensitivity to Bacitracin and Vancomycin alongside G458S. This particular deletion occurred at the very beginning of a β-strand predicted in TMBETA in a region of high conservation (Figure

7). Mutations in the corresponding region in pIV also resulted in a severe leaky phenotype. It is possible that deleted residues are important in the conformational changes involved in the opening and closing of the GATE structure. Deletion of these four amino acids may restrict GATE movement, jamming it in an opened state.

The largest deletion mutant, Δ513-522, removed much of the GATE2 region of PulD. Despite 10 residues being removed from the PulD protein, this mutant was still capable of forming multimers allowing growth on maltopentaose. Alignment of a set of type 2 secretins revealed the GATE2 region as poorly conserved (Figure 7). This low level of conservation is common in loop structures and may explain why the protein was capable of accommodating such a large deletion. Coincidentally, a similar deletion mutant within the GATE2 region of pIV was constructed by site-directed mutagenesis (Spagnuolo et al., 2010). Cryo-EM and single particle analysis of purified mutant revealed a slight change in the septum of the ΔGATE2 mutant when compared to the wild type multimer. It was thought that the GATE2 serves as either structural support for the central septum or controls opening and closing of the channel. Deletion of this region would then result in the multimer being unable to completely close the channel allowing the entry of large molecules such as maltopentaose. In order to confirm this, wild type PulD and Δ513-522 mutant would have to be compared using Cryo-EM and single particle analysis, as well as electrophysiology experiments to indicate whether the central septum was altered. It was also observed that the ΔGATE2 pIV mutant was incapable of exporting f1 filamentous phage. PulA secretion assays carried out on wild-type, Δ513-522 and other leaky PulD mutants would reveal if removal of the GATE2 region in PulD also compromised the secretin's function.

Figure 7: Alignment of the secretin homology domain of 5 type 2 secretins. From top to bottom: PulD of *Klebsiella oxytoca*, GspD of *Escherichia coli* O1:K1, EpsD of *Escherichia coli* K12, EtpD of *Escherichia coli* 0157, and EpsD of *Vibrio cholerae*. Conserved residues indicated as black bands, similar residues indicated as dark grey, and nonconserved residues indicated as light grey. Leaky mutations of PulD indicated in blue, TMBETA β-sheet predictions for PulD indicated in red.

4.2 TMBETA predictions

Most OM channel proteins contain β-strands arranged in a β-barrel conformation. The TMBETA algorithm predicts the presence of β-strands within these proteins (Gromiha et al., 2005). When put through the TMBETA algorithm, several of these regions were predicted for PulD. When aligned to the Pfam secretin homology domain consensus, two regions of conservation were observed either side of the GATEs which corresponded to four β-strand predictions (Drummond et al., 2010). Using this information; a topological picture could be built of PulD in the outer membrane (Figure 8). The two GATE regions, which are thought to form the central septum of the secretin multimer, are situated with the β-sheets flanking either side of these regions: GATE1 at residues 414-432 and 477-491, and GATE2 at 501-512 and 535-548. It is also known that the C-terminus of PulD contains the S-domain which is responsible for interacting with the pilotin PulS. It then stands to reason that this region should be situated in the periplasm so that it may remain bound to the pilotin (Guilvout et al., 2006). The TMBETA data indicated another β-strand was present downstream from the last GATE2 strand. Taking this into account, another extracellular loop, between two β-strands, can be included placing the S domain back within the periplasm. The first GATE1 β-strand placed the PulD N-domains outside the cell. TMBETA predicted another β-strand upstream from GATE1 and indicated the presence of a large loop which would place the N-domains firmly within the periplasm. This is further supported by sequence alignment of a number of Type 2 secretins (Drummond et al., 2010). The two predicted β-strands are shown to be in conserved regions containing an area of little to no conservation between them, indicative of a loop.

