Interactions between ExeA and Peptidoglycan in the Type II Secretion System of *Aeromonas hydrophila*

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By

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ABSTRACT

Aeromonas hydrophila uses the type II secretion system to transport protein toxins across the outer membrane. The trans-envelope system is comprised of more than ten proteins, including ExeA and ExeB, which form a complex in the inner membrane and are required for assembly of the ExeD secretion channel multimer, called the secretin, into the outer membrane. A putative peptidoglycan binding domain (Pfam protein families database number PF01471) is present in the periplasmic region of ExeA (pExeA), leading to the hypothesis that ExeA generates gaps in peptidoglycan, a barrier for trans-envelope transport and apparatus assembly, to allow ExeD to assemble into the outer membrane.

In this study, interactions between ExeA and peptidoglycan were examined both *in vivo* and *in vitro*. Wild type ExeA, but not the mutants containing substitution mutations of three highly conserved amino acid residues in the putative peptidoglycan binding domain, was cross-linked to peptidoglycan *in vivo* with DTSSP. Furthermore, the presence of wild type ExeA was also required for co-crosslinking of ExeB and ExeC to peptidoglycan. *In vitro* cosedimentation revealed that purified pExeA was able to bind to highly purified peptidoglycan. The protein assembled into large multimers in the presence of peptidoglycan fragments, as shown in cross-linking and co-gel filtration experiments. The requirement of peptidoglycan for multimerization was abrogated when the protein was incubated at temperatures above 25 °C. Two pExeA constructs, which disrupted the putative peptidoglycan binding domain, greatly reduced the cosedimentation, accompanied by decreased multimerization in the presence of peptidoglycan fragments. These results provide evidence that the putative peptidoglycan binding domain of ExeA is involved in physical contact with peptidoglycan. The interactions cause ExeA to multimerize, possibly forming a ring-like structure on the peptidoglycan, to generate a gap large enough to accommodate the secretion apparatus and/or to form an assembly scaffold.

The putative peptidoglycan binding domain of ExeA was also analyzed by comparing its amino acid sequence with that of other homologues. The highly conserved amino acid residues were found to cluster at one pocket on the surface in the crystal structure of hydrolase metallo (Zn) DD-peptidase that also contains this domain. We propose that this pocket is the binding site for the peptidoglycan ligand.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
Ap	Ampicillin
AT	Autotransporter
ATP	Adenosine 5´-triphosphate
BLAST	Basic local alignment search tool
C	Carboxy
CBB	Coomassie Brilliant Blue
Cm	Chloramphenicol
CP	D,D-carboxypeptidase
D	Dalton
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DTSSP	3´-Dithiobis[sulfosuccinimidylpropionate]
EDTA	Ethylenediaminetetraacetic acid
EP	D,D-endopeptidase
GFP	Green fluorescent protein
GlcNAc	N-acetylglucosamine
GSP	General secretion pathway
GST	Glutathione S-transferase
HPLC	High-pressure liqid chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase pairs
Km	Kanamycin
LB	Luria-Bertani
LPP	Major lipoprotein
LPS	Lipopolysaccharide
LT	Lytic transglycosylase
m-A_2pm	*Meso*-2,6-diaminopimelic acid
m-DAP	*Meso*-2,6-diaminopimelic acid
MMP	Matrix metalloprotein
MS	Mass spectrometry
MurNAc	N-acetylmuramic acid
N	Amino
Nal	Nalidixic acid
NTA	Nitrilotriacetic acid
NTP	Nucleoside 5´-triphosphate

PAGE	Polyacrylamide gel electrophoresis
PBD	Research Collaboratory for Structural Bioinformatics protein data bank
PBP	Penicillin-binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pExeA	C-terminal periplasmic region of ExeA
Pfam	Protein families database
PMSF	Phenylmethylsulphonyl fluoride
psi	Pounds per square inch
PVDF	Poly vinylidene difluoride
R_H	Hydrodynamic radius
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
St	Streptomycin
T1SS	Type I secretion system
T2SS	Type II secretion system
T3SS	Type III secretion system
T4SS	Type IV secretion system
T5SS	Type V secretion system
T6SS	Type VI secretion system
Tat	Twin-arginine translocation
Tc	Tetracycline
T-DNA	Tumer-inducing nucleoprotein particles
TG	Monofunctional transglycosylase
TG/TP	Bifunctional transglycosylase and transpeptidase
TP	Monofunctional transpeptidase
TPS	Two-partner secretion
Tris	Tris(hydroxymethyl)aminomethane
V	Volts
WT	Wild type

1. INTRODUCTION

1.1 Protein toxin secretion systems in Gram-negative bacteria

Bacteria are enclosed by membrane barriers to preserve the integrity of the cells. However, the barriers must be somehow made selectively permeable to allow certain molecules to travel into and out of the cells, for example for the acquisition of nutrients, the secretion of extracellular enzymes and toxins and the assembly of trans-envelope organelles such as pili and flagella (Holland, 2004). Bacteria have developed many trans-membrane machineries for translocation of specific molecules across the inner (cytoplasmic) membrane, the outer membrane, or both. The Sec pathway, which recognizes the N-terminal signal sequence of polypeptides, is the major route for transport of unfolded proteins into and across the cytoplasmic membrane and is evolutionary conserved in all kingdoms of life (Veenendaal *et al.*, 2004). Recently, twin-arginine translocation (Tat) system, which recognizes an N-terminal signal sequence containing a twin-arginine consensus motif, was identified (Natale *et al.*, 2008). The most remarkable feature of the Tat system is that it is able to transport folded proteins across the cytoplasmic membrane. In Gram-positive bacteria, protein secretion is generally carried out by the Sec and Tat systems across the cytoplasmic membrane, although a multicomponent system, called the Type VII secretion system, has been identified recently in a few Gram-postitive bacteria (van Wely *et al.*, 2001; Abdallah *et al.*, 2007). In addition, Gram-negative bacteria have developed several pathways to deliver extracellular proteins across the outer membrane into the external medium or directly into their target cells. Six secretion systems, from type I to type VI (T1SS to T6SS), have been identified to date and will be summarized briefly in the following sections. The T2SS is the subject of this thesis and will be addressed in detail in section 1.2.

1.1.1 Type I secretion system

The T1SS transports unfold proteins from the cytoplasm to extracellular medium in a single step, independent of the Sec pathway. The apparatus forms a trans-envelope structure, including an inner membrane ABC (ATP-binding cassette) protein, a channel-forming outer membrane protein (OMP) and a membrane fusion protein (MFP) which connects the above two proteins (Buchanan, 2001), as illustrated in Fig. 1. The ABC protein belongs to a large family of ABC transporters, which are involved in transport of a diverse range of substrates

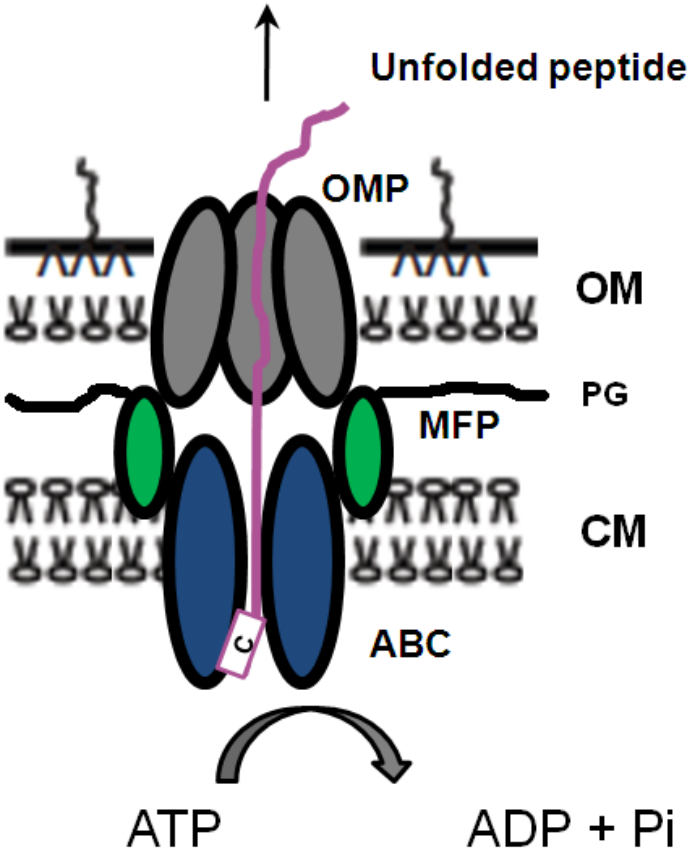


Fig. 1 Model of the T1SS system. The T1SS apparatus is comprised of an inner membrane protein ABC, an outer membrane protein OMP and a membrane fusion protein MFP. The secretion signal is located at the C-terminus of unfolded secreted protein. CM: cytoplasmic (inner) membrane. OM: outer membrane. The figure is modified from Delepelaire (2004).

into and out of the cell, for example, uptake of nutrients, secretion of toxins and efflux of antibiotics. ATP hydrolysis by this protein is required to provide energy for transport of these molecules against a concentration gradient. OMP forms a long trimeric channel, including a β-barrel in the outer membrane and an α-helical hovel extending into the periplasmic space, as illustrated by the crystal structure of TolC (Koronakis *et al.*, 2000). The assembly of the three-protein apparatus is proposed to be triggered by binding of the protein substrate to the ABC protein and consequent interactions between the ABC, MFP and OMP (Delepelaire, 2004). The secretion signal of the protein substrate is located at the C-terminal end and tends to form an α-helical structure. Three possible signals have been identified, including: (1), an essential amphiphilic α-helix followed by a nonessential α-helix and an unstructured C-terminal domain favouring non-positively charged residues; (2), a negatively charged residue followed by three hydrophobic residues; and (3), glycin-rich repeats (Delepelaire, 2004). The glycin-rich repeats (GGXGXDXXX) are very remarkable in that the residues G2, G4 and D6 in one repeat and G1, X3 and D6 in another come together in a β-roll structure to bind a Ca^{2+} ion (Baumann *et al.*, 1993).

1.1.2 Type III secretion system

The T3SS features a trans-envelope needle-like structure that is able to deliver bacterial protein effectors to the extracellular medium or directly into host cells (He *et al.*, 2004), as illustrated in Fig. 2. About 20 genes encoding T3SS components are usually found in large pathogenicity islands that often have different G+C composition compared to the rest of the chromosome, suggesting that they are acquired by horizontal transfer (Hacker and Kaper, 2000). The T3SS needle-like structure shares striking similarities with flagella in core protein component sequences, assembly, secretion mechanism and morphology. Some reports even show that the two systems can secrete the same proteins under certain conditions (Young and Young, 2002; Lee and Galán, 2004). It has been proposed that the T3SS is evolved from an ancient flagella assembly system, or the virulence-associated T3SS and flagella both belong to the T3SS families evolved from a common ancestor (Gophna *et al.*, 2003). One of the differences between the two systems is that the T3SS apparatus contains an outer membrane multimer, called secretin, which is also conserved in T2SS, type IV pilus and filamentous phage secretion but not in flagella (c.f. secretin in section 1.2).

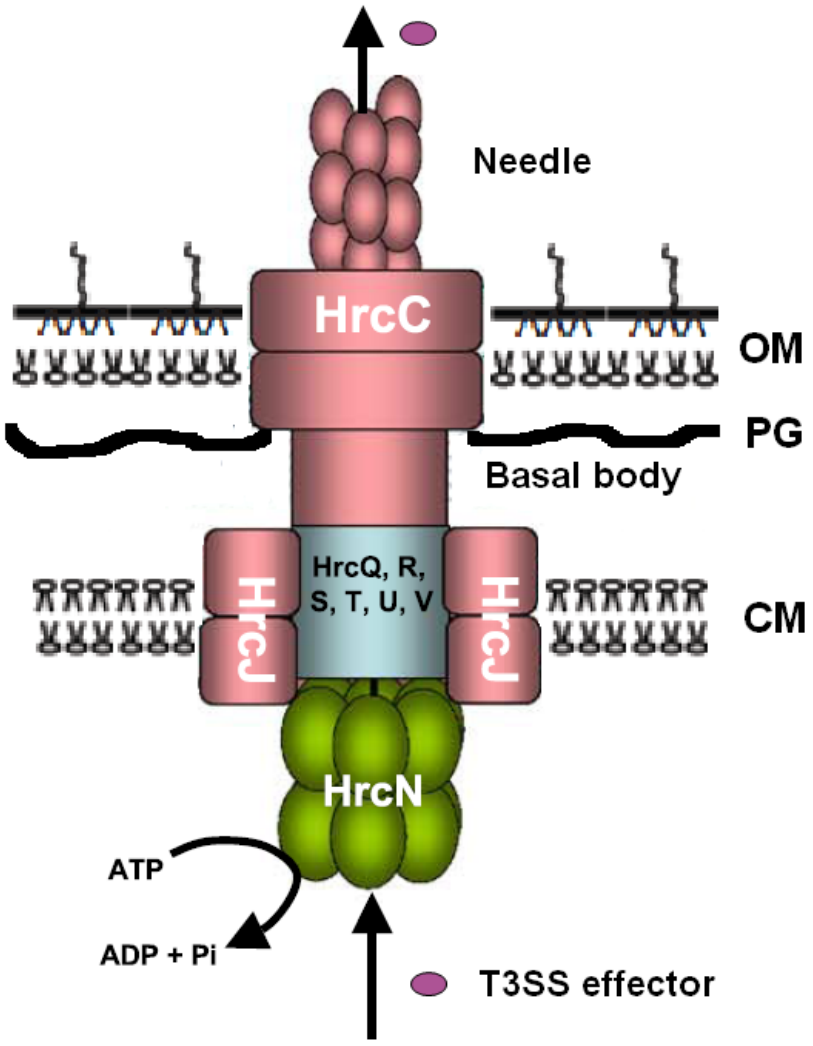


Fig. 2 Model of the T3SS system in plant pathogenic bacteria. The needle-like T3SS apparatus is comprised of about 20 proteins, ressembling the structure of flagellum. In plant pathogen *Pseudomonas syringae*, the T3SS is encoded by a cluster of hypersensitive response and pathogenicity (*hrp*) genes that are required to trigger hypersensitive response (a plant defense response) or to cause disease in plants. The figure is modified from He *et al.* (2004) with permission from the Elsevier Limited.

Protein secretion by T3SS is Sec-independent. They are transported directly from the cytoplasm out of the cell through the trans-envelope needle-like channel. The signal for secretion was first reported to be located at the N-terminal 15-17 amino acid residues of the proteins traversing the T3SS; however, controversial results showed that the 5' region of mRNAs encoding the secreted proteins actually contained the signal recognized by T3SS (Sory *et al.*, 1995; Anderson and Schneewind, 1997 and 1999). The most recent reports suggest that the amphipathic peptide sequence, not mRNA, is actually recognized by the T3SS machinery (Lloyd *et al.*, 2002).

1.1.3 Type IV secretion system

The T4SS was firstly known as the 'adapted conjugation' system (Christie and Vogel, 2000). In recent reviews, T4SS members include all conjugation and related systems mediating DNA or protein translocation (Christie, 2004). The T4SS apparatus, encoded by more than 10 genes, features an envelope-spanning channel and an extracellular pilus (mating pair formation structure) that is able to establish direct cell to cell contact. In *Agrobacterium tumefaciens*, the same trans-envelope structure transports both DNA and proteins, assisted by different cytoplasmic chaperones (Christie, 2004), as illustrated in Fig. 3. The apparatus delivers substrates across the envelope in one step to the medium or directly into recipient cells. The *Bordetella pertussis* system is an exception in that it transports pertussis toxin in two steps, involving Sec-dependent translocation across the inner membrane and T4SS-dependent translocation across the outer membrane. Comparison of known T4SS substrates has failed to show a common sequence for secretion. Some protein-fusion studies showed that the signal for secretion is located at the C-terminal end of secreted proteins. It is further found that many T4SS substrates share an Arginine-rich or positive charge-abundant C-terminus. How positive charges are involved in substrate recognition is not yet clear.

1.1.4 Type V secretion system

The T5SS is also known as autotransporter (AT)/two-partner secretion (TPS) pathway, which is widely spread especially among pathogenic bacteria (Henderson and Nataro, 2001; Jacob-Dubuisson *et al.*, 2001; Jacob-Dubuisson *et al.*, 2004). The AT pathway is the simplest

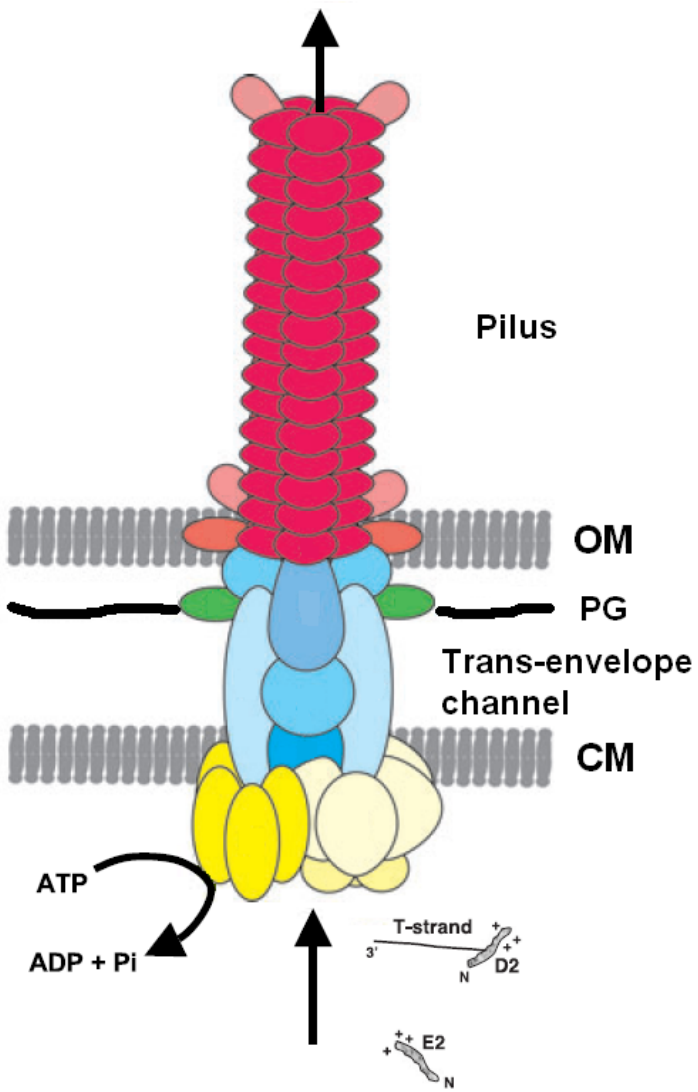


Fig. 3 Model of the T4SS system in *A. tumefaciens*. The T4SS apparatus is comprised of an extracellular pilus (mating pair formation structure) and a trans-envelope channel. In *A. tumefaciens*, the system is able to transport both DNA and proteins, such as D2-T-strand and E2 shown in the figure. The figure is modified from Juhas *et al.* (2008).

secretion system in that the substrates can mediate their own secretion across the outer membrane (Fig. 4). AT proteins are synthesized with an N-terminal signal peptide, which is recognized by the Sec machinery and cleaved after the proteins are transported across the inner membrane. They also contain two distinct modules, an N-terminal passenger module (following the signal peptide), which performs a specific function after they are secreted, and a C-terminal translocation module which functions to transport the passenger module out of the cell. It has been suggested that the C-terminal translocation domain is able to insert itself into the outer membrane as a β-barrel, in a similar way as porins do, to form a conduit to allow passage of the passenger domain. After transport, the passenger domain may remain on the cell surface or be released by proteolytic cleavage. The TPS system functions similarly as the AT system except that two TPS proteins (TpsA and TpsB) play the roles of passenger domain and transport domain, respectively. In addition, TpsA contain a TPS domain after the signal peptide which interacts with TpsB for secretion.

1.1.5 Type VI secretion system

The T6SS is the most recently described and least understood system but is found widespread in bacterial pathogens (Bingle *et al.*, 2008). The system is able to establish close contact with eukaryotic cells and release effectors directly into the cell. The apparatus is comprised of 12 to more than 20 proteins but little is known about the protein-protein interactions in the machinery. The current model describes it as a trans-envelope structure that delivers protein effectors from the cytoplasm out of the cell or directly into the host cell in one step (Cascales, 2008), as shown in Fig. 5. However, some data suggest that the secretion may contain two steps because one secreted protein was found to accumulate in the periplasm in a T6SS mutant strain (Mougous *et al.*, 2007). One interesting feature of the *Vibrio cholerae* T6SS is that it may assemble an outer membrane needle-like structure that shows homology with the bacteriophage T4 base-plate. The needle-like structure is comprised of trimeric VgrG and may puncture the host cell and release the activity domain of VgrG from the needle tip (Pukatzki *et al.*, 2007).