Chami *et al.* (2005) predicted that the C-terminal domain of PulD contained a lower level of β-strands than most OM proteins (27%) and only a small portion of the protein would remain within the OM lipid bilayer, suggesting that much of the PulD C-domain extends far beyond the OM and penetrates both the periplasm and lipopolysccaride and capsule layers on the cell surface. The TMBETA predictions of both PulD and those provided for pIV by Spanguolo *et al.,* (2011) indicated a much larger loop between the first and second β-strands in PulD (45 residues) than was predicted for pIV (24 residues). Should this loop be situated on the surface of the cell, it would extend into the

lipopolysaccharide and capsule layers (Chami et al., 2005). This is corroborated by the prediction of β-strands on either side of the GATE1 region (strands 2 and 3) which seems to indicate that the GATE1 region is 15 residues larger in PulD than in pIV. If this were the case, it would explain why PulD was observed to have a relatively small number of residues involved in β-sheets: The presence of a large GATE1 region and a large extracellular loop would mean that the overall proportion of transmembrane residues would be lower than expected for OM proteins. It should be noted that the TMBETA algorithm tends to over predict the trans-membrane β-strands in outer membrane proteins that have large periplasmic domains (e.g. TolC) (Koronakis et al., 2000).

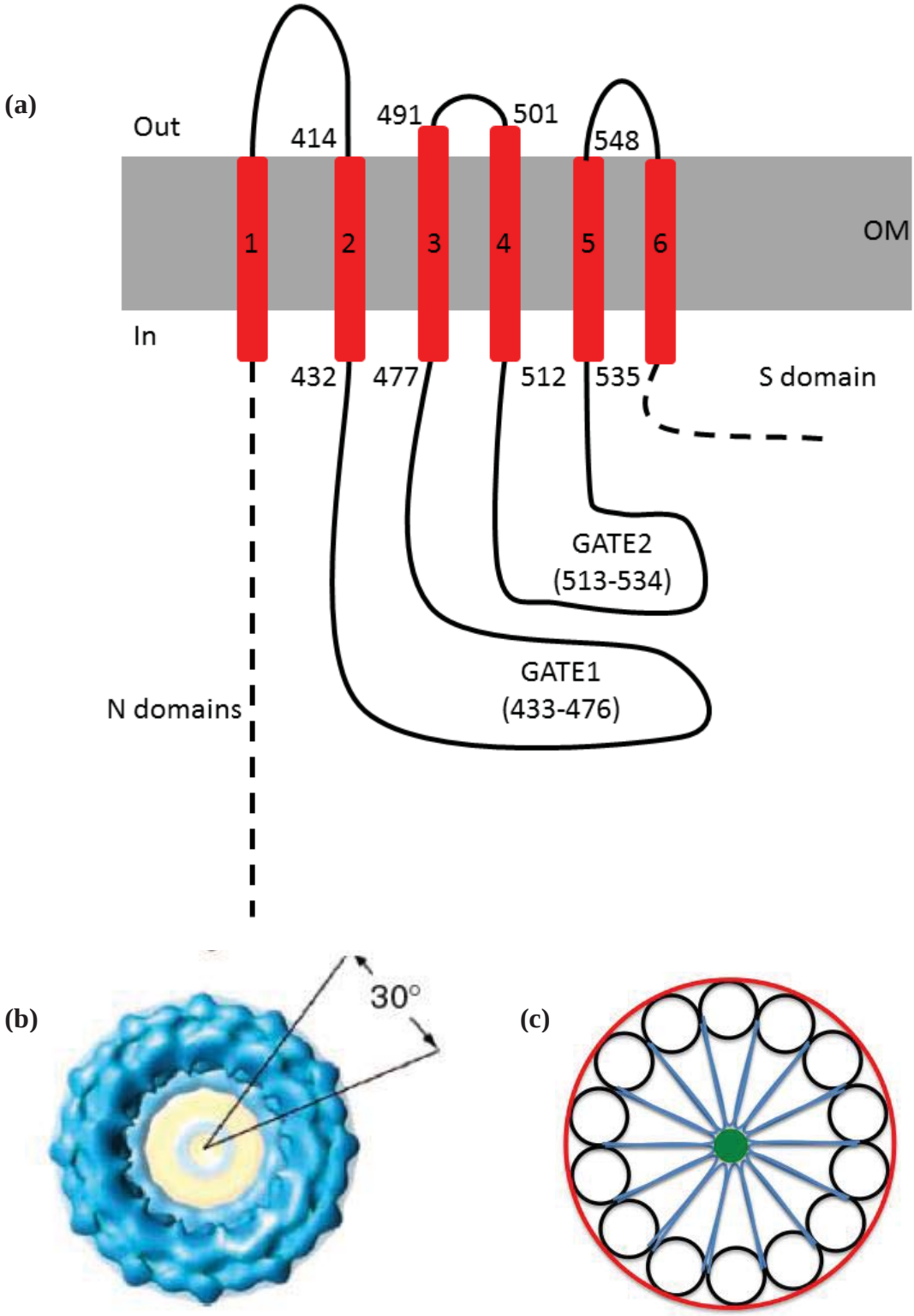

Figure 8: (a) Proposed topology model of PulD OM-spanning β-sheets surrounding the two GATE regions. Black dashed lines indicate regions of unknown conformation, N-domains and the C-terminal S domain. Modified with permission from Reichow *et al.* 2010, and Spagnuolo *et al.* 2010. **(b)** Basal view of the GspD multimer. **(c)** Schematic of PulD monomers arranged radially around a central axis. Blue represents GATE1 loops extending into the centre of the multimer forming the gate.

5 Conclusions

This work has located the two GATE regions in the primary sequence of the secretin PulD confirming the suspected conservation of these regions within the secretin homology domains of PulD and pIV. This information gives insight into the primary sequence of secretins as a whole and provides a basis for future investigation into the biochemistry, structure, and function of this family of proteins. This work has also produced two large deletion mutants of PulD which may be used to shed light on the how the gate operates within the central channel.

6 Future work

6.1 Secretin mutagenesis