1.2 The type II secretion system in Gram-negative bacteria

The type II secretion system (T2SS) has long been known as the main terminal branch

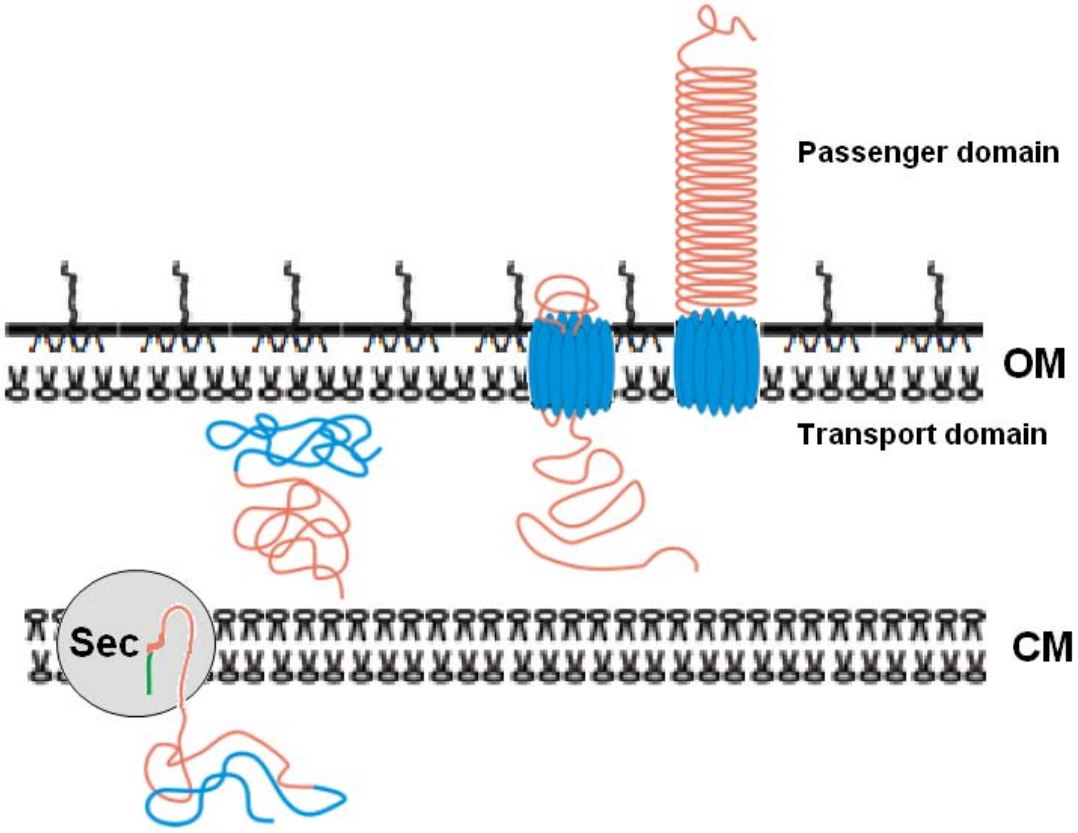


Fig. 4 Model of the T5SS system. The AT protein contains a signal peptide (green), which is required for transport across the innter membrane via the Sec system, an N-terminal passenger domain and a C-terminal transport domain. The transport domain is able to insert into the outer membrane as a β-barrel, through which the passenger domain is transported. After transport, the passenger domain is folded and may remain on the cell surface or be released by proteolytic cleavage. The TPS system functions similarly, except that two proteins TpsA and TpsB play the roles of passenger domain and transport domain, separately (not shown in the figure). The figure is modified from Wells *et al.* (2007).

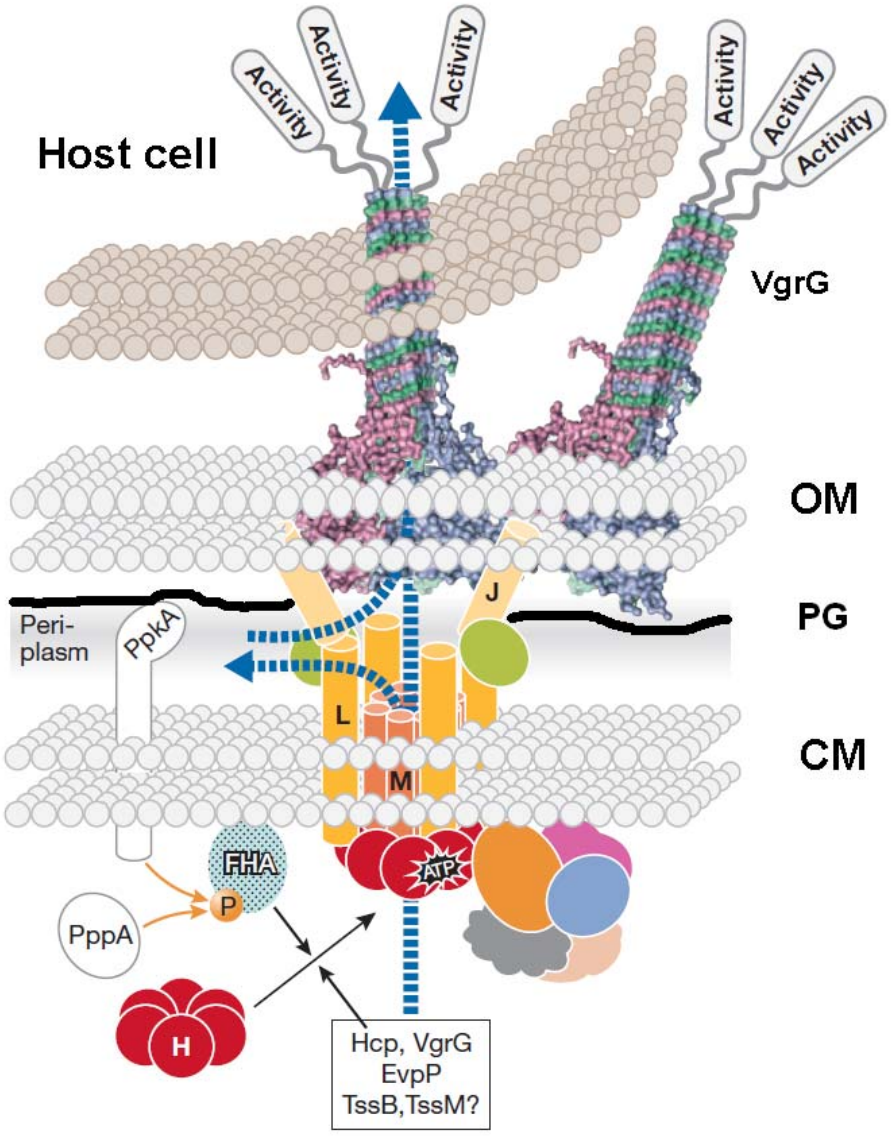


Fig. 5 Model of the T6SS system. The T6SS apparatus is proposed to be a multicomponent trans-envelope structure that is able to inject protein effectors directly in to host cell. VgrG forms a unique trimeric needle-like structure that assembles the bacteriophage T4 base-plate. The needle-like structure may puncture into the host cell and release the activity domain from the needle tip. The figure is modified from Cascales (2008) with permission from the Nature Publishing Group.

of the general secretion pathway (GSP), recognized as the main route for extracellular protein translocation in Gram-negative bacteria (Pugsley, 1993a; Cao *et al.*, 2003). The GSP describes a two-step secretion pathway in which exoproteins cross the inner membrane and the outer membrane successively. In the first step, the precursor proteins containing a cleavable N-terminal signal peptide are recognized by Sec machinery and transported across the inner membrane (Veenendaal *et al.*, 2004). The proteins are processed and fold in the periplasm. In the second step, the exoproteins are recognized and transported across the outer membrane. The second step is called the terminal branch of the GSP. T2SS is known as the main terminal branch, one of several terminal branches identified. Recently, T2SS was also demonstrated to be able to secrete Tat-dependent substrates (Voulhoux *et al.*, 2001). The T2SS machinery involves 12-16 proteins which assemble into a complex structure, termed the secreton, spanning both the inner membrane and the outer membrane.

The T2SS was first discovered in *Klebsiella oxytoca*, and called the Pul system (d'Enfert *et al.*, 1987). Homologues have since been demonstrated in numerous Gram-negative bacteria, including *Pseudomonas aeruginosa* (Xcp system), *Erwinia chrysanthemi* (Out), *Vibrio cholerae* (Eps) and *Aeromonas hydrophila* (Exe). Important virulence factors of bacterial pathogens are T2SS substrates, for example in the secretion of cholera toxin, the major cholera diarrhea-causing factor, by the Eps system of *V. cholerae* (Spangler, 1992; Johnson *et al.*, 2006). Homologous proteins in different systems are named with the same letters, e.g. PulC of *K. oxytoca* is a homologue of EpsC of *V. cholerae*. The designation of the Xcp system in *P. aeruginosa* is an exception for historical reasons, for example, XcpP is a homologue of PulC and EpsC. Currently Gsp is the common name for different T2SSs, for example, the above homologues belong to the GspC family.

The most significant feature of T2SS is that it resembles the type IV pilus biogenesis system in that a pilus-like structure, called pseudopilus, is present in the T2SS (Köhler *et al.*, 2004; Peabody *et al.*, 2003, Vignon *et al.*, 2003). A homologue of type IV prepilin peptidase is also required to process the pseudopilins (Nunn and Lory, 1992 and 1993; Pugsley, 1993b). In addition, several components of T2SS share similarities in both sequence and structure with those of the type IV pilus (see more details below). The two systems are thus proposed to have evolved from a same ancestor (Nunn, 1999).

1.2.1 Genes encoding Gsp proteins

The Gsp system is encoded by 12-16 genes that are usually clustered in operons *gspC-O*, *gspAB* and *gspS*, as shown in Fig. 6 (Filloux, 2004). *gspC-N* is conserved in most T2SSs. In some bacteria, *gspO* is substituted by the prepilin peptidase of type IV pilus, for example, *pilD* in *P. aeruginosa* and *tapD* in *A. hydrophila* (Nunn and Lory, 1991; Pepe *et al.*, 1996). *gspAB* and *gspS* are not present in all bacteria, although they are absolutely required in *A. hydrophila* and *K. oxytoca*, respectively (Jahagirdar and Howard 1994; Hardie *et al.*, 1996).

1.2.2 Structure of the T2SS machinery

The complicated trans-envelope structure of T2SS and how this structure is assembled are not fully understood yet. The experimental data from protein-proteins interactions between T2SS components, subcellular localization, electron microscopy and crystallography studies have suggested dissecting the machinery into three parts: an inner membrane platform, a periplasmic pseudopilus and an outer membrane channel (Johnson *et al.*, 2006). A model of T2SS is illustrated in Fig. 7.

The inner membrane platform is composed of GspE, F, L and M (Py *et al.*, 2001). GspE is a cytoplasmic protein which is associated with the inner membrane platform through interactions with the membrane protein GspL (Sandkvist *et al.*, 1995; Py *et al.*, 1999). GspE is a member of a large family of secretion nucleoside triphosphatases that contain conserved motifs of Walker A box, Walker B box, a histidine box and an aspartate box (Possot and Pugsley, 1994). EpsE from *V. cholerae* has been demonstrated to be a Mg^{2+}-dependent ATPase *in vitro* (Camberg and Sandkvist, 2005). The purified protein formed a small fraction of hexamers that showed increased ATPase activity, consistent with the crystallography studies in which the protein was modeled into a hexameric ring (Robien *et al.*, 2003). Similar hexameric structure was also obtained in the crystallography studies of secretion ATPase family members HP0525 of *Helicobacter pylori* and PilT of the type IV pilus (Yeo *et al.*, 2000; Forest *et al.*, 2004). It is likely that the functional form of GspE *in vivo* is a hexameric ring that provides energy for secretion/assembly of the T2SS and/or forms a channel to allow transport. Other components of the inner membrane platform are membrane proteins GspF, L and M. Interactions among these proteins include GspE-GspL interactions, GspL-GspM interactions and GspE-F interactions (Sandkvist *et al.*, 1995; Py *et al.*, 1999;

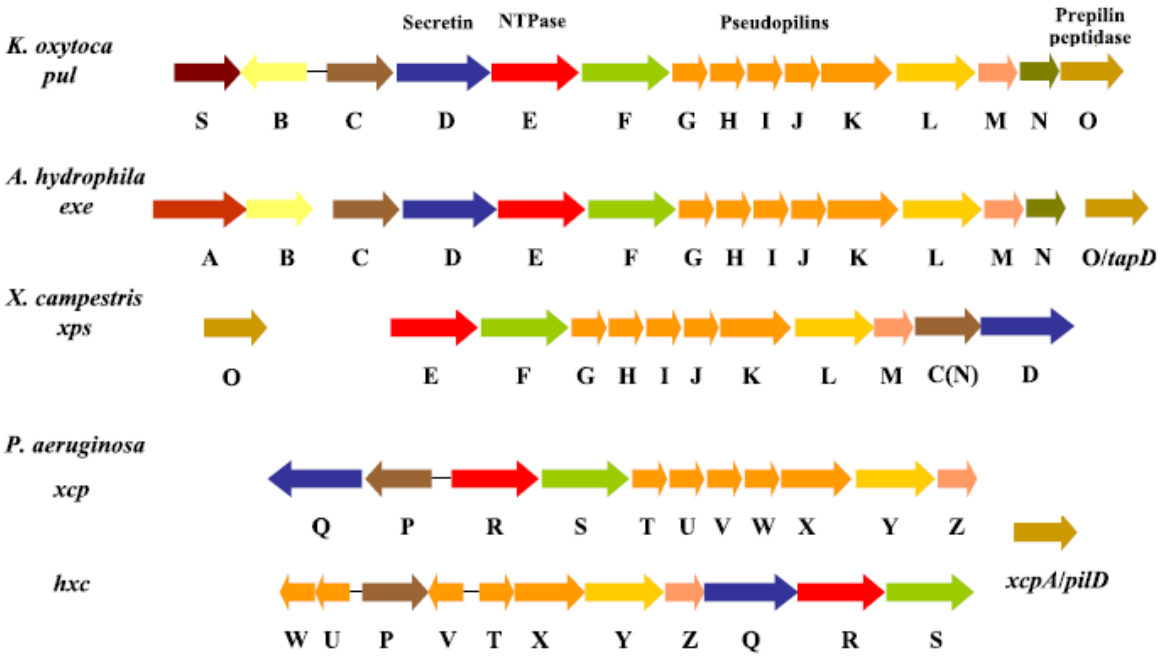


Fig. 6 Genes encoding the T2SSs in *K. oxytoca* (*pul*), *A. hydrophila* (*exe*), *X. campestris* (*xps*) and *P. aeruginosa* (*xcp* and *hxc*). Homologous genes are designated with the letters A-O and S. *P. aeruginosa* is an exception, as P-Z and A are used. In *A. hydrophila* and *P. aeruginosa*, *exeO* and *xcpA* are substituted by the type IV pilus prepilin peptidase encoding genes *tapD* and *pilD*, respectively. The figure is modified from Filloux (2004) with permission from the Elsevier Limited.

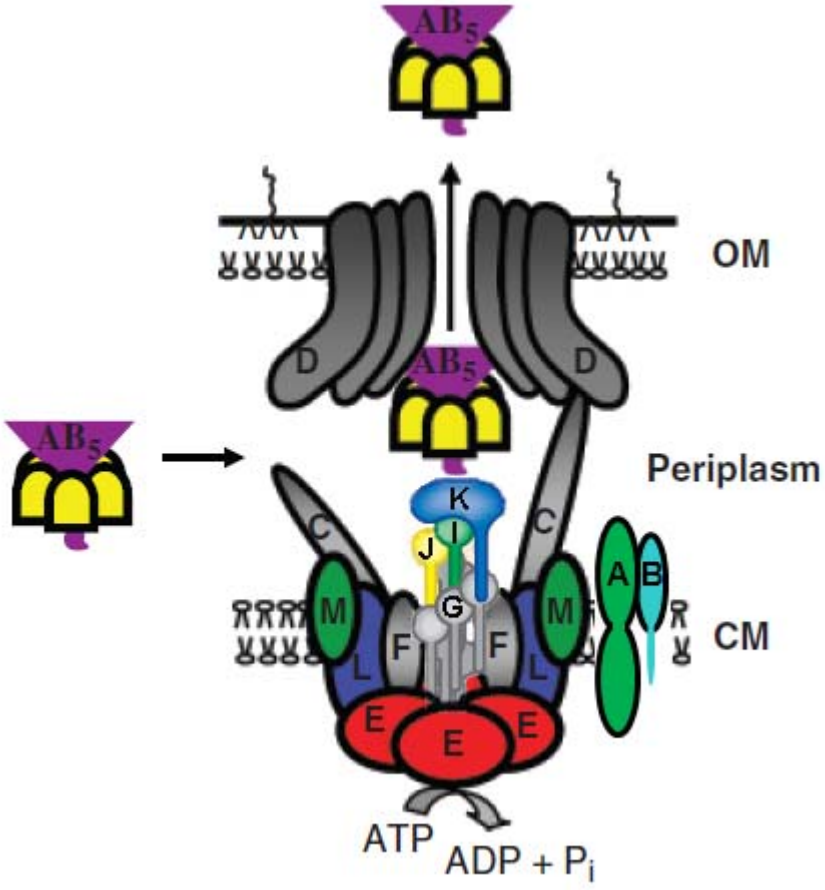


Fig. 7 Model of the T2SS system. The T2SS apparatus is comprised of 12-16 proteins, forming an inner membrane platform of GspE-F-L-M, a periplasmic pseudopilus of GspG-H-I-J-K, and an outer membrane channel GspD secretin. GspC is proposed to connect the inner membrane and the outer membrane complexes. GspA and B form an inner membrane complex and their assembly in the apparatus will be studied in this study. GspN and S are not illustrated. AB_5: cholera toxin secreted by the T2SS in *V. cholerae*. CM: cytoplasmic (inner) membrane. OM: outer membrane. The figure is modified from Johnson *et al.* (2006).

Scott *et al.*, 2001; Crowther *et al.*, 2004 and 2005). The inner membrane platform complexes of Out and Xcp systems could be co-immunoprecipitated and co-purified, respectively (Py *et al.*, 2001; Robert *et al.*, 2005).

The periplasmic pseudopilus is composed of GspG, H, I, J and K. These proteins share sequence and structural similarities with the pilins of type IV pilus and thus are called pseudopilins (Nunn and Lory, 1992 and 1993; Pugsley, 1993b). They all have a special N-terminal leader peptide for transport across the inner membrane and require GspO or prepilin peptidase PilD for processing. GspO and PilD have a high degree of identity. PilD is a bifunctional peptidase, cleaving the leader peptide of prepilins and N-methylating the generated N-termini before the pilin subunits are assembled into type IV pilus (Lapointe and Taylor, 2000). In *A. hydrophila* and *P. aeruginosa* that do not contain GspO, the prepilin peptidases TapD and PilD, respectively, process both type IV prepilins and T2SS pseudopilins (Nunn and Lory, 1991; Pepe *et al.*, 1996). Among the pseudopilins, GspG is most abundant, thus called the major pseudopilin; others are minor pseudopilins (Nunn and Lory, 1993; Pugsley, 1993b; Hu *et al.*, 2002). Overproduction of GspG resulted in long pilus-like structures on the cell surface under transmission electron microscopy (Durand *et al.*, 2003). Together with the crystal structure of GspG, the pseudopilus has been modeled into a left-handed structure with the hydrophobic N-terminal α-helix of GspG packed into the core of the pseudopilus (Köhler *et al.*, 2004). A greater understanding of the roles of the minor pseudopilins has been obtained through crystallography studies, especially the crystal structure of the heterotrimeric complex of GspI-J-K (Korotkov and Hol, 2008). The pseudopilins all contain a conserved N-terminal α-helix, a conserved C-terminal β-sheet and a variable region between them. GspK is special in that it contains a large α-helical domain in the β-sheet region, preventing upward addition of pseudopilins but allowing downward addition of GspI, J and G. The GspJ-I-K complex may thus form the “arrow head” of the pseudopilus with the α-helical domain of GspK interacting with the outer membrane channel or secreted proteins (Forest, 2008).

The outer membrane channel is comprised of a 12-14 member multimer of GspD, called secretin (Chen *et al.*, 1996; Hardie *et al.*, 1996; Sandkvist, 2001). GspD is a member of the secretin family of outer membrane channel-forming proteins required in T2SS, type IV pilus, T3SS and filamentous phage extrusion (Genin and Boucher, 1994). They contain a variable

N-terminal domain and a highly conserved C-terminal domain. The C-terminal domain contains putative amphipathic β-strands that are believed to insert in the outer membrane and form the wall of the conduit. One remarkable feature of secretin is that it forms a stable, heat and detergent-resistant structure that runs on SDS-PAGE as a mega Dalton band just able to enter into the gel (Hardie *et al.*, 1996; Ast *et al.*, 2002). Two T2SS and one phage secretion secretins have been characterized by electron microscopy (Bitter *et al.*, 1998; Nouwen *et al.*, 1999 and 2000; Opalka *et al.*, 2003; Chami *et al.*, 2005). The C-terminal domain of GspD forms a proteolysis-resistant 12-14 multimeric ring-like structure with a central pore of 50-100 Å in diameter which is occluded with a plug. The N-terminal domain of secretin protrudes into the periplasm and is proposed to interact with other components of the machinery or secreted proteins (Chami *et al.*, 2005). In *K. oxytoca* and *Erwinia* species, lipoprotein GspS is required for assembly of GspD secretin into the outer membrane and probably is a part of the secretin complex through the interactions between GspS and the C-terminal end of GspD (Hardie *et al.*, 1996; Shevchik and Condemine, 1998). GspD also interacts with two inner membrane proteins GspC and GspN, which in turn interact with the inner membrane complex GspM-L (Gérard-Vincent *et al.*, 2002; Lee *et al.*, 2004; Possot *et al.*, 1999). GspC and GspN may thus have a function of connecting the inner membrane platform GspE-F-L-M and the outer membrane GspD secretin. In addition, GspD may interact with the inner membrane proteins GspAB. GspA and GspB are not present in all T2SSs, although they are absolutely required in *A. hydrophila* (Jahagirdar and Howard, 1994). In this bacterium, ExeAB functions to locate/assemble ExeD secretin into the outer membrane, a similar role to that of GspS in GspAB-absent bacteria (Ast *et al.*, 2002). The assembly of GspD secretin and the roles of GspAB in this process are the main objectives of this dissertation and will be addressed in more details in section 1.3.3.

1.2.3 Secretion mechanism of the T2SS

Protein secretion via the T2SS is a two-step process (Poquet *et al.*, 1993; Sandkvist, 2001). The exoproteins are first transported across the inner membrane via the Sec or Tat pathway and then recognized by the T2SS machinery and transported across the outer membrane. In T2SS mutants, the exoproteins are accumulated in the periplasm instead of secreted out of the cell (Pugsley *et al.*, 1991; Jiang and Howard, 1991).

The mechanism of protein secretion by T2SS is highly specific in two aspects. Firstly, the machinery is able to discriminate exoproteins from periplasmic proteins for secretion. Secondly, exoproteins of one bacterium are usually not recognized by other bacteria, with a few exceptions (de Groot *et al.*, 1991; Wong *et al.*, 1990; Frenken *et al.*, 1993; Gerritse *et al.*, 1998). This suggests that the proteins targeted by T2SS must contain specific signals for secretion. However, the exoproteins do not have identifiable consensus sequences either among species or within species except the signal peptide which is cleaved after export via the Sec machinery. Fusion protein technology has been used by many researchers to identify the secretion signal; however, the results are controversial. For example, a fusion protein containing a.a. 60-120 of exotoxin A and the periplasmic β-lactamase was secreted in *P. aeruginosa* (Lu and Lory, 1996). When this region was removed from exotoxin A, however, the protein was still secreted (MacVay and Hamood, 1995). One unique feature of the T2SS is that exoproteins are folded in the periplasm before secretion (Hirst and Holgmgren, 1987; Pugsley *et al.*, 1991; Hardie *et al.*, 1995). It is thus proposed that the signal for secretion might therefore be a 3-D structure created during folding of the protein (Sandkvist, 2001). The tertiary structure property of a secreted protein, but not the primary structure, may be the secretion determinant. To support this notion, the B-subunit of *E. coli* heat-labile enterotoxin, which shares only 11% similarity in sequence but high similarity in tertiary structure with the B-subunit of cholera toxin, was secreted by the T2SS of *V. cholerae* (Connell *et al.*, 1995).

The crystal structures of several T2SS-dependent exoproteins have been solved. Aerolysin, elastase, lipase, neuraminidase, pectate lysase C and cholera toxin B-subunit do not share amino acid similarity and have distinctive functions. They are also very diverse in crystal structure and no apparent common structural motif can be identified. One property of these structures is that they all have a high content of β-sheet; however, no reliable evidence is available to suggest a function for β-sheets during secretion (Sandkvist, 2001). It is thought that some physicochemical properties of the structure, such as charge and hydrophobicity distribution, might be the important factors for recognition by T2SS (Filloux, 2004).

How the T2SS exoproteins find their way to dock at the secretion channel for transport is not clear. The finding that the GspD secretin contains a periplasmic structure, comprised

of the species-specific N-terminal domain, suggests that it may interact with the exoproteins (Chami *et al.*, 2005). The crystal structure of pseudopilin heterotrimeric GspI-J-K indicates that they are likely the "arrow head" of the pseudopilus with the α-helical domain of GspK on the top (Korotkov and Hol, 2008). It is therefore proposed that the α-helical domain of GspK may have a role in the recruitment of exoproteins to the secretin (Forest, 2008). In addition, dedicated chaperones may be involved in the docking process (Filloux, 2004).

The structural similarity between the T2SS pseudopilus and the type IV pilus has implied a mechanism for how exoproteins pass through the secretin channel. The type IV pili are involved in twitching motility which bacteria use to move on solid surface (Burrows, 2005). This involves cycles of extension and extraction of the pili, achieved by assembly and disassembly of pilin subunits through NTPase PilB and NTPase PilT, respectively, of the inner membrane platform (Filloux, 2004; Burrows, 2005). The T2SS pseudopilus is thus proposed to have similar actions to a piston, to push exoproteins through the secretin (Sandkvist, 2001; Forest, 2008). GspE, shown to be an ATPase, shares high similarity with PilB and would probably have the same function (Camberg and Sandkvist, 2005). To support the hypothesis, overexpression of the major pseudopilin GspG is found to result in pilus-like fibers exposed on cell surface, accompanied by interference with secretion (Hu *et al.*, 2002; Sauvonnet *et al.*, 2000; Durand *et al.*, 2003). This implies that GspG is able to assemble into a pilus-like structure; the pseudopilus is directed to the secretion channel and may occlude the channel upon over-assembly. Alternatively, the pseudopilus may have a function to gate the secretin pore rather than actively push the exoproteins (Forest, 2008). Since the pore of the secretin is large (50-100 Å) for passage of folded proteins, it is assumed to be closed to protect the integrity of the cell except when an open channel is required during transport. The retractable pseudopilus may thus control the open or closed status of the secretin. It is also likely that the pseudopilus may induce conformational changes in the secretin to open the pore through interactions with the secretin once the exoproteins are docked.

1.3 The type II secretion system in *A. hydrophila*

1.3.1 Proteins secreted by the T2SS of *A. hydrophila*

A. hydrophila is a Gram-negative bacillus well known as a pathogen of many cold blooded aquatic animals, whereas occasional infections in mammals have been reported

(Hazen *et al.*, 1978; Altwegg and Geiss, 1989). The T2SS of *A. hydrophila* secretes a variety of extracellular protein toxins and degradative enzymes, including aerolysin, amylase, lipase, protease and deoxyribonuclease (Jiang and Howard, 1991).

In particular, aerolysin is an important virulence determinant in rainbow trout and mouse infection models (Chakraborty *et al.*, 1987; and Cascon *et al.*, 2000). This toxin is synthesized as preproaerolysin with an N-terminal signal sequence. After transport across the inner membrane by Sec machinery, the signal sequence is cleaved and the protein folds into dimeric proaerolysin. The inactive proaerolysin is secreted by the T2SS to the extracellular environment where it matures into active aerolysin by proteolytic removal of a C-terminal fragment, either by *A. hydrophila*-secreted or exogenous proteases (Howard and Buckley, 1985). The active dimeric aerolysin is able to bind to eukaryotic cell protein receptors that have a common glycosylphosphatidylinositol (GPI) membrane anchor. Aerolysin dimers then undergo conformational changes to form heptamers that insert into the membranes of the eukaryotic cell, creating large open channels to kill the cell (Buckley, 1991; Rossjohn *et al.*, 1998). The crystal structure of proaerolysin shows a dimeric structure with a high β-sheet content (Parker *et al.*, 1994). Based on electron microscopy, the aerolysin heptamer has been modeled into a mushroom-shaped structure with its stalk spanning the membrane and a central pore of 17 Å in diameter (Rossjohn *et al.*, 1998).

In our laboratory, lysis of red blood cells by aerolysin is used as the major method to examine the function of T2SS in *A. hydrophila*. Other secreted proteins including lipase and protease are also assayed for functional analysis.

1.3.2 *exe* operons encoding the T2SS of *A. hydrophila*

In *A. hydrophila*, the *gsp* genes encoding T2SS components are termed *exe* genes (Jiang and Howard, 1991; Jahagirdar and Howard, 1994). They are clustered at two separate chromosomal locations as operon *exeC-N* and operon *exeAB* as shown in Fig. 6 (page 12). They were identified by complementation studies of aerolysin secretion mutants isolated by Tn5-751 transposon insertion mutagenesis. One of the mutant strains is C5.84, which contains the insertion in the *exeAB* operon and is used in this thesis to study the functions of ExeAB. The *gspO* gene is not found with the *exe* clusters; instead, *tapD*, encoding the prepilin peptidase of type IV pilus, is required to process the T2SS pseudopilins (Pepe *et al.*,

1996). In addition, genes encoding homologues of GspS are not found in *A. hydrophila*.

1.3.3 Roles of ExeA and ExeB in the T2SS of *A. hydrophila*

Although homologues of ExeAB are not found in all bacteria, they are essential for normal type II secretion in *A. hydrophila* (Jahagirdar and Howard, 1994). Insertion of Tn5-751 transposon in *exeA* eliminated the production of both ExeA and ExeB. However, marker exchange mutagenesis confirmed that both of the proteins are required for secretion.

The hydrophobicity profile as well as cell localization and alkaline phosphatase reporter studies showed that both ExA and ExeB are trans-inner membrane proteins with domains exposed on the periplasmic and cytoplasmic sides of the membrane (Jahagirdar and Howard, 1994; Howard *et al.*, 1996). The 60 kDa ExeA is comprised of a 31 kDa N-terminal cytoplasmic domain, a short central hydrophobic inner membrane domain and a 28 kDa C-terminal periplasmic domain. The 25 kDa ExeB resides mainly in the periplasm with only a small N-terminal domain exposed on the cytoplasmic side. The two proteins form a complex, demonstrated by co-stabilization, cross-linking, co-immunoprecipitation and co-purification (Howard *et al.*, 1996; Schoenhofen *et al.*, 1998).

The role of the ExeAB complex was first implied from the finding that the cytoplasmic domain of ExeA contains Walker A and Walker B motifs, which are conserved in Walker superfamily of ATPases (Walker *et al.*, 1992; Howard *et al.*, 1996). In addition, this domain also contains a motif kinase-3a that is commonly conserved in kinases. Mutagenesis studies showed that this putative ATP binding site is required for normal function of ExeA (Howard *et al.*, 1996; Schoenhofen *et al.*, 1998). The cytoplasmic domain of ExeA was then purified and shown to have ATPase activity *in vitro* (Schoenhofen *et al.*, 2005). ExeB shares a low sequence similarity with TonB, which is required for energy transduction in the uptake of siderophores and other molecules across the outer membrane (Howard *et al.*, 1996; Krewulak and Vogel, 2008). ExeA and ExeB were thus proposed to act together to transduce metabolic energy to the secretion process, for example, in opening the channel of the ExeD secretin or in assembly of the secretion apparatus (Howard *et al.*, 1996). However, GspE, a critical component conserved in all T2SSs, has also been found to have ATPase activity and suggested to provide the energy required for secretion (Camberg and Sandkvist, 2005). It is therefore necessary to explain the redundant energy sources in *A. hydrophila*, especially

when GspAB is absent in many T2SSs. It is likely that the energy provided by ExeAB is not for the general secretion process, but for an event that can be achieved in distinct ways by different bacteria.

A breakthrough in understanding of the role of ExeAB was provided by the studies of Ast *et al.*, 2002. The ExeAB complex was found to play an important role in ExeD secretin assembly. In ExeAB mutants, ExeD accumulated in the inner membrane as a monomer instead of being assembled into the outer membrane as the multimeric secretin. ExeAB may not be involved in the secretion process itself, because when ExeD was overproduced, resulting in a few multimers assembled in the outer membrane through leakage or non-specific pathways, secretion was restored even in the absence of ExeAB. The role of exeAB in secretin assembly may be achieved through interactions between ExeA and peptidoglycan, a barrier to trans-envelope apparatus assembly, since a putative peptidoglycan-binding motif has been identified in the periplasmic C-terminus of ExeA (Howard *et al.*, 2006). Mutations at the putative peptidoglycan-binding motif could abolish ExeD secretin assembly and therefore aerolysin secretion without abrogation of ExeAB complex formation. The role of ExeAB in the assembly of ExeD secretin is therefore proposed to involve local remodelling of peptidoglycan, either by direct hydrolysis or interference with normal peptidoglycan biogenesis, to allow ExeD to traverse across the peptidoglycan barrier and assemble in the outer membrane (Howard *et al.*, 2006).

Homologues of GspAB are found in some but not all species that have the T2SS. In *Erwinia* species and *K. oxytoca*, only GspB homologues OutB and PulB, respectively, but not GspA homologues, are present (Condemine and Shevchik, 2000; Possot *et al.*, 2000). OutB is required for normal secretion. In an OutB mutant, secretion was restored when OutD was overproduced, a phenomenon also observed in ExeA mutants of *A. hydrophila* (Condemine and Shevchik, 2000; Ast *et al.*, 2002). PulB, in contrast, was not required for pullulanase secretion when the *K. oxytoca* Pul system was expressed in *E. coli* (Possot *et al.*, 2000). However, we have to notice that the *pul* genes expressed in *E. coli* were encoded in multi-copy plasmids under the control of maltose-inducible promoters. This might cause overproduction of PulD, which has been shown to suppress GspAB/B mutations in *A. hydrophila* and *E. chrysanthemi* (Condemine and Shevchik, 2000; Ast *et al.*, 2002). It is therefore necessary to control the expression level of *pul* genes in order to better understand

the role of PulB. GspAB homologues are also found in *Vibrio* species and *E. coli* (Francetic and Pugsley, 1996; Howard *et al.*, 2006). Their role in the T2SS needs to be further clarified.

In bacteria that do not have GspA, GspS may perform a similar function. In *E. chrysanthemi*, an OutS mutant failed to insert the OutD secretin into the outer membrane (Shevchik and Condemine, 1998). In addition, OutS was found to interact with the 62 C-terminal amino acids of OutD. Consistent with this, PulS was reported to play a role in piloting PulD secretin into the outer membrane (Hardie *et al.*, 1996; Guilvout *et al.*, 2006). The PulS-interacting site was also identified at the 65 C-terminal residues of PulD (Daefler *et al.*, 1997). These findings are quite similar to those observed for ExeA of *A. hydrophila*, although GspS and GspA are distinct proteins. GspS is a lipoprotein that is associated with the outer membrane (d'Enfert and Pugsley, 1989; Shevchik and Condemine, 1998). It is proposed that GspD in the inner membrane is co-sorted into the outer membrane by the lipoprotein sorting pathway, a system transporting lipoproteins to the outer membrane (Tokuda and Matsuyama, 2004; Guilvout *et al.*, 2006). These facts may therefore explain why GspA is not required in these bacteria. In bacteria that do not have GspA and GspS, for example, *X. campestris* and *P. aeruginosa*, other pathways may be involved in sorting GspD into the outer membrane.

The exact role(s) of GspAB and GspS are not clear. Do they solely translocate the GspD into the outer membrane or are they also involved in assembly of the secretin? It has been reported that PulD was able to insert into the inner membrane and formed ring-like particles corresponding to the secretin as viewed by electron microscopy (Guilvout *et al.*, 2006). They further showed that PulD inserted and formed secretin-like structures in lecithin lipids when the protein was produced in a cell-free *in vitro* transcription–translation system (Guilvout *et al.*, 2008). It was therefore concluded that PulD has an inherent ability to insert as multimers into lipid bilayers. However, we have to note that these findings were based on overexpression of PulD either *in vivo* or *in vitro*. They might therefore not present the physiological facts *in vivo*. A few reports indicate that the assembly of the T2SS secretin requires other factors. One of these factors is the outer membrane protein Omp85/YaeT, which is required for general outer membrane protein insertion and/or multimerization, for example, for porins, TolC and the PilQ secretin (Voulhoux *et al*., 2003; Doerrler and Raetz, 2005; Werner and Misra, 2005). Other factors include outer membrane lipoproteins QilW

and Tgl, which are required for PilQ secretin assembly in *Neisseria meningitides* and *Myxococcus xanthus*, respectively (Carbonnelle *et al.*, 2005; Nudleman *et al.*, 2006). Lipoprotein YscW homologues are required for T3SS secretin assembly (Daefler and Russel, 1998; Crago and Koronakis, 1998; Burghout *et al.,* 2004; Cornelis, 2006). In addition, special components of the outer membrane, such as lipopolysaccharide (LPS), may be involved in the assembly process.

1.4 Peptidoglycan as a barrier in trans-envelope apparatus assembly

1.4.1 Structure of peptidoglycan

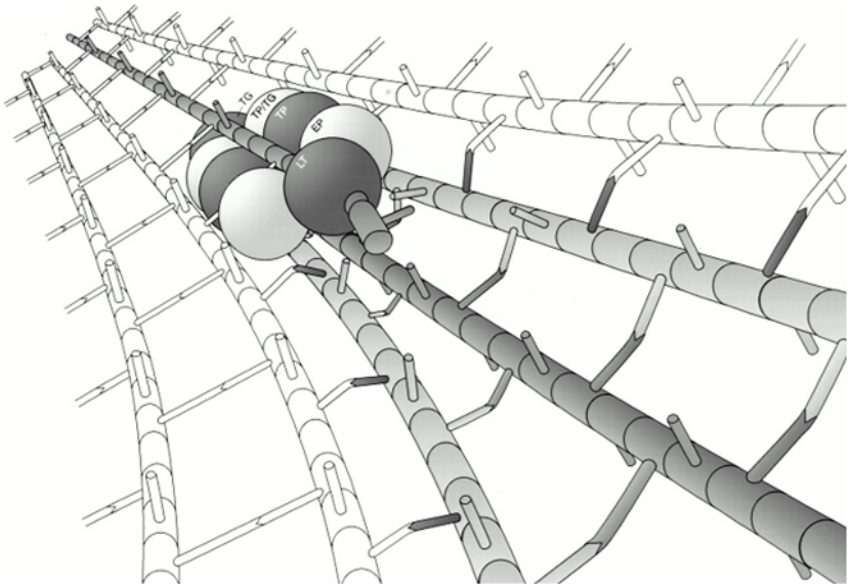
Peptidoglycan, also known as murein, is a net-like structure made of glycan strands cross-linked by short peptides and can be hydrolyzed by mutanolysin or other N-acetylmuramidases into N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) disaccharide peptide subunits called muropeptides (Schleifer and Kandler, 1972; Glauner, 1988; Nakimbugwe *et al.*, 2006). The sugars are linked with β-1,4 glycosidic bonds. The peptides are distinctive in that they contain D-amino acid residues not found in proteins. Certain modifications or variations may be present in peptidoglycan, for example, N-deacetylation, O-acetylation, N-glycolylation, peptide cross-linking variations and number of layers (Vollmer *et al.*, 2008). The structures of *E. coli* peptidoglycan and the commonest muropeptide disaccharide tetrapeptide are illustrated in Fig. 8. Peptidoglycan usually contains *meso*-2,6-diaminopimelic acid (*m*-A_2pm or *m*-DAP) to D-Ala cross-links and is a multi-layer structure located outside of the cytoplasmic membrane in Gram-positive bacteria, a thin layer in the periplasm in Gram-netative bacteria. One exception is *Staphylococcus aureus*, in which the peptidoglycan contains Lys-penta-Gly cross-links.