Further mutagenesis and selection for leaky mutations in PulD is required for complete coverage of the GATE1 and GATE2 regions. It is important to identify the boundaries of the two GATE regions so that further structural and biochemical investigation can take place. It has also been observed by Spagnuolo *et al.* 2010 that several leaky mutations appear in the N-domains of pIV. So far, no such N-domain leaky mutations have been observed for PulD. Should such residues be important in PulD, they may have been missed by this first round of mutagenesis.

6.2 Testing functionality of PulD mutant in the context of T2SS

Many of the leaky mutations exhibited a phenotype similar to that of G458S, which has been previously characterised as a multimerization-deficient mutant (Guilvout et al., 2011). Since the mutant G458S was able to facilitate growth of Δ*lamB E. coli* on maltopentaose, it is likely that this particular mutant is able to form sufficient number of multimeric channels in the OM *in vivo.* By assessing secretion efficiency of the PulD substrate Pullulanase (PulA) by the G458S and other leaky PulD mutants, the efficiency of multimerization can be observed. The lack of secretion does not necessarily mean that the channel is not assembled; some leaky mutants of pIV were found not to be able to secrete the substrate.

6.3 Cryo-EM imaging

Structural analysis of the pIV ΔGATE 2 deletion mutant by cryo-EM and single-particle analysis in Spagnuolo *et al.* 2010 indicated a possible minor change in the structure of the central septum of the pIV multimer and walls of the C-ring. A similar deletion mutant was discovered in PulD: Δ513-522. Cryo-EM imaging of this mutant may indicate to what extent this deletion changes the structure of the central septum. Further imaging of a second deletion mutant, Δ477-480, will also give insight into what structural changes occur when a portion of the GATE1 region is removed and how that may differ from GATE2 deletion.

6.4 Electrophysiological characterization

Characterization of the wild-type pIV and subsequently PulD secretin indicated tight gating of these channels, which requires high energy (Voltage) to force the opening (Marciano, 1999; Nouwen et al., 1999). A pIV leaky mutant (S324G within GATE2) analysed by electrophysiology required much lower voltage to open and has opened more frequently than the wild-type pIV, but the diameter of the open channel was unchanged. Such characterization of a number of leaky PulD mutants would serve to indicate whether the mutations simply reduce the electrical potential required to open the channel or if the mutations result in a more drastic, permanently open channel, as well as to determine the diameter of constitutively open channel.

7 References

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