1.4.2 Biosynthesis of peptidoglycan

Peptidoglycan is assembled with disaccharide muropeptide units (reviewed by Höltje, 1998). The precursors of muropeptide are synthesized via a linear pathway of 12 steps from fructose-6-P to lipid intermediates in the cytoplasm (reviewed by van Heijenoort, 2007). The lipid intermediates include Lipid I and Lipid II. Lipid I is comprised of MurNAc-pentapeptide linked with diphosphate undecaprenyl. The hydrophobic undercaprenyl moiety

Fig. 8 Structures of *E. coli* peptidoglycan and muropeptide. A. Net-like peptidoglycan structure with a peptidoglycan synthesis complex in the "three-for-one" model. The shaded triple glycan strands beneath the synthesis complex are newly synthesized and replace the top existing glycan strand called the docking strand. TG, transglycosylase; TP/TG, bifunctional transpeptidase-transglycosylase; TP, transpeptidase; EP, endopeptidase; LT, lytic transglycosylase. B. Glycan strands of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) disaccharide peptides (muropeptides) cross-linked by *m*-A_2pm-D-Ala peptide bonds. *m*-A_2pm, 2,6-diaminopimelic acid. C. Structure of the most abundant muropeptide disaccharide tetrapeptide. The figures are modified from Höltje (1998), Scheffers and Pinho (2005) and Vollmer and Bertsche (2008) with permissions from the American Society for Microbiology and the Elsevier Limited.

A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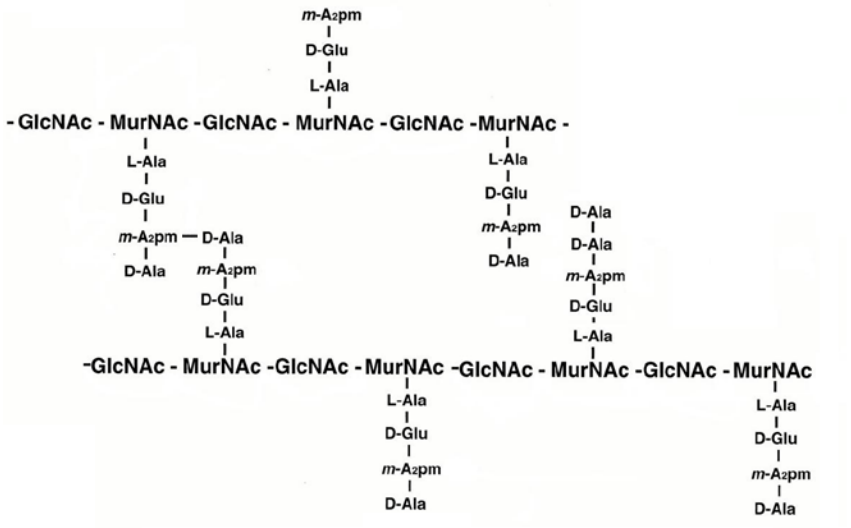
TPTG
TP
EP
LT

B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D-Ala
m-A2pm
D-Glu
L-Ala
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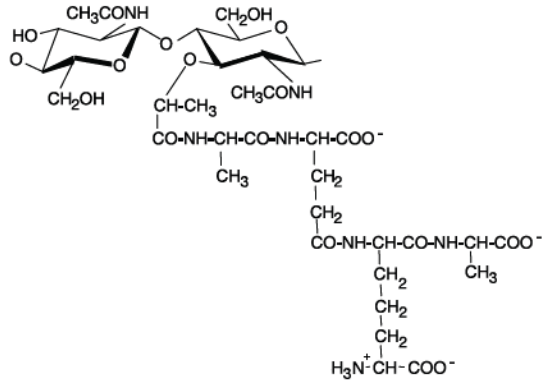
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CH2OH
HO
O
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CH-CH3
CH3CONH
CO-NH-CH-CO-NH-CH-COO-
CH3
CH2
CH2
CO-NH-CH-CO-NH-CH-COO-
CH3
CH2
CH2
CH2
H3N+CH-COO-

is anchored in the cytoplasmic membrane, with the hydrophilic moiety MurNAc-pentapeptide facing the cytoplasm. Addition of GlcNAc to the MurNAc of Lipid I yields Lipid II, which is then transferred by an unknown pathway or flipase across the cytoplasmic membrane with the disaccharide pentapeptide moiety exposed to outside of the membrane for peptidoglycan assembly.

Polymerization of the disaccharide muropeptide involves two major reactions: transglycosylation for synthesis of glycan stands and transpeptidation for formation of peptide cross-links (reviewed by Höltje, 1998). The two reactions are catalyzed by transglycosylases and transpeptidases, respectively. One interesting fact is that bacteria have multiple enzymes that carry one or both of the above activities, for example, monofunctional transglycosylase (TG), monofunctional transpeptidase (TP), bifunctional transglycosylase and transpeptidase (TG/TP). In addition, peptidoglycan lytic enzymes are also required for release and recycling of old peptidoglycan and shape control of the peptidoglycan sacculus. These enzymes include D,D-endopeptidase (EP), D,D-carboxypeptidase (CP), lytic transglycosylase (LT) and other lytic enzymes. It has been questioned why bacteria have so many "redundant" enzymes in peptidoglycan biosynthesis, as illustrated by the presence of 12 penicillin-binding proteins (PBPs) in *E. coli* (Macheboeuf *et al.*, 2006). PBPs, which have long been the targets of penicillin and other β-lactam antibiotics, are transpeptidases, endopepidases and carboxypeptidases that recognize and act on the D-Ala-D-Ala moiety of muropeptides, which share a similar structure with β-lactam antibiotics (Fisher *et al.*, 2005). The functions of these enzymes are not the subject of this thesis, but the complexity of the structure, modifications and enzymology illustrate that the biogenesis of peptidoglycan is not a simple process and that peptidoglycan is not a homogenous structure. Peptidoglycan synthesis, hydrolysis and modification are dynamic activities involved in cell elongation, septation and trans-envelope structure assembly.

The well-accepted model for murein growth is the "three-for-one" model (Höltje, 1998). In this model, new triple glycan strands are synthesized and attached underneath an existing glycan strand called the docking strand, followed by removal of the docking strand by hydrolases to insert of the triplet (Fig. 8A, page 23). The insertion of three glycan strands and removal of one docking strand enable the cell to double in length if every second glycan strand serves as a docking strand. This model follows the principle of "make-before-break",

in which new peptidoglycan should be synthesized before old peptidoglycan is removed in order to maintain cellular integrity (Koch, 1995). It also explains the high murein turnover rate in which half of the peptidoglycan is broken down in each cell generation in *E. coli* (Goodell, 1985). The major peptidoglycan synthesis is proposed to be carried out by a membrane-bound multi-enzyme complex, comprised of transglycosylase, transpeptidase, bifunctional transglycosylase/transpeptidase, endopeptidase and lytic transglycosylase for different functions, such as synthesis of the central glycan strand of the triplet, synthesis of the side glycan strands of the triplet and attachment of the triplet to the docking strand by formation of peptide cross-links and removal of the docking strand (Höltje, 1998). The complex may include different enzymes for peptidoglycan synthesis at different cell locations (septum and pole) and interact with the cell cytoskeleton for cell shape maintenance (Scheffers and Pinho, 2005).

During the synthesis of new glycan strands, Lipid II is used as the substrate to attach the disaccharide pentapeptide via β-1,4 glycosidic bond to the growing glycan strand that will reach a mean length of 25-35 disaccharide units in *E. coli* (Harz *et al.*, 1990). Glycan strand growth is terminated by cleavage of the diphosphate undecaprenyl moiety and release of the glycan strand with a 1,6-anhydroMurNAc end. It should be noted, however, that peptidoglycan synthesis is a continuous progress in growing cells. According to the "three-for-one" model, if glycan strands are evenly synthesized during cell life, three of every five glycan strands (including the non-docking stand) are immature and still linked with the lipid moiety. Given the average length of glycan strands in *E. coli*, 1.7%-2.4% of muropeptides are linked with the lipid moiety. Taking into account that bacterial cells have two growth phases of elongation and septation, the actual amount of lipid moiety-linked muropeptides may be lower than the above numbers; however, they should not be neglected in peptidoglycan-related studies.

1.4.3 The peptidoglycan barrier in protein transport and large apparatus assembly

The rigid peptidoglycan structure is essential to protect the cell from osmotic burst; however, it also presents a problem in the transport of proteins or the assembly of large trans-envelope machineries (Dijkstra and Keck, 1996). In *E. coli*, the holes of peptidoglycan are believed no larger than 4 nm and can only pass globular proteins smaller than 50 kDa

(Demchick and Koch, 1996). It was therefore postulated that local hydrolysis of peptidoglycan could be involved in assembly of a trans-envelope apparatus and transport of macromolecules (Koraimann, 2003). To support this hypothesis, many peptidoglycan hydrolyzing proteins have been found to play such a function, for example, FlgJ in flagellar assembly, P19 in conjugation system, PleA in pili assembly and PtlE in T4SS (Nambu *et al.*, 1999; Hirano *et al.*, 2001; Bayer *et al.*, 2001; Pucciarelli and Garcia-del Portillo, 2003; Viollier and Shapiro, 2003). In addition, protein-peptidoglycan interactions were also reported in T3SS (Rambow-Larsen and Weiss, 2002). To create gaps on peptidoglycan should also be involved in T2SS apparatus assembly, for example, the role of ExeAB to allow ExeD traverses into the outer membrane where the secretin is assembled (Howard *et al.*, 2006).

It should be noted that direct hydrolysis of peptidoglycan by these assembly-specific enzymes, for example, FlgJ and other lytic transglycosylases, is a simple way but not the only way to create gaps on peptidoglycan (Koraimann, 2003; Scheurwater *et al.*, 2008). Some non-hydrolytic proteins may play such a role by recruiting peptidoglycan-lytic enzymes to the assembly site. Alternatively, locally inhibition of peptidoglycan-synthetic enzymes may generate gaps during peptidoglycan growth. Other routes may include modification of peptidoglycan to change its susceptibility to lytic and synthetic enzymes.

1.5 Purpose of this dissertation

The previous studies in Dr. Howard's laboratory have shown that the role of ExeAB in the T2SS of *A. hydrophila* is to locate and/or assemble the ExeD secretin into the outer membrane (Ast *et al.*, 2002). The C-terminal domain of ExeA was found to contain a putative peptidoglycan binding motif. Mutagenesis of this motif abolished the normal function of this protein without interfering with ExeAB complex formation (Howard *et al.*, 2006). The ExeA-peptidoglycan interactions were thus proposed to play a role in the assembly of ExeD secretin. The focus of this research is to use biochemical and bioinformatical approaches to examine the interactions between ExeA and peptidoglycan *in vivo* and *in vitro*. In addition, the interactions between ExeA and other Exe components are also addressed.

2. EXPERIMENTAL PROCEDURES

2.1 Bacterial strains, plasmids and culture conditions

A. hydrophila Ah65 is the prototypical wild type stain used in our laboratory and in this thesis. Its derivative C5.84 is an *exeA*::Tn5-751 insertion mutant that is unable to produce ExeA (Jahagirdar and Howard, 1994). Because of a polar effect, this strain cannot produce ExeB either. *E. coli* BL21 (DE3) was used for expression of constructed C-terminal/periplasmic domains of ExeA and its derivatives (pExeAs). *E. coli* XL1-Blue was used for production of plasmid constructs. For purification of peptidoglycan, *A. hydrophila* C5.84, *E. coli* BL21 and *Bacillus subtilis* ATCC 6633 were used. See Table 1 for a complete list of strains.

Plasmid constructs encoding *exeAB* were pRJ31.1 (wild type, Jahagirdar and Howard, 1994) and pCGs that contain substitution mutations for three highly conserved amino acids in the putative peptidoglycan binding motif in the C-terminal domain of ExeA (pCG1: F487S, pCG2: L493S and pCG3: G500D, Howard *et al.*, 2006), with IPTG inducible promoters in the wild-host-range vector pMMB207 (Morales *et al.*, 1991). Plasmid constructs encoding His tagged wild type and mutant pExeAs are pN-His-pExeA, pC-His-ExeA, pN-His-pCGs and pC-His-pCGs in pET30a vector with an IPTG inducible promoter (This study). See Table 2 for a complete list of plasmids.

A. hydrophila strains were grown at 30 ºC to an OD_{600} of 1.8-2.0 in Luria-Bertani (LB) media buffered with 30 mM Na_2HPO_4, 30 mM K_2HPO_4, 16.5 mM NaH_2PO_4, 16.5 mM KH_2PO_4, 0.75 mM $(NH_4)_2SO_4$ and 0.4 mM $MgSO_4$ in cross-linking studies (Ast *et al.*, 2002). IPTG (0.04 mM) was added to induce plasmid-encoded ExeAB. For production of pExeAs, *E. coli* was grown at 35 ºC in 2xYT media to an OD_{600} of 0.6-0.8 and induced for two hours by adding 0.4 mM IPTG. For purification of peptidoglycan, *A. hydrophila*, *E. coli* and *B. subtilis* were grown in 2xYT to an OD_{600} of 1.5-1.8 at 30 ºC (*A. hydrophila*) or 37 ºC. Appropriate antibiotics were used at the following concentrations: ampicillin (Ap), 100 μg ml^{-1}; chloramphenicol (Cm), 1.25 μg ml^{-1}; kanamycin (Kn), 50 μg ml^{-1}; tetracycline (Tc), 10 μg ml^{-1}.

2.2 Construction of *pexeA* plasmids

DNA fragments encoding N-His tagged and C-His tagged pExeAs were amplified by

Table 1. Bacterial strains used in this study.

Strain	Genotype/phenotype	Source
A. hydrophila		
Ah65	Wild type; Nal^r; St^r; Ap^r	This laboratory
C5.84	Ah65 *exeA*::Tn5-751; Km^r; Nal^r; St^r; Ap^r	This laboratory
E. coli		
BL21 (DE3)	F^- *ompT hsdS*$_B$(r_B-m_B-) *gal dem* (DE3)	Novagen*
XL1-Blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' *proAB lacI*^{q}*ZΔM15* Tn10]; Tc^r	Stratagene*
B. subtilis		Dept. Microbiology and
ATCC 6633	Wild type	Immunology, U of S

Navagen*, Madison, USA; Stratagene*, La Jolla, USA.

Table 2. Plasmids used in this study.

Plasmid	Description	Source
PMMB207	Low-copy-number, wide-range-host plasmid; *tac* promoter; Cm^r	Morales *et al.*, 1991
pBluescript II SK/KS+	High-copy-number phagemid; *lac* promoter; Ap^r	Stratagene
pET30a	oriColE1; T7*lac* promoter; Km^r	Novagen
pRJ31.1	*exeAB* 2.5 kb *Bst*X1 in *Sma*I of pMMB207	Jahagirdar and Howard, 1994
pCG1	*exeA* F487S of pRJ31.1	Howard *et al.*, 2006
pCG2	*exeA* L493S of pRJ31.1	Howard *et al.*, 2006
pCG3	*exeA* G500D of pRJ31.1	Howard *et al.*, 2006
pN-His-pExeA	*N-His pexeA Nde*I-*Xho*I PCR fragment from pRJ31.1 in pET30a	This study
pN-His-pCG1	*N-His pexeA* F487S *Nde*I-*Xho*I PCR fragment from pCG1 in pET30a	This study
pN-His-pCG2	*N-His pexeA* L493S *Nde*I-*Xho*I PCR fragment from pCG2 in pET30a	This study
pN-His-pCG3	*N-His pexeA* G500D *Nde*I-*Xho*I PCR fragment from pCG3 in pET30a	This study
pC-His-pExeA	*C-His pexeA Nde*I-*Xho*I PCR fragment from pRJ31.1 in pET30a	This laboratory
pC-His-pCG1	*C-His pexeA* F487S *Nde*I-*Xho*I PCR fragment from pCG1 in pET30a	This study
pC-His-pCG2	*C-His pexeA* L493S *Nde*I-*Xho*I PCR fragment from pCG2 in pET30a	This study
pC-His-pCG3	*C-His pexeA* G500D *Nde*I-*Xho*I PCR fragment from pCG3 in pET30a	This study

PCR with Pfu DNA polymerase (Fermentas, Burlington, Canada) from pRJ31.1 or pCGs with the following primers. *Nde*I, *Xho*I, hexa Histidine-tag, and stop codon are underlined accordingly.

For N-His tagged,

US18 (lig) 5'-CATATGCACCATCATCATCATCATCAGTTCTTCGGCTTCTTCCCC

UR154 5'-CTCGAGTCAGGAAGCCTCCTCCGACAATGTG

For C-His tagged,

UR156 5'-CATATGCAGTTCTTCGGCTTCTTCCCCGAAC

UR124 5'-CTCGAGGGAAGCCTCCTCCGACAATGTGAC

The PCR was performed with the following program: 95 ºC for 1 min, 55 ºC for 1 min and 72 ºC for 1 min, 28 cycles, followed by 72 ºC for 3 min. After agarose-purification, the PCR fragments were inserted into pBluescript II SK+ vectors with blunt ends at the *Eco*RV site by T4 DNA ligase (Invitrogen, Carlsbad, USA). The ligation mixtures were electroporated to *E. coli* XL1-Blue cells and screened for white colonies on LB agar plates supplemented with IPTG and X-gal. The recombinant plasmids were extracted and the *Nde*I-*Xho*I fragments cut out with the restriction enzymes, followed by insertion of the agarose-purified fragments into pET30 vectors and electroporation into XL1-Blue cells. The recombinant plasmids, as identified by restriction digestion, were sequenced to rule out the possibility of unwanted mutations and then electroporated into *E. coli* BL21 (DE3) cells for protein production. The plasmid construct for C-His pExeA was made by Melody Harrington, a former summer student in Dr. Howard' laboratory. Minipreparations of plasmid DNA were obtained using the alkaline lysis method (Sambrook *et al.*, 1989). PCR and restriction DNA fragments were isolated in 0.8 % agarose gels and purified with an Ultraclean 15 kit (Mo Bio Laboratories, Carlsbad, USA).

2.3 Purification of pExeAs

E. coli BL21 (DE3) containing *pexeA* plasmids were grown at 35 °C to an OD_{600} of 0.6-0.8 and induced with 0.4 mM IPTG. After two hours, the bacteria were harvested by centrifugation and stored at -20 °C. The pellets were resuspended in NTA buffer A (20 mM Tris, pH 8.0, 250 mM NaCl, 10 mM imidazole and 10% glycerol) at 10 ml per 200 ml cell culture. After addition of 100 µg/ml RNase, 1 µg/ml DNase and 1 protease inhibitor cocktail

tablet (Roche, Mississauga, Canada) per 50 ml cell resuspension, the cells were disrupted by two passes through a French Press at 14,000 lb/in^2, followed by centrifugation at 40,000 x *g* for 40 min to separate the pExeA-containing supernatants from cell debris. The samples were applied to HisTrap affinity columns (GE Healthcare, Giles, UK) and pExeAs were eluted with a gradient of NTA buffer B (20 mM Tris, pH 8.0, 250 mM NaCl, 500 mM imidazole and 10% glycerol). After desalting into ion exchange buffer A (20 mM Tris, pH 8.0, 25 mM NaCl, 0.2 mM PMSF, 1 mM EDTA and 10% glycerol), the samples were applied to a Resource Q column (GE Healthcare), eluted with a gradient of ion exchange buffer B (20 mM Tris, pH 8.0, 500 mM NaCl, 0.2 mM PMSF, 1 mM EDTA and 10% glycerol). The chromatography was performed on a FPLC system (GE Healthcare) at 4 °C. The protein preparations were examined by SDS-PAGE for purity. Protein concentrations were determined by measuring absorbance at UV_{280} with theoretical extinction coefficients obtained with ProtParam software. The proteins were stored at 4 °C and used in *in vitro* studies.

2.4 SDS-PAGE, CBB staining and immunoblotting

Protein samples were electrophoresed on 12% SDS-PAGE gels (Laemmli, 1970), except that 10% gels were used for cross-linked samples. For Coomassie Brilliant Blue R250 (CBB) staining, the gels were soaked in stain solution (0.125% CBB, 25% v/v isopropanol and 10% v/v acetic acid) for half an hour, followed by destain solution 1 (0.0025% CBB, 25% isopropanol and 10% acetic acid) for one hour, destain solution 2 (0.0005% CBB, 10% isopropanol and 10% acetic acid) overnight and destain solution 3 (10% acetic acid) until the background was destained satisfactorily. For immunoblotting, proteins were transferred to PVDF membranes in cold Tris-glycine transfer buffer containing 20% methanol. Visualization of transferred proteins was achieved by blocking the membranes in 5% casein, incubating with appropriate rabbit antisera, incubating with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Sigma) and developing with HRP chemiluminescent substrate luminal and enhancer (SuperSignal West Pico kit, Pierce, Rockford, USA) or ECL Advance (ECL Advance kit, GE Healthcare).

2.5 *In vivo* cross-linking

Cleavable cross-linker 3,3′-dithiobis[sulfosuccinimidylpropionate] (DTSSP, Pierce) was used for cross-linking analysis of ExeA and peptidoglycan *in vivo*. *A. hydrophila* cells grown to an OD_{600} of 1.8-2.0 were washed and resuspended in phosphate buffered saline (PBS, pH 7.5), and adjusted to an OD_{600} of 2.0. Concentrated fresh DTSSP solutions were made in 5mM sodium citrate (pH 5.0) and added to a final concentration of 0.5 mM (Schoenhofen *et al.*, 1998). The cross-linking mixtures were incubated at room temperature for 0 to 10 min and quenched by adding 50 mM Tris (pH 8.0). The cross-linked cells were added to an equal volume of boiling 8% SDS solution and incubated for 15 min in a boiling water bath. After cooling to room temperature, the samples were ultracentrifuged at 130,000 x *g* for one hour at room temperature to pellet peptidoglycan (Hancock and Poxton, 1998). The pellets were resuspended in water and the SDS-washing step was repeated two additional times. The peptidoglycan samples were resuspended in water to 10% of the original culture volume. Before loading on SDS-PAGE gels, the whole cell and peptidoglycan samples were mixed with 2 x SDS sample buffer containing 0 or 10% β-mercaptoethanol and heated at 95 °C for 5 min.

2.6 Purification of peptidoglycan

A. hydrophila C5.84 cells were grown in 2xYT at 30 °C to an OD_{600} of 1.8-2.0. To purify non-denatured peptidoglycan, the cells were disrupted by one pass through a French Press at 14,000 lb/in^2 (psi), followed by a low speed spin of 5,000 x *g* for 15 min to remove unbroken cells and a high speed spin of 40,000 x *g* for one hour to pellet cell envelopes (Sprott *et al.*, 1994). The cell envelopes were washed with 2% Triton X-100 containing 20 mM $MgCl_2$ and then 2% Triton X-100 containing 5 mM EDTA to remove membrane lipids (Schoenhofen *et al.*, 1998). After each wash, peptidoglycan was collected by ultracentrifugation at 130,000 x *g* for one hour. After four washes with water, the peptidoglycan was resuspended in water and stored at 4 °C. All these steps were performed at 4 °C to reduce peptidoglycan autolysis.

Boiling SDS and pronase treated peptidoglycan samples were prepared as described by Glauner, 1988 with some modifications. *A. hydrophila* or *E. coli* cells were resuspended in 10 ml boiling 4% SDS solution per 250 ml cell culture and incubated with boiling for one

hour with vigorous stirring. After standing at room temperature overnight with continuous stirring, the peptidoglycan was collected by ultracentrifugation at 130,000 x *g* for one hour, followed by an additional wash in boiling 4% SDS for 15 min. The peptidoglycan pellets were washed four times with water to remove SDS, followed by resuspension in 10 ml water per litre cell culture and incubation with 100 µg/ml α-amylase (Roche) in 10mM Tris (pH 7.0) at 37 °C for two hours to remove glycogen trapped in the peptidoglycan sacculi. Pre-incubated pronase was then added to a final concentration of 200 µg/ml and the samples incubated at 60 °C for 90 min to hydrolyze proteins associated with the peptidoglycan. The pronase (Roche) was pre-incubated at 60 °C for two hours to inactivate possible muramidase contamination. The samples were treated with boiling 4% SDS solution for 15 min. After four washes with water, the peptidoglycan pellets were resuspended in water and stored at 4 °C. All of the above steps were performed at room temperature unless otherwise indicated to avoid SDS precipitation.

B. subtilis peptidoglycan was purified as described by Bacher *et al.* (2001) with some modifications. The cells were washed with boiling 4% SDS solution as described in *A. hydrophila* peptidoglycan purification except that the peptidoglycan was collected by centrifuge at 40,000 g. After four washes with water, the samples were treated with 2 mg/ml pre-incubated pronase in 50 mM Tris (pH 7.0) at 60 °C for 90 min. After one wash with water, the pellets were resuspended in 2 ml concentrated hydrofluoric acid per litre cell culture and incubated overnight on ice to remove anionic polymers such as lipoteichoic, teichoic and teichuronic acids covalently bound to peptidoglycan. The hydrofluoric acid was neutralized by adding 12 volumes of cold 2 M KOH solution and briefly cooled on ice, due to heat generated in the neutralization reaction. The samples were washed three times with water to remove the salts and then incubated with 10 µg/ml RNase and 10 µg/ml DNase in 10 ml 50 mM Tris (pH 7.5) with 2.5 mM $MgCl_2$ and 0.2 mM $CaCl_2$ per litre cell culture to degrade nucleic acids trapped in the peptidoglycan sacculi. The enzymes and degraded nucleic acids were removed by one wash with boiling 1% SDS solution at 100 °C for 15 min. After four washes with water, the peptidoglycan pellets were resuspended in water and stored at 4 °C. All of the above steps were performed at room temperature unless otherwise indicated to prevent SDS precipitation.

2.7 Muramic acid assay of peptidoglycan

Peptidoglycan was quantitated by measuring its muramic acid content with the method of Hadzija (1974), modified by Hoijer *et al.* (1995). 80 µl peptidoglycan samples were hydrolyzed with an equal volume of 5 M H_2SO_4 at 90 °C for two hours. After adding 360 µl H_2O and 140 µl 10 M NaOH, the samples were incubated at 37 °C for 30 min to release lactic acid from the muramic acid residues of the peptidoglycan. The lactic acid concentration was then assayed in the following colorimetric reaction. Samples of 300 µl were moved to new glass tubes in duplicate. After 2 ml concentrated H_2SO_4 (18.8 M) was added, the tubes were incubated in a boiling water bath for 5 min and then cooled in a room-temperature water bath. $CuSO_4 \cdot 5H_2O$ (20 µl 4% w/v in H_2O) and 4-phenylphenol (Sigma, 40 µl 1.5% w/v in ethanol) were added, followed by incubation at 30 °C for 30 min to develop a blue colour measured at 570 nm wavelength. Muramic acid (Sigma) solutions (0 to 1 mM) were used as standards. The assays were performed in duplicate and the average absorbance was used to calculate muramic acid concentrations, except for the assays of peptidoglycan gel filtration fractions, in which single measurement was used.

2.8 Cosedimentation and cosedimentation inhibition assays

100 µM muramic acid units of peptidoglycan preparations were incubated with purified pExeA proteins of various concentrations in 150 µl 40 mM sodium phosphate buffer (pH 6.5), 0.05% Tween 20 and 10 µg/ml bovine albumin at 4 °C for one hour. The mixtures were centrifuged at 21,000 x *g* at 4 °C for one hour to pellet the peptidoglycan. The mixture, supernatant and pellet samples were applied to SDS-PAGE gels and immunoblotted with ExeA antiserum. In cosedimentation inhibition assays, pExeA proteins were incubated with peptidoglycan fragments at 4 °C for one hour before intact *A. hydrophila* peptidoglycan was added.

2.9 Dynamic Light Scattering

Dynamic Light Scattering (DLS) was carried out on a Protein Solutions/DynaPro instrument (Wyatt, Santa Barbara, USA) at the Saskatchewan Structure Science Centre (SSSC). N-His pExeA (2 mg/ml) in 40 mM sodium phosphate (pH 6.5 or 8.0) and 150 mM NaCl were filtered through 10 µm membranes before being added to the cuvette.

Measurements were performed at a range of temperatures from 4 ºC to 37 ºC with 5 min incubation at each temperature. At least 20 valid readings were recorded for each measurement. The protein sample was also incubated at 37 ºC for one hour for multimerization analysis by DLS. The average particle size was calculated with Dynamics software. The particle size distribution was analyzed with DynaLS software.

2.10 Gel filtration chromatography

All gel filtration experiments were performed at 4 °C with a flow rate of 0.5 ml/min on a FPLC system (GE Healthcare). For self-multimerization studies, 2 mg/ml N-His pExeA in 40 mM sodium phosphate (pH 6.5) and 150 mM NaCl was incubated at 37 °C for one hour, followed by incubation at 4 °C overnight. 0.5 ml samples were applied to a Superdex 200 10/300 GL column (GE Healthcare) in the same buffer. For gel filtration of peptidoglycan fragments, 1ml of 7 mM muramic acid units of *B. subtilis* peptidoglycan were hydrolyzed by 1,000 units of mutanolysin from *Streptomyces globisporus* (Sigma) in 40 mM sodium phosphate (pH 6.5) at 37 °C overnight, followed by incubation at 95 °C for 20 min to inactivate the enzyme. Insoluble material was removed by centrifugation at 21,000 x *g* at 4 °C for one hour. 0.5 ml of the supernatant that contained peptidoglycan fragments was applied to a Superose 6 10/300 GL column (GE Healthcare) in the same buffer and fractionated into 0.5 ml fractions. For multimerization of pExeA in the presence of intact peptidoglycan, N-His pExeA (42 nM) was incubated with 250 µM muramic acid units of *A. hydrophila* peptidoglycan in 40 mM sodium phosphate (pH 6.5) and 0.05% Tween 20 at room temperature for one hour, followed by incubation with 50 µg/ml lysozyme at room temperature for four hours. After centrifugation at 21,000 x *g* for one hour to remove unhydrolyzed peptidoglycan, 0.5 ml of the sample or the non-peptidoglycan control were applied to a Superdex 200 10/300 GL column (GE Healthcare) in the same buffer and fractionated into 0.5 ml fractions. The fractions were then immunoblotted to determine the pExeA distribution. For multimerization of pExeAs in the presence of peptidoglycan fragments, 80 nM protein concentrations were incubated with the peptidoglycan gel filtration fractions 38-40 (200 µl of each, total 650 µl) in 40 mM sodium phosphate (pH 6.5) and 0.05% Tween 20 at 4 °C for one hour. 0.5 ml of the mixtures were applied to a Superose 6 10/300 GL column (GE Healthcare) in the same buffer and collected into 1 ml fractions. The

fractions were immunoblotted to determine the pExeA distribution. Both of the columns were calibrated with a molecular weight marker kit (Sigma). 2000 kDa: blue dextran; 669 kDa: thyroglobulin (bovine); 440 kDa: apoferritin (horse spleen); 200 kDa: β-amylase (sweet potato); 150 kDa: alcohol dehydrogenase (yeast); 66 kDa: bovine serum albumin; 29 kDa: carbonic anhydrase (bovine erythrocytes); 12.4 kDa: cytochrome c (horse heart).

2.11 Native gradient PAGE

Native gradient PAGE was performed as described by Schagger (2001) with some modifications. The electrophoresis was run in a Mini-PROTEAN II system (Bio-Rad, Hercules, USA). Three-stage gradient acrylamide gels were made by adding the following solutions in sequence. A: 2.5 ml 18% T, B: 0.5 ml 1:1 mixed 18% T + 3% T, C: 3% T. 18% T and 3% T were prepared with 30% T 3% C (T = Total acrylamide-bisacrylamide; C = Crosslinker bisacrylamide) solution and 6 x gel buffer (150 mM imidazole, adjusted to pH 7.0 with HCl). 18% T also contained 20% glycerol. After polymerization was complete, the gels were electrophoresed at 4 °C. N-His pExeA samples were mixed with 2 x sample buffer containing 100 mM imidazole/HCl, pH 7.0, 0.002% Coomassie Blue G-250 and 20% glycerol before loading. The anode buffer contained 25 mM imidazole/HCl, pH 7.0 and the cathode buffer contained 50 mM Tricine and 7.5 mM imidazole (resulting in a pH of approximately 7.0). For the electrophoresis, 100 V was applied for two hours. The proteins were visualized by Coomassie Brilliant Blue staining as described for SDS-PAGE gels.

2.12 *In vitro* cross-linking

N-His pExeA (60 nM) was incubated with 10 µl peptidoglycan gel filtration fragments in 15 µl 40 mM sodium phosphate (pH 6.5) and 0.05% Tween 20 at 4 °C for one hour. After an equal volume of 50 mM sodium phosphate, pH 8.5 containing 0 to 5 mM DTSSP (Pierce) was added, resulting in a final pH of 7.2, the mixtures were incubated on ice for 30 min and mixed with 2 x SDS-PAGE sample buffer (no β-mercaptoethanol added) to stop the cross-linking. The samples were applied to 10 % SDS-PAGE gels, transferred to poly vinylidene difluoride (PVDF) membranes and immunoblotted with ExeA antiserum.

3. RESULTS

3.1 *In vivo* cross-linking of ExeA, ExeB and ExeC to peptidoglycan

3.1.1 Cross-linking of ExeA to peptidoglycan

The presence of a putative peptidoglycan binding domain (Pfam number PF01471, analyzed in more detail in section 3.6) at the C-terminus of ExeA suggests that this protein may functionally interact with peptidoglycan. This hypothesis was examined by *in vivo* cross-linking. Wild type *A. hydrophila* Ah65 cells were incubated with 0.5 mM cleavable cross-linker DTSSP for 0 to 10 min, followed by extraction of peptidoglycan with boiling SDS (Hancock and Poxton, 1988). The peptidoglycan samples, as well as the whole cell samples, were immunoblotted for the presence of ExeA (Fig. 9A). In the absence of reducing agent, the cross-linked whole cell samples showed that the ExeA monomer band was shifted into much higher molecular weight bands, which might include the ExeAB complex as demonstrated previously (Schoenhofen *et al*., 1998). In the presence of β-mercaptoethanol, the high molecular weight ExeA-containing bands disappeared and the monomer band appeared again in the immunoblot, confirming the cross-linking by DTSSP. The immunoblot also shows that ExeA was found in the peptidoglycan samples isolated from cross-linked cells in the presence of β-mercaptoethanol but not in its absence or in the non-cross-linked control. This suggests that ExeA was cross-linked to peptidoglycan by DTSSP. Because peptidoglycan cannot enter SDS-PAGE gels due to its large size, ExeA cross-linked to peptidoglycan remained out of the gel unless the cross-linker was broken by treatment with β-mercaptoethanol before electrophoresis. As a control, the major lipoprotein (Lpp), a known peptidoglycan associated protein, was also found to cross-link to peptidoglycan (Fig.9A).

To control for the possibility that the cross-linking of ExeA with peptidoglycan was an artifactual linkage of nonassociated components, *A. hydrophila* cells expressing mutant ExeAs that contained substitution mutations in the putative peptidoglycan binding motif (pCG1: F487S, pCG2: L493S and pCG3: G500D) were also used in the cross-linking studies (Fig. 9B). In this experiment, the wild type ExeA was again present in the cross-linked peptidoglycan, however, the three mutants were present in much reduced quantities.

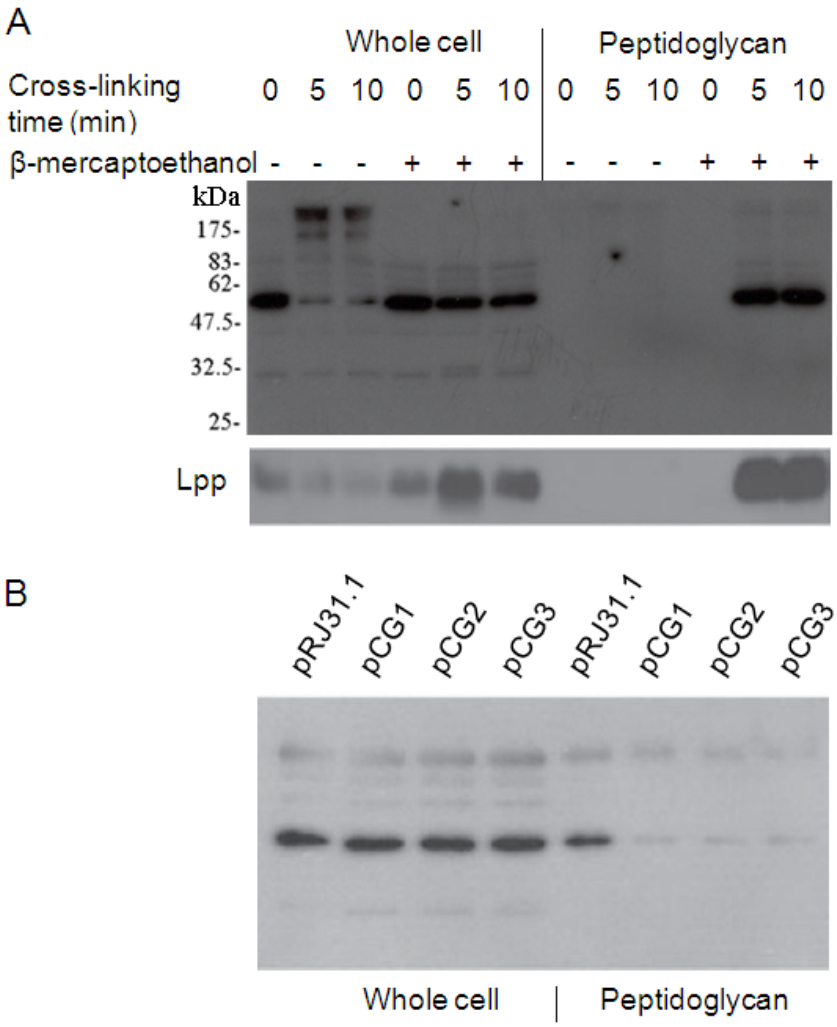


Fig. 9 *In vivo* cross-linking of ExeA to peptidoglycan. A. Cross-linking of ExeA to peptidoglycan. *A. hydrophila* Ah65 cells were cross-linked with 0.5 mM DTSSP for 0 to 10 min, followed by isolation of peptidoglycan by boiling SDS treatment. Whole cell and peptidoglycan samples were treated with or without β-mercaptoethanol before being applied to SDS-PAGE and immunoblotted with ExeA antiserum. The samples were also immunoblotted for the major lipoprotein (Lpp) at the bottom of the panel. B. Cross-linking of ExeA variants to peptidoglycan. *A. hydrophila* C5.84 cells expressing wild type (pRJ31.1) or putative peptidoglycan binding motif mutant ExeAs (pCG1, pCG2 and pCG3) were cross-linked with 0.5 mM DTSSP for 5 min. Whole cell and peptidoglycan samples were treated with β-mercaptoethanol before electrophoresis and anti-ExeA immunoblot.

This demonstrates that the cross-linking of ExeA to peptidoglycan was specific and required the putative peptidoglycan binding domain.

3.1.2 Co-crosslinking of ExeB and ExeC to peptidoglycan

ExeA and ExeB have been shown to cross-link into a heterodimeric complex *in vivo* (Schoenhofen *et al*., 1998). We therefore examined if ExeB was also present in the peptidoglycan samples isolated from cross-linked cells. The immunoblot shows that ExeB was detected in the peptidoglycan sample of wild type ExeA (pRJ31.1) but not in that of mutant ExeA (pCG3) (Fig. 10B). This result confirms the ExeAB complex reported previously (Schoenhofen *et al*., 1998). It also suggests that ExeB was co-crosslinked to peptidoglycan through ExeA, or alternatively, the cross-linking of ExeB to peptidoglycan required a functional ExeA. Similarly, ExeC was found co-crosslinked to peptidoglycan in the presence of wild type ExeA (pRJ31.1) but not in its absence (pCG1, pCG2 and pCG3) (Fig. 10A). These cross-linking studies suggest an ExeA-B-C complex associated with peptidoglycan.

3.2 *In vitro* cosedimentation of pExeAs with peptidoglycan

3.2.1 Construction and purification of pExeAs

Since ExeA is an insoluble membrane protein, the C-terminal/periplasmic domain of ExeA (pExeA), from a.a. 296 to a.a. 547, was cloned with a His-tag at the N-terminal end (N-His pExeA) or at the C-terminal end (C-His pExeA). pExeAs with substitution mutations of three highly conserved amino acid residues at the putative peptidoglycan binding motif (N-His pCG1, N-His pCG2, N-His pCG3, C-His pCG1, C-His pCG2 and C-His pCG3) were also constructed. The linear maps of the pExeA variants are shown in Fig. 11. *pexeA* fragments were PCR amplified from pRJ31.1 or pCGs and inserted into the *Nde*I-*Xho*I sites of the pET30a vector. All constructs were sequenced to rule out the possibility of unwanted mutations before being electroporated into *E. coli* BL21 (DE3) cells for expression. The plasmid for C-His pExeA was constructed by Melody Harrington, a former summer student in Dr. Howard's laboratory.

Cells producing pExeAs were disrupted by French Press and the proteins were purified by His-tag affinity chromatography and ion exchange chromatography. All pExeA

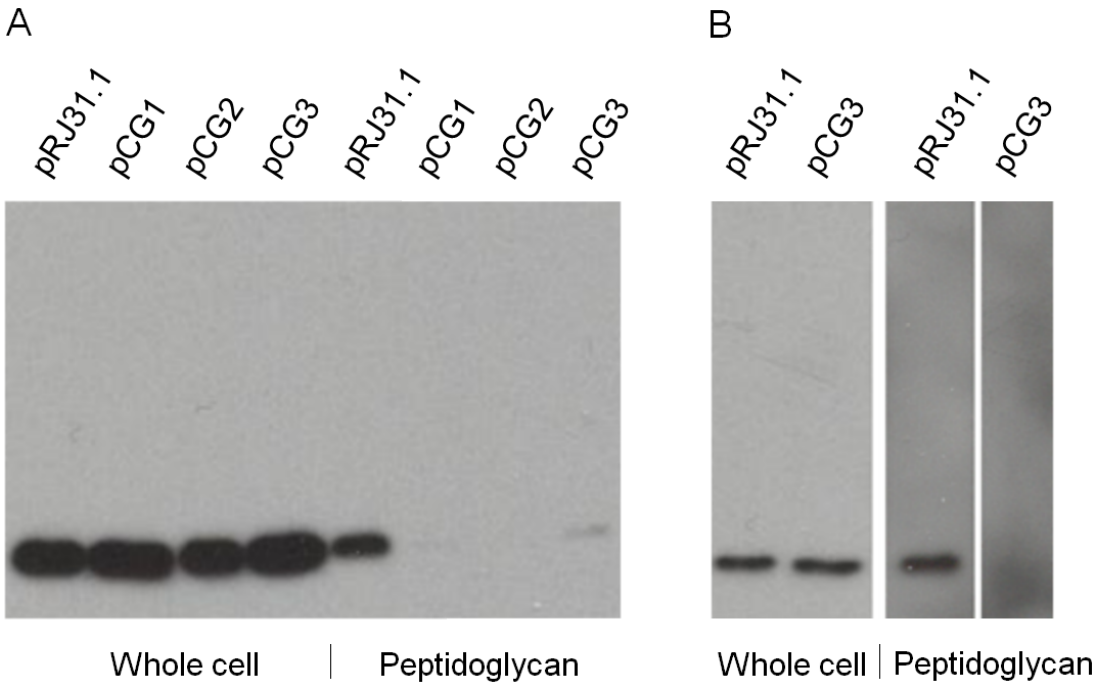


Fig. 10 Co-crosslinking of ExeB and ExeC to peptidoglycan. *A. hydrophila* C5.84 strains expressing wild type ExeA (pRJ31.1) and mutant ExeAs (pCG1, pCG2 and pCG3) were cross-linked with 0.5 mM DTSSP before isolation of peptidoglycan by boiling SDS treatment. Whole cell and peptidoglycan samples were treated with β-mercaptoethanol before SDS-PAGE and immunoblotting with ExeC antiserum (A) and ExeB antiserum (B).

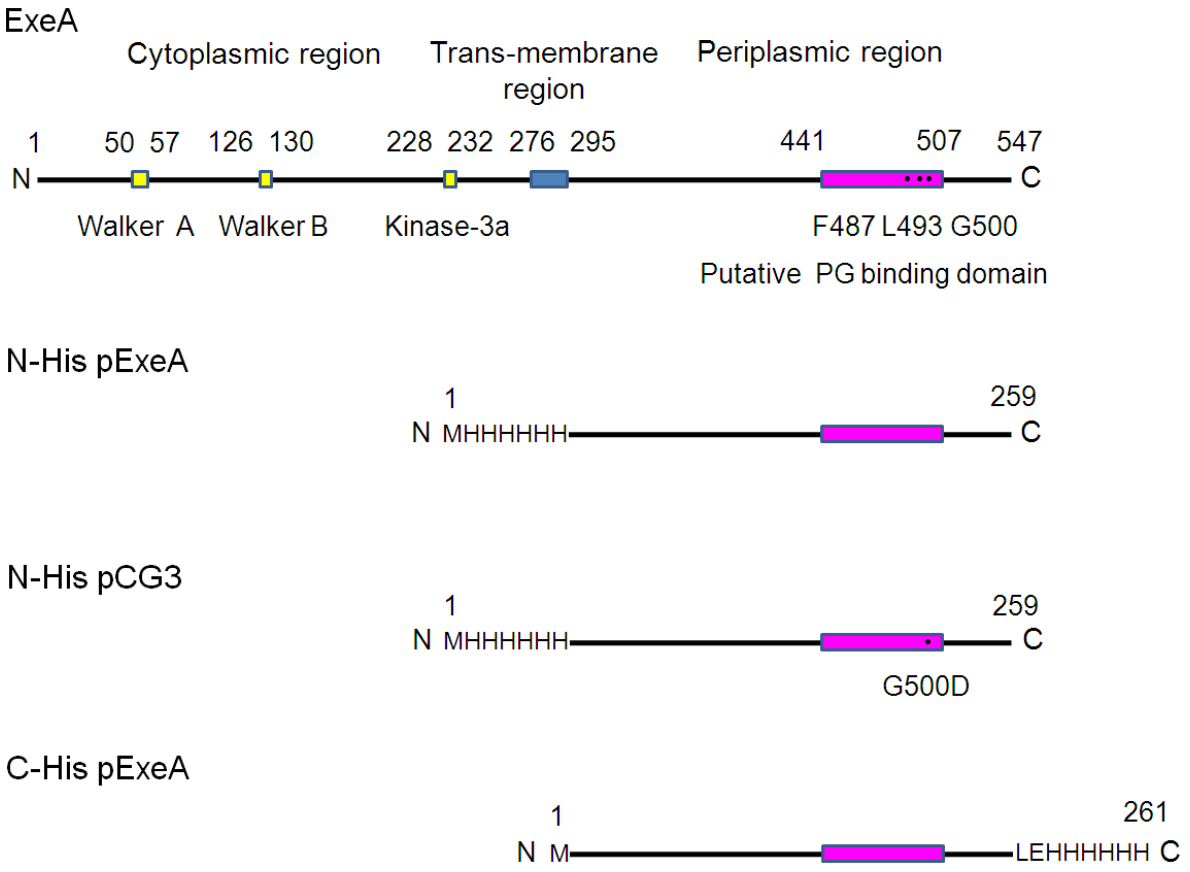


Fig. 11 Linear maps of ExeA and pExeA constructs. The cytoplasmic region, trans-membrane region and periplasmic region of ExeA are drawn to scale with amino acid residue numbers indicated. The ATP binding/hydrolysis motifs Walker A, Walker B and kinase-3a are indicated in yellow. The hydrophobic trans-membrane region is marked in blue. The putative peptidoglycan binding domain is marked in pink. The three highly conserved amino acid residues subjected to mutagenesis are indicated, corresponding to pCG1 (F487S), pCG2 (L493S) and pCG3 (G500D), respectively. N- or C-His tagged pExeA constructs containing the C-terminal 296-547 residues of ExeA are also illustrated.

preparations were examined on SDS-PAGE for purity. Their theoretical extinction coefficients at 280 nm were obtained with ProtParam software and used to calculate protein concentrations. Purified N-His pExeA, C-His pExeA and N-His pCG3 are shown in Fig. 12. N-His pCG1, N-His pCG2, C-His pCG1 and C-His pCG2 were found to be unstable and fractionated as multiple peaks in ion exchange chromatography (data not shown). It is likely that the two mutations F487S and L493S interfered with the normal folding of this protein (see more details in section 3.6.2). They were therefore not used in the following binding studies.

3.2.2 Non-denaturing and denaturing purification of peptidoglycan

The routine method to purify peptidoglycan involves extensive treatment in boiling SDS solutions (Glauner 1988; Hancock and Poxton, 1988). The initial *in vitro* cosedimentation studies of pExeA and SDS-purified peptidoglycan failed to show convincing interactions between them (data not shown). To address the possibility that the interactions required peptidoglycan-associated proteins, non-denatured peptidoglycan was isolated and used in cosedimentation studies to compare with denatured peptidoglycan for ability to interact with pExeA. ExeAB mutant stain *A. hydrophila* C5.84 was used to purify peptidoglycan samples to avoid possible interference caused by native ExeA.

Non-denatured peptidoglycan was prepared by washing cell envelopes with cold 2% Triton X-100 solutions containing 20 mM $MgCl_2$ and 5 mM EDTA, separately, to remove membrane lipids (Schoenhofen *et al*., 1998). Theoretically, covalently bound proteins, such as the major lipoprotein, and tightly non-covalently bound proteins should remain in the peptidoglycan sample. Denatured peptidoglycan was prepared by extensive washing in boiling 4% SDS and treatment with pronase to remove all proteins linked to peptidoglycan, as described by Glauner, 1988. Aliquots of Triton X-100-treated, SDS-treated and SDS/pronase-treated peptidoglycan preparations were hydrolyzed overnight with lysozyme before analysis on SDS-PAGE, because proteins linked to peptidoglycan could not to enter the gel. As shown in Fig. 13, the Triton X-100-treated peptidoglycan sample contained many protein contaminants; in contrast, little or no protein contamination was found in the SDS-treated and SDS/pronase-treated samples.

To quantitate peptidoglycan samples, a colorimetric method was used to measure the

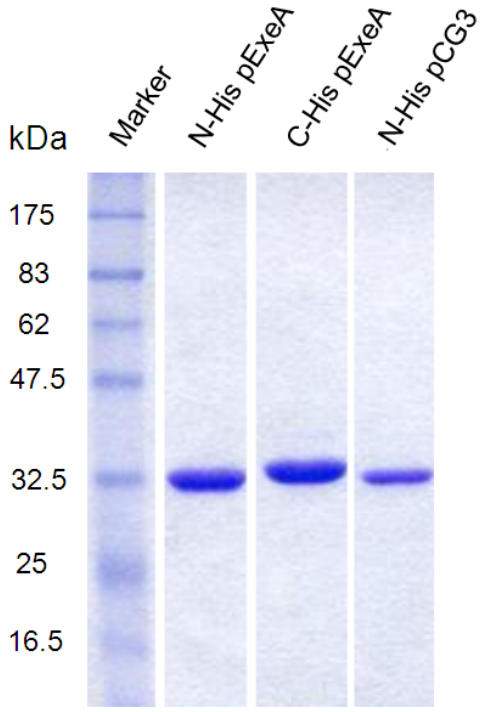


Fig. 12 Coomassie Brilliant Blue stained SDS-PAGE gel of purified pExeAs. His-tagged pExeA proteins N-His pExeA, C-His pExeA and N-His pCG3 were purified by His-tag affinity chromatography and ion exchange chromatography. The samples were applied to a SDS-PAGE gel and stained with Coomassie Brilliant Blue R250.

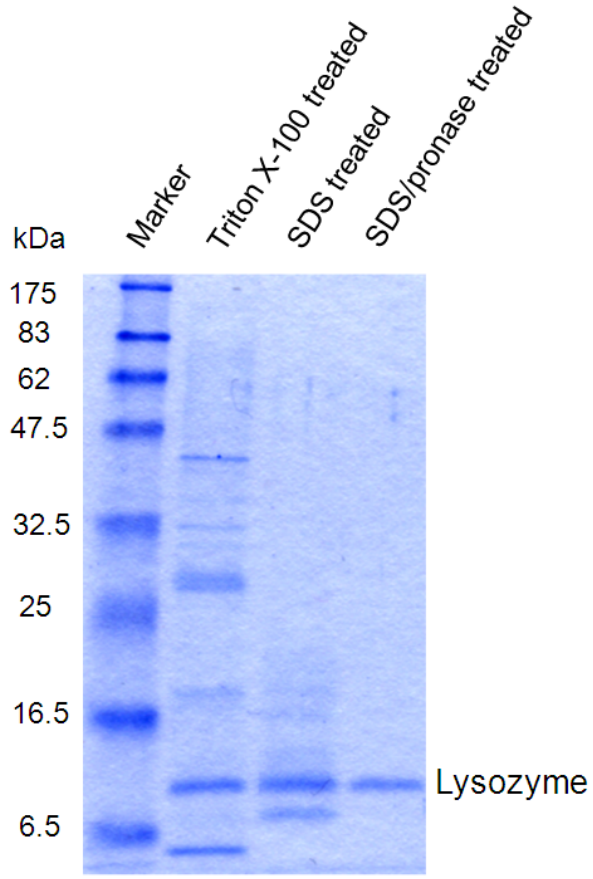


Fig. 13 Coomassie Brilliant Blue stained SDS-PAGE gel of non-denatured and denatured peptidoglycan preparations. Non-denatured peptidoglycan sample was isolated from *A. hydrophila* C5.84 cells by treatments with 2% Triton X-100 solutions containing 20 mM $MgCl_2$ and 5 mM EDTA, separately. Denatured peptidoglycan samples were isolated by boiling 4% SDS treatment and pronase digestion treatment (Glauner 1988). The samples were hydrolyzed with lysozyme before SDS-PAGE. The gel was stained with Coomassie Brilliant Blue R250.

lactic acid released from the muramic acid residues of peptidoglycan upon acidic and alkaline treatments (Hadzija, 1974; Hoijer *et al.*, 1995). A blue colour is developed when copper is reduced by the lactic acid in the presence of 4-phenylphenol in concentrated H_2SO_4. The yield of *A. hydrophila* peptidoglycan was about 2 millimole muramic acid units per litre per OD of cell culture, or 2 mg $L^{-1} \cdot OD^{-1}$ if the average muropeptide molecular weight is 1 kDa, comparable to the yield of 2.08 mg $L^{-1} \cdot OD^{-1}$ from *E. coli* reported by Glauner (1988).

In addition, peptidoglycan samples were also purified from *E, coli* and the Gram-positive *Bacillus subtilis* with boiling SDS treatment for *in vitro* pExeA-peptidoglycan studies. The yield of *B. subtilis* peptidoglycan was about 25 mg $L^{-1} \cdot OD^{-1}$.

3.2.3 Cosedimentation of pExeAs with non-denatured peptidoglycan

Peptidoglycan purified with boiling SDS treatment was first used in cosedimentation studies with pExeAs. In 40 mM sodium phosphate (pH 6.5), wild type N-His pExeA and mutant N-His pCG3 were both able to cosediment with the denatured peptidoglycan preparation, detected by SDS-PAGE and Coomassie Brilliant Blue stain (data not shown). However, when 0.05% Tween 20 was added, no cosedimentation could be observed with either protein (data not shown), suggesting non-specific interactions in the cosedimentation experiments. It is possible that the pExeA-peptidoglycan interactions were too weak to detect by Coomassie Brilliant Blue stain. It is also possible that the interactions required peptidoglycan-associated proteins which were denatured during SDS treatment. To examine these possibilities, non-denatured peptidoglycan and more sensitive immunoblot were used in the following cosedimentation studies.

Wild type (N-His pExeA) and mutant (N-His pCG3) pExeA proteins (10 nM) were incubated with 100 μM muramic acid units of non-denatured *A. hydrophila* peptidoglycan in 40 mM sodium phosphate containing 0.05% Tween 20 at 4 °C for one hour, followed by centrifugation to recover peptidoglycan. The mixture, supernatant and pellet samples were examined by immunoblot with ExeA antiserum. Fig. 14 shows that N-His pExeA was found in the pellet sample; however, N-His pCG3 cosedimented to a much lower degree, suggesting specific cosedimentation and involvement of the putative peptidoglycan binding domain in these interactions.

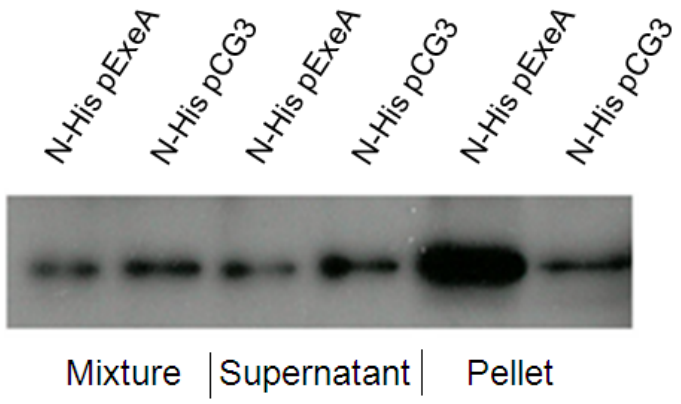


Fig. 14 Cosedimentation of pExeAs with non-denatured peptidoglycan. 10 nM N-His ExeA (wild type) and N-His pCG3 (mutant) were mixed with 100 μM muramic acid units of *A. hydrophila* peptidoglycan, purified by Triton X-100 treatments, in 40 mM sodium phosphate (pH6.5) and 0.05% Tween 20 at 4 °C for one hour, followed by centrifugation to pellet the peptidoglycan. The mixture, supernatant and pellet samples were applied to SDS-PAGE and immunoblotted with ExeA antiserum. The pellet samples were 30 times concentrated during resuspension.

3.2.4 Cosedimentation of pExeAs with denatured peptidoglycan

To examine if proteins, lipids or other impurities included in the non-denatured peptidoglycan sample were actually involved in the interactions with pExeA, peptidoglycan samples prepared with treatments known to denature or degrade proteins, such as boiling SDS and pronase hydrolysis (Glauner, 1988), were used in the cosedimentation assays. The effects of these treatments were confirmed in Fig. 13 (page 45) as little or no proteins were found in these samples. The result of cosedimentation with the pure peptidoglycan sample is shown in Fig. 15. Once again, N-His pExeA, but not the mutant N-His pCG3, was present in the pellet sample of Triton X-100 treated *A. hydrophila* peptidoglycan. A similar amount of N-His pExeA was also found in the pellet sample of SDS/pronase treated peptidoglycan; however, the mutant protein bound much less. This indicates that pExeA interacted with the peptidoglycan backbone but not with proteins or lipids associated with peptidoglycan. It was noticed that the mutant pExeA cosedimented much more with the pure peptidoglycan, possibly because of elimination of impurities that prevent weak interactions. SDS/pronase treatment was therefore used in all peptidoglycan purifications in the following studies.

These results imply that the ExeA-binding component of peptidoglycan accounts for only a small part of the peptidoglycan, because a 10,000 fold excess of muramic acid units of peptidoglycan are required to bind the small amount of pExeA detected on the immunoblot. This could also be caused by very low affinity pExeA-peptidoglycan interactions. Our trials for affinity analysis did not yield a satisfactory binding curve. This was partially due to the limitations of the cosedimentation assay, in which the peptidoglycan forms large pellets that could non-specifically trap proteins, because of its rigid net-like structure. It may also have been caused by the complicated nature of the pExeA-peptidoglycan interactions, which have been shown to be accompanied by multimerization of the protein (see section 3.5).

To examine if pExeA interacts with a universal peptidoglycan structure, peptidoglycan samples extracted from *E. coli* and the Gram-positive *B. subtilis* were also used in cosedimentation assays. Fig. 15 shows that similar results for N-His pExeA and N-His pCG3 were obtained with the *E. coli* peptidoglycan preparation. However, the *B. subtilis* sample failed to cosediment the protein (data not shown). Further analysis indicated that N-His pExeA could interact with *B. subtilis* peptidoglycan after it had been hydrolyzed by

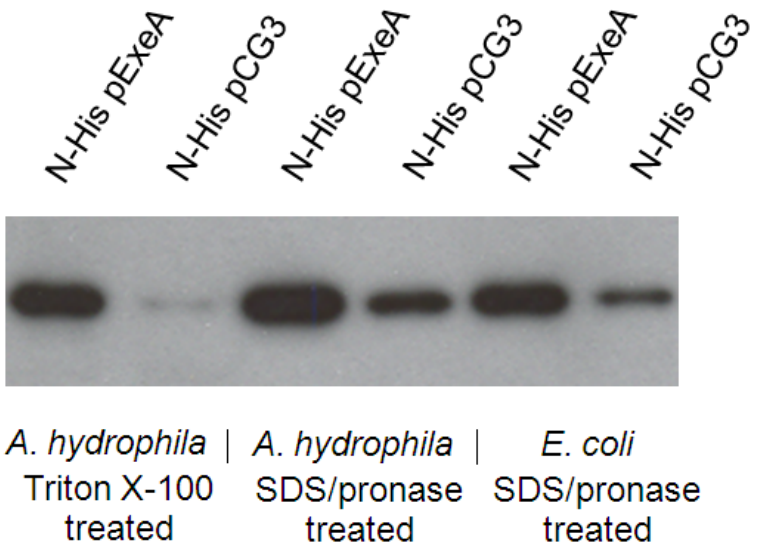


Fig. 15 Cosedimentation of pExeAs with different peptidoglycan preparations. Peptidoglycan samples were prepared by Triton X-100 treatment or SDS/pronase treatment from *A. hydrophila* or *E. coli* cells as indicated. The cosedimentation was performed as described previously, except that only pellet samples were analyzed by SDS-PAGE and ExeA immunoblot.

mutanolysin into small fragments (see later in sections 3.4 and 3.5). A possible explanation could be that the multi-layer structure of *B. subtilis* peptidoglycan prevented access of the protein to the binding sites or its multimerization. These results suggest that pExeA could interact with a peptidoglycan structure conserved in different bacteria, consistent with the widespread occurrence of the putative peptidoglycan binding domain in a variety of species.

C-His tagged pExeA was also examined for its ability to interact with peptidoglycan (Fig. 16). Surprisingly, the addition of the tag to the C-terminal side of the protein almost completely abrogated cosedimentation, so that it bound much less than even the mutant N-His pCG3. This may be because the putative peptidoglycan binding domain (a.a. 441–507) is located near the C-terminal end of pExeA (a.a. 296–547). The C-His tag might hinder peptidoglycan from accessing its binding site on pExeA, or alternatively, affect the normal folding and 3-D structure of this domain. In the following studies, C-His pExeA was therefore used as a negative control that had lost its ability to interact with peptidoglycan.

3.2.5 Cosedimentation at various pHs and salt concentrations

Cosedimentation of pExeA and peptidoglycan was examined in buffers of different pHs and salt concentrations. Fig. 17 shows that the cosedimentation was pH-dependent. At pH 7.4 or above, very little N-His pExeA cosedimented with peptidoglycan, whereas at pH 6.0, the cosedimentation was highest. These results were not due to precipitation of the protein at lower pH, as indicated by non-peptidoglycan controls (data not shown). The theoretical pI of pExeA is 4.8. At lower pHs, the protein should be less negatively charged. The charges may possibly affect pExeA-peptidoglycan interactions or pExeA-pExeA interactions. The latter will be analyzed in more detail in sections 3.3 and 3.5. The periplasmic environment is slightly acidic, as protons diffuse into the periplasm from outside of the cell due to a Donnan equilibrium that causes uneven distribution of charges across the outer membrane (negative inside), resulting in a pH of around 6.5 (Dhungana *et al.*, 2003). In addition, bacteria are known to release protons into the periplasm to establish protonmotive force as an energy source (Mitchell, 1966). The pH requirement observed for pExeA-peptidoglycan interactions is therefore consistent with the physiological environment.

The cosedimentation was also affected by salt concentration (Fig. 18). N-His pExeA showed decreased cosedimentation at 50 mM NaCl and above. It should be noted that the

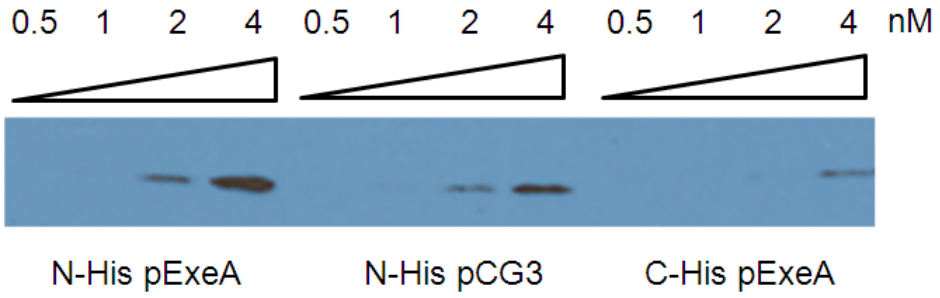


Fig. 16 Cosedimentation analysis of N- and C-His tagged pExeAs. N-His pExeA, N-His pCG3 and C-His pExeA (0.5 to 4 nM) were incubated with 100 μM muramic acid units of *A. hydrophila* peptidoglycan in the cosedimentation assay as described previously, except that only pellet samples were analyzed on SDS-PAGE and immunoblotted with ExeA antiserum.

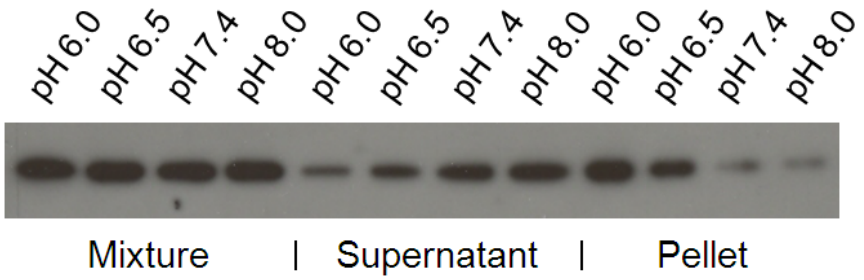


Fig. 17 Cosedimentation of pExeA with peptidoglycan at various pHs. N-His pExeA (10 nM) was incubated with 100 μM muramic acid units of *A. hydrophila* peptidoglycan in 40 mM sodium phosphate of pH 6.0, 6.5, 7.4 and 8.0 with 0.05% Tween 20. After centrifugation, the pellets were resuspended in a same volume of buffer. The mixture, supernatant and pellet samples were applied to SDS-PAGE and immunoblotted with ExeA antiserum.

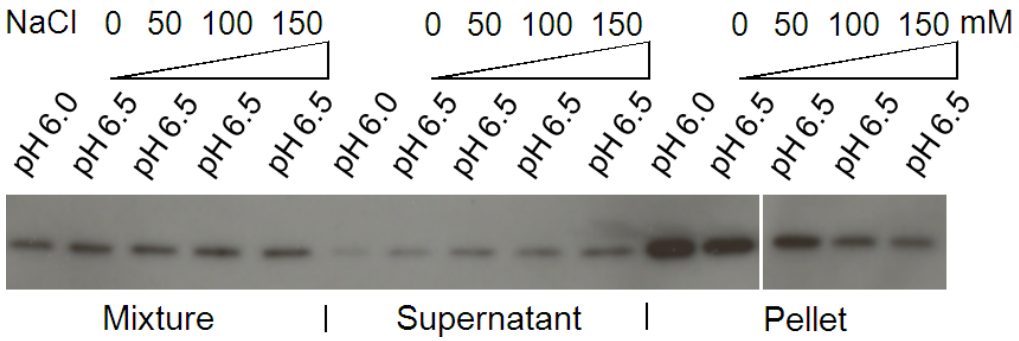


Fig. 18 Cosedimentation of pExeA with peptidoglycan at various salt concentrations. N-His pExeA (10 nM) was incubated with 100 µM muramic acid units of *A. hydrophila* peptidoglycan in 40 mM sodium phosphate of pH 6.0 and 6.5 with 0.05% Tween 20 and 0 to 150 mM NaCl. The pellets samples were five times concentrated during resuspension. The mixture, supernatant and pellet samples were applied to SDS-PAGE and immunoblotted with ExeA antiserum.

buffers used in these assays contained 40 mM sodium phosphate, weakening the possibility that the cosedimentation was caused by non-specific interactions.

3.3 Multimerization of pExeA at elevated temperatures

3.3.1 Analysis of pExeA multimerization by DLS

Multimerization of pExeA was first observed when the protein was examined with Dynamic Light Scattering (DLS) for crystallization purpose. DLS analyzes scattered light intensity fluctuation to derive the hydrodynamic radius (R_H) of molecules. R_H is then used to calculate molecular mass (Murphy, 1997). N-His pExeA samples in 40 mM sodium phosphate buffers of pH 6.5 and 8.0, 150 mM NaCl were measured at the range of temperatures from 4 ºC to 37 ºC with 5 min incubation at each temperature point. The result of temperature scanning is shown in Fig. 19. At 4 °C, the 29 kDa N-His pExeA showed an average R_H of 3 nm or an apparent molecular weight of 58.4 kDa, suggesting that dimers were prevalent under this condition. However, the R_H was increased when the temperature was raised above 25 °C. An R_H of 6-7 nm was observed at 37 °C, indicating that large multimers were formed. The multimerization of this protein was favoured by low pH, as shown by delayed multimerization at pH 8.0 compared to pH 6.5. Because pExeA has a pI of 4.8, the protein was less negatively charged at lower pH, possibly resulting in increased accessibility for multimerization.

The particle size distribution of N-His pExeA was analyzed using DynaLS software, shown in Fig. 20. At 4 ºC, the protein showed a single peak at R_H 3 nm. After the sample was incubated at 37 ºC for one hour followed by storage at 4 ºC overnight, the protein showed a second peak at 10 nm, suggesting formation of large multimers. Since R_H is highly dependent on particle shape, accurate molecular weights of the multimers were difficult to derive by this method.

3.3.2 Analysis of pExeA multimerization by gel filtration

To confirm that the multimerization of N-His pExeA observed in DLS was not caused by non-specific aggregation at elevated temperature, the protein was also examined by gel filtration. 2 mg/ml N-His pExeA was applied to a Superdex 200 10/300 GL column (GE Healthcare) in 40 mM sodium phosphate (pH 6.5) and 150 mM NaCl at 4 ºC (Fig. 21).

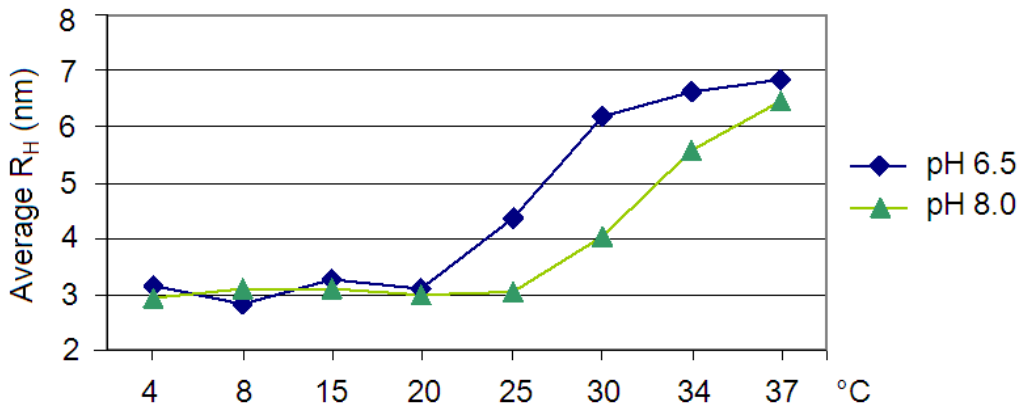


Fig. 19 Multimerization of pExeA at elevated temperatures by DLS. 2 mg/ml N-His pExeA were measured for average hydrodynamic radius (R_H) in 40 mM sodium phosphate of pH 6.5 or 8.0, 150 mM NaCl at the range of temperatures from 4 °C to 37 °C by DLS. The samples were incubated at each temperature for 5 min before average R_H was measured.

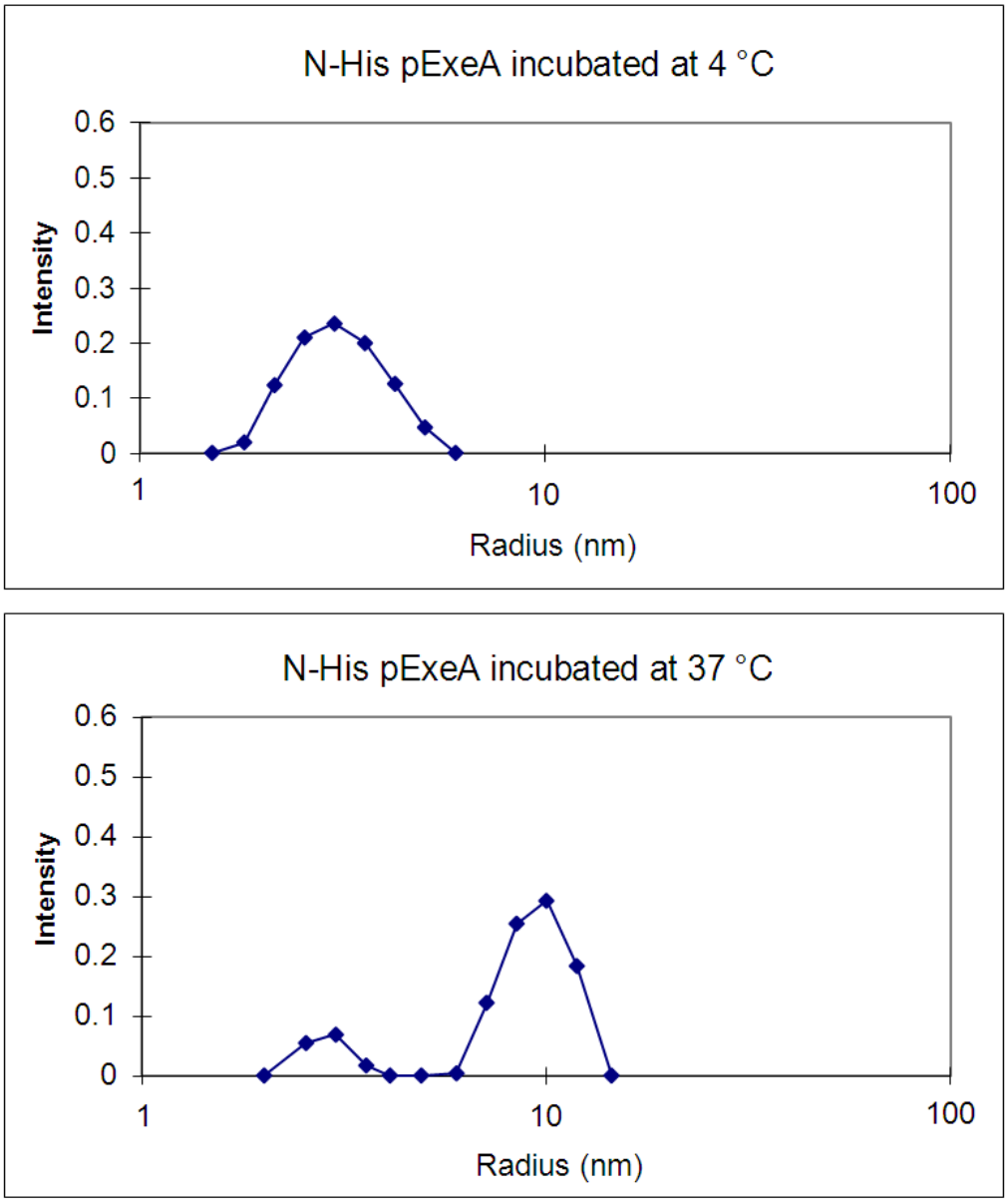
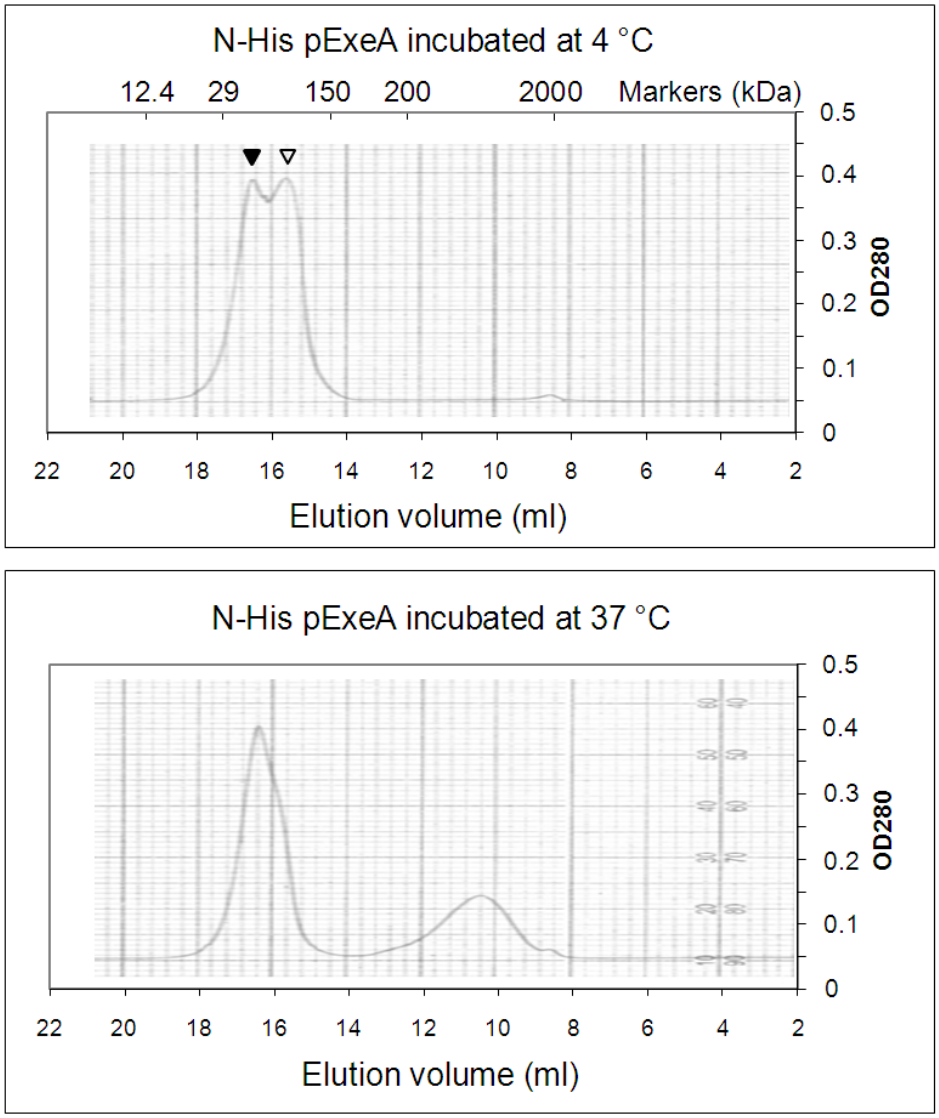


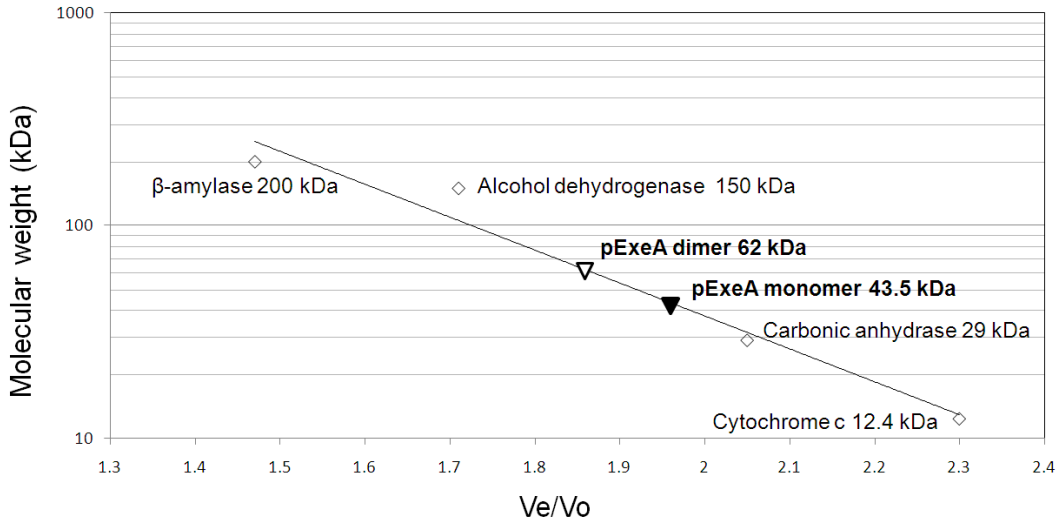
Fig. 20 Size distribution of pExeA by DLS. 2 mg/ml N-His pExeA in 40 mM sodium phosphate (pH 6.5) and 150 mM NaCl was incubated at 37 ºC for one hour followed by sitting at 4 ºC overnight. The incubated sample and a sample without incubation were measured at 4 ºC by DLS. The particle size distribution for hydrodynamic radius (R_H) was analyzed using DynaLS software.

Fig. 21 Size distribution of pExeA by gel filtration. A. Gel filtration of pExeA. 2 mg/ml N-His pExeA with or without incubation at 37 ºC for one hour were applied to a Superdex 200 10/300 GL column (GE Healthcare) in 40 mM sodium phosphate (pH 6.5) and 150 mM NaCl. The gel filtration was performed at 4 ºC with a flow rate of 0.5 ml/min. Protein markers (Sigma) were also applied in a parallel experiment. Their elution positions are indicated at the top of the graph. B. Size determination of pExeA. The molecular weight vs. the Ve/Vo (elution volume/void volume) for each protein marker is plotted using a semi-logarithmic scale. The Ve/Vo values for the pExeA peaks (▼ and ▽) are plotted and their apparent molecular weights indicated, accordingly. 2000 kDa (void volume): blue dextran; 200 kDa: β-amylase (sweet potato); 150 kDa: alcohol dehydrogenase (yeast); 29 kDa: carbonic anhydrase (bovine erythrocytes); 12.4 kDa: cytochrome c (horse heart).

A


N-His pExeA incubated at 4 °C
12.4 29 150 200 2000 Markers (kDa)
0.5
0.4
0.3
0.2
0.1
0
OD280
22 20 18 16 14 12 10 8 6 4 2
Elution volume (ml)
N-His pExeA incubated at 37 °C
0.5
0.4
0.3
0.2
0.1
0
OD280
22 20 18 16 14 12 10 8 6 4 2
Elution volume (ml)

B


1000
100
10
Molecular weight (kDa)
β-amylase 200 kDa
Alcohol dehydrogenase 150 kDa
pExeA dimer 62 kDa
pExeA monomer 43.5 kDa
Carbonic anhydrase 29 kDa
Cytochrome c 12.4 kDa
1.3 1.4 1.5 1.6 1.7 1.8 1.9 2 2.1 2.2 2.3 2.4
Ve/Vo

Without incubation at 37 ºC, the protein eluted as closely spaced, double peaks. By comparison with protein standards, the two peaks correspond to pExeA dimers and monomers. After the protein was incubated at 37 ºC for one hour, the dimer peak disappeared and a new peak with an apparent molecular weight of 600 kDa appeared. This peak eluted 2 ml after a small peak at the void volume of 8.4 ml. It was thus not likely caused by protein precipitation.

3.3.3 Analysis of pExeA multimerization by native gradient PAGE

Native gradient PAGE was used to further analyze the pExeA multimers induced at elevated temperatures. The method was modified from the blue native PAGE reported by Schagger (2001), in which Coomassie Brilliant Blue G-250 is added in the cathode buffer to give the proteins negative charge for electrophoresis. In the initial trials, N-His pExeA multimers ran as smears with the blue dye in the cathode buffer (data not shown). The electrophoresis was improved in the absence of the blue dye as shown in Fig. 22. N-His pExeA has a pI of 4.8 and therefore under the running conditions (pH 7.0), does not need to bind the dye to become negatively charged. N-His pExeA (2 mg/ml) was incubated at 37 ºC for one hour and then 4 ºC for one hour before being electrophoresed at 4 ºC. The presence of distinctive multiple pExeA bands in the native PAGE gel suggests that the components of the multimers had confined structures, and thus that the multimers were not likely to be caused by denaturation and aggregation. To examine the stability of the pExeA multimers, one sample was stored at 4 ºC overnight before being loaded on the gel. It showed that the amount of multimer in the sample was decreased by approximately half, suggesting that the multimerization is at least partially reversible.

3.4 Separation and identification of peptidoglycan fragments interacting with pExeA

3.4.1 Cosedimentation inhibition analysis of peptidoglycan fragments

Since peptidoglycan is a cell-sized structure (the sacculus), hydrolysis of peptidoglycan into small fragments was required to further characterize the pExeA-peptidoglycan interactions. *A. hydrophila* peptidoglycan was incubated at 37 ºC overnight with mutanolysin (Sigma), an N-acetylmuramidase that cleaves peptidoglycan at the β-1,4 glycosidic bonds into muropeptides. The sample was centrifuged to remove unhydrolyzed peptidoglycan. The

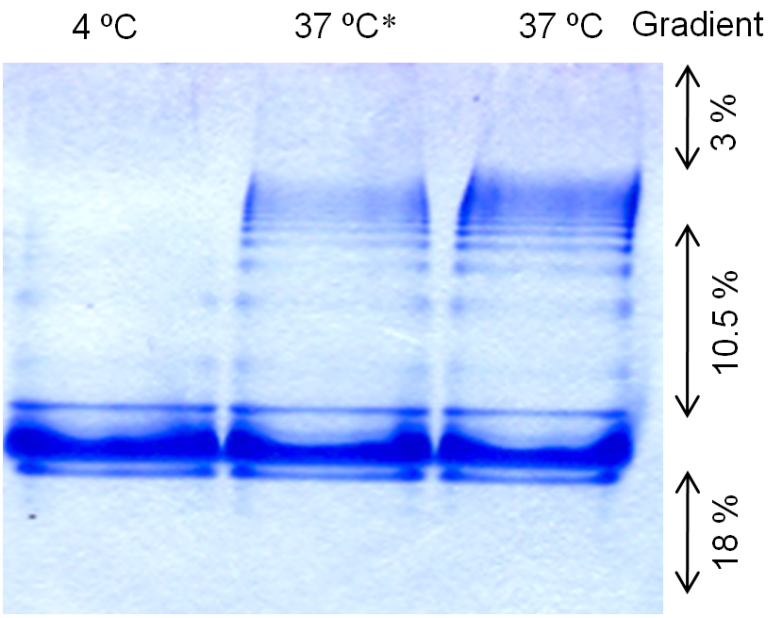


Fig. 22 Native gradient PAGE of pExeA multimers. 2 mg/ml N-His pExeA samples were incubated at 37 ºC for one hour, followed by incubation at 4 ºC for one hour or overnight* before electrophoresis at 4 ºC in a native three-stage gradient PAGE gel. A sample without incubation at 37 ºC was also included (4 ºC). The proteins were visualized by Coomassie Brilliant Blue stain as for SDS-PAGE gels.

peptidoglycan fragments were examined for their ability to interact with pExeA by cosedimentation inhibition assays. N-His ExeA was first incubated with the peptidoglycan fragments before intact peptidoglycan was added. Any interactions of the fragments with N-His pExeA would compete with the protein for binding and cosedimentation with the intact peptidoglycan. To further investigate the interactions, a commercial preparation of muropeptide, N-acetyl-D-glucosaminyl-(β1,4)-N-acetylmuramyl-L-alanyl-D-isoglutamine (Calbiochem), which contains only the first two amino acids of the peptide side chain, was also assayed. Fig. 23 shows that the peptidoglycan fragments, added at 4 times the muramic acid concentration of peptidoglycan in the assay, could inhibit the cosedimentation; whereas the commercial muropeptide, added at 16 times the muramic acid concentration of the peptidoglycan, could not. These data suggest that the peptidoglycan fragments were still able to interact with pExeA; however, muropeptides with longer peptides, larger structures, or special modifications are required for the interactions.

3.4.2 Separation of peptidoglycan fragments by gel filtration

The huge mass of peptidoglycan required cleavage by mutanolysin into small fragments, followed by gel filtration, to separate and identify the fragments that interacted with pExeA. However, the low yield of *A. hydrophila* peptidoglycan and the sensitivity limit of the muramic acid assay hindered this process in the initial trials. Peptidoglycan from the Gram-positive *B. subtilis* was therefore purified and used as an alternative for the following reasons. Firstly, the yield of peptidoglycan from *B. subtilis* was more than 10 times that from *A. hydrophila* (25mg $L^{-1}\cdot OD^{-1}$ vs. 2mg $L^{-1}\cdot OD^{-1}$). Secondly, the components of the peptidoglycan of *Bacillus* species have been extensively studied by HPLC and MS, which may assist in the identification of ExeA-binding fragments (Bacher *et al.*, 2001).

B. subtilis peptidoglycan was prepared by boiling SDS, pronase and concentrated hydrofluoric acid treatment to remove all proteins, lipids and anionic polymers attached to the peptidoglycan (Bacher *et al.*, 2001), plus an additional treatment with DNase and RNase to reduce nucleic acid contamination (Antignac *et al.*, 2003). The purified peptidoglycan

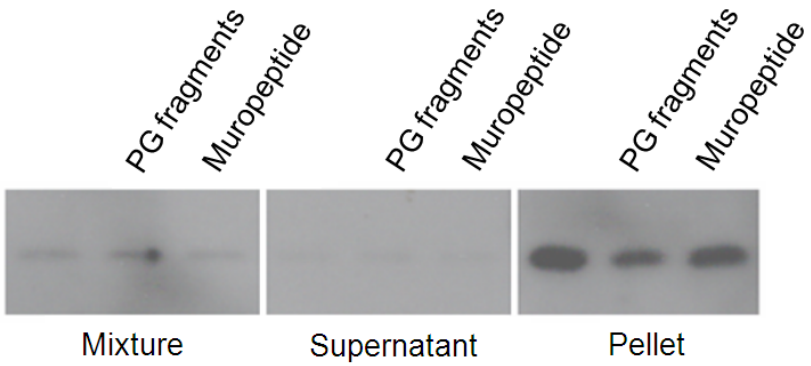


Fig. 23 Cosedimentation inhibition assay of peptidoglycan fragments. N-His pExeA (0.8 nM) was incubated with buffer or 200 µM muramic acid units of mutanolysin hydrolyzed *A. hydrophila* peptidoglycan fragments or 0.8 mM commercial muropeptide* (Calbiochem) at 4 ºC for one hour before 50 µM muramic acid units of intact peptidoglycan were added. After centrifugation, the pellet samples were 30 times concentrated during resuspension. The samples were applied to SDS-PAGE and immunoblotted with ExeA antiserum.

*: N-acetyl-D-glucosaminyl-(β1,4)-N-acetylmuramyl-L-alanyl-D-isoglutamine

sample was hydrolyzed overnight by mutanolysin at 37 ºC overnight, followed by inactivation of the enzyme at 95 ºC, and centrifuged to separate the supernatant that contained the peptidoglycan fragments. The supernatant was able to inhibit N-His pExeA from cosedimentation with *A. hydrophila* peptidoglycan (Fig. 25), indicating that the pExeA-binding components were present in the *B. subtilis* peptidoglycan fragments.

The *B. subtilis* peptidoglycan fragments were applied to a Superose 6 10/300 GL column for gel filtration. The fractions 15-48 (0.5 ml fraction size) were assayed for muramic acid concentration as shown in Fig. 24. A single muramic acid peak occurred in fraction 43 or an elution volume of 21.25 ml, after a 12.4 kDa protein marker eluting at a volume of 20.1 ml, suggesting that the peptidoglycan was almost completely hydrolysed into small fragments.

3.4.3 Identification of peptidoglycan fractions interacting with pExeA

The peptidoglycan gel filtration fractions were examined for their ability to interact with pExeA by cosedimentation inhibition assays. N-His pExeA was first incubated with peptidoglycan fragments at 4 ºC for one hour before intact *A. hydrophila* peptidoglycan was added. After centrifugation, the pellet samples were analyzed by anti-ExeA immunoblot, shown in Fig. 25. Fractions 36-42, but not the muramic acid peak fractions 43-44 that contained most of the muropeptides, could inhibit the cosedimentation of N-His pExeA with intact peptidoglycan, suggesting that larger peptidoglycan fragments were involved in the interactions.

3.5 Multimerization of pExeA in the presence of peptidoglycan

3.5.1 Multimerization of pExeA in the presence of intact peptidoglycan

The ability of pExeA to self-multimerize at elevated temperature and its interactions with peptidoglycan made it of interest to examine the multimerization status of the protein in the presence of peptidoglycan. N-His pExeA (42 nM) was incubated with 250 μM muramic acid units of *A. hydrophila* peptidoglycan at room temperature for one hour, followed by the addition of lysozyme to fragment the peptidoglycan. The sample was centrifuged to remove uncleaved peptidoglycan before being applied to a Superdex 200 10/300 GL column for gel filtration. The resulting fractions were analyzed by immunoblot with ExeA antibodies.

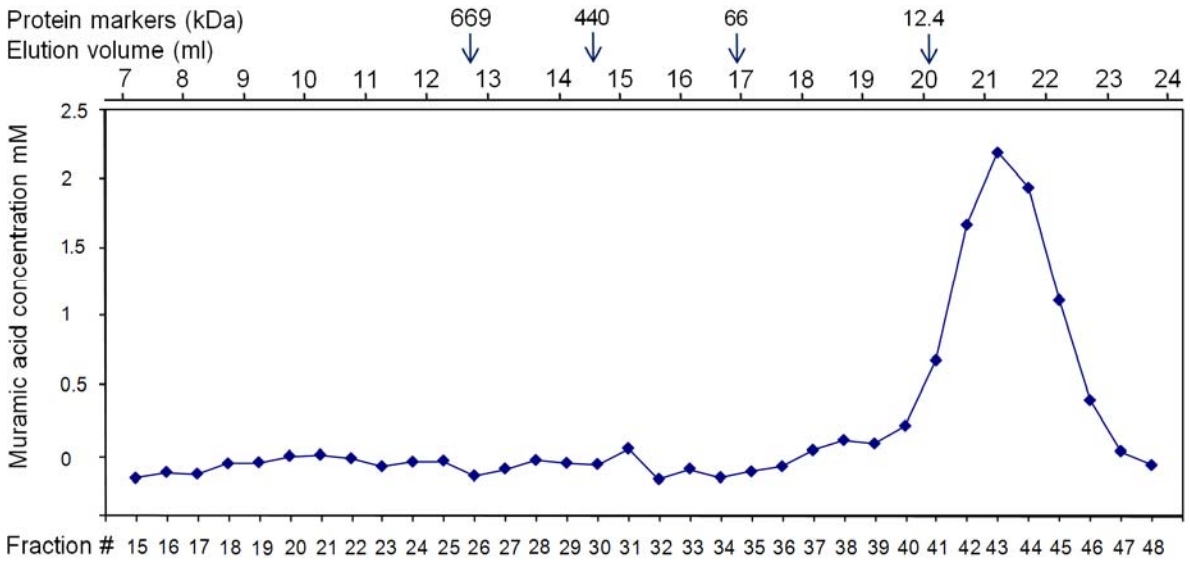


Fig. 24 Muramic acid distribution of mutanolysin-digested *B. subtilis* peptidoglycan gel filtration fractions. 1 ml of 7 mM muramic acid units of *B. subtilis* peptidoglycan were hydrolysed with 1,000 units of mutanolysin (Sigma) overnight at 37 ºC. After centrifugation, 0.5 ml of the supernatant was applied to a Superose 6 10/300 GL column (GE Healthcare) in 40 mM sodium phosphate (pH 6.5) at 4 ºC and collected into 0.5 ml fractions. Fractions 15-48 were assayed for muramic acid concentration. Protein markers (Sigma) were also applied in a parallel experiment. 669 kDa: thyroglobulin (bovine); 440 kDa: apoferritin (horse spleen); 66 kDa: bovine serum albumin; 12.4 kDa: cytochrome c (horse heart).

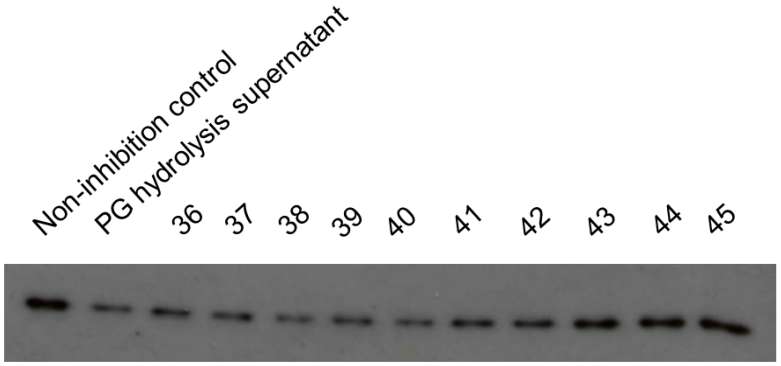


Fig. 25 Cosedimentation inhibition assay of mutanolysin-digested *B. subtilis* peptidoglycan gel filtration fractions. The mutanolysin-digested *B. subtilis* peptidoglycan fractions (Fig. 24, page 64) were assayed for their ability to interact with pExeA by inhibition of this protein from cosedimentation with intact *A. hydrophila* peptidoglycan. N-His pExeA (1 nM) was incubated with 100 µl peptidoglycan fractions 36-45 (total 150 µl) at 4 ºC for one hour before adding 230 µM muramic acid units of intact *A. hydrophila* peptidoglycan. After centrifugation to precipitate the peptidoglycan, the pellet samples were applied to SDS-PAGE and immunoblotted with ExeA antiserum.

Fig. 26 shows that in the absence of peptidoglycan, the 29 kDa protein eluted in fractions 31-33 (0.5 ml fraction size), or an elution volume of 16 ml, consistent with the previous gel filtration result (Fig. 21, page 57). In the presence of peptidoglycan, however, some of the protein also eluted in fractions 16 and 17, or the void volume of 8.4 ml. The column has a separation range of 10 to 600 kDa for globular proteins. The protein was thus apparently included in very large complexes, corresponding to multimers of above 600 kDa containing about 20 monomers of N-His pExeA, depending on the shape of the multimers. This indicated that N-His pExeA multimerized upon interactions with peptidoglycan. However, it is also possible that the large complexes were due to a small amount of large peptidoglycan fragments that were not hydrolyzed by the lysozyme.

3.5.2 Cross-linking analysis of pExeA multimerization in the presence of peptidoglycan fragments

In vitro cross-linking was used to examine the interactions between N-His pExeA and the mutanolysin-digested *B. subtilis* peptidoglycan fragments. If N-His pExeA interacted with the peptidoglycan fragments and consequently multimerized, the protein would be cross-linked in different patterns than the non-peptidoglycan control and offer a direct way to examine the interactions of the peptidoglycan fragments with N-His pExeA. N-His pExeA (80 nM) was incubated with peptidoglycan fraction 37, which showed interactions with the protein in the cosedimentation inhibition assay (Fig. 25, page 65), at 4 ºC for two hours and cross-linked with 0 to 5 mM DTSSP on ice for 30 min. A sample with no peptidoglycan and an N-His pExeA sample induced multimerization at 37 ºC were also cross-linked as controls. Fig. 27 shows that the protein was cross-linked into closely spaced double bands as well as larger bands in the presence of the peptidoglycan fragments, as was observed for the N-His pExeA multimers induced at 37 ºC, whereas the non-peptidoglycan control showed little or no cross-linking. The closely spaced double bands corresponded to dimers by comparison of their apparent molecular weights with the protein markers. This result suggests interactions between N-His pExeA and the fragments in the peptidoglycan digest fraction 37, confirming the cosedimentation inhibition result. It also shows that the protein was self-crosslinked, not to peptidoglycan fragments, indicating that the peptidoglycan fragments induced the protein to form dimers or multimers in a similar arrangement as that of the temperature-induced

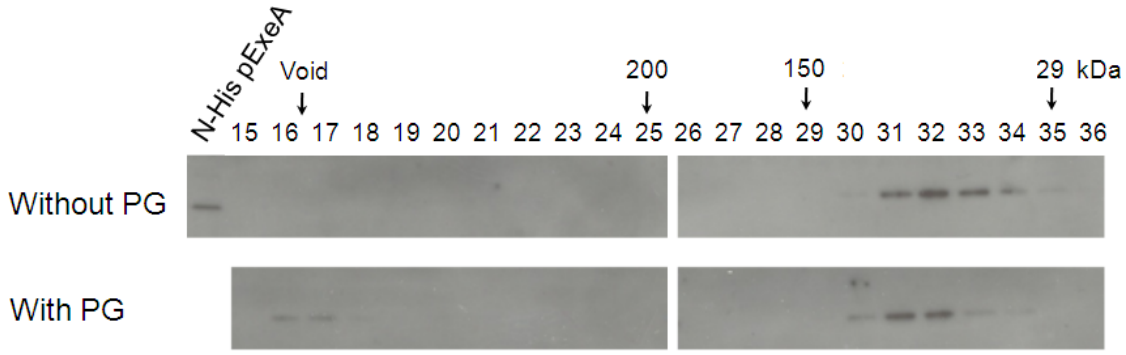


Fig. 26 Multimerization of pExeA in the presence of intact peptidoglycan. N-His pExeA (42 nM) was incubated with 250 µM muramic units of *A. hydrophila* peptidoglycan at room temperature for one hour, followed by hydrolysis of the peptidoglycan with 50 µg/ml lysozyme at room temperature for four hours. After centrifugation to remove unhydrolyzed peptidoglycan, the sample was applied to a Superdex 200 10/300 GL column (GE Healthcare) for gel filtration at 4 ºC. The fractions (0.5 ml fraction size) were examined on SDS-PAGE and immunoblotted with ExeA antiserum. A control without peptidoglycan was also included in the experiment. The column had a separation range of 10 to 600 kDa and an exclusion limit of 1300 kDa for globular proteins. The positions of protein markers (Sigma) are indicated. 2000 kDa (void): blue dextran; 200 kDa: β-amylase (sweet potato); 150 kDa: alcohol dehydrogenase (yeast); 29 kDa: carbonic anhydrase (bovine erythrocytes).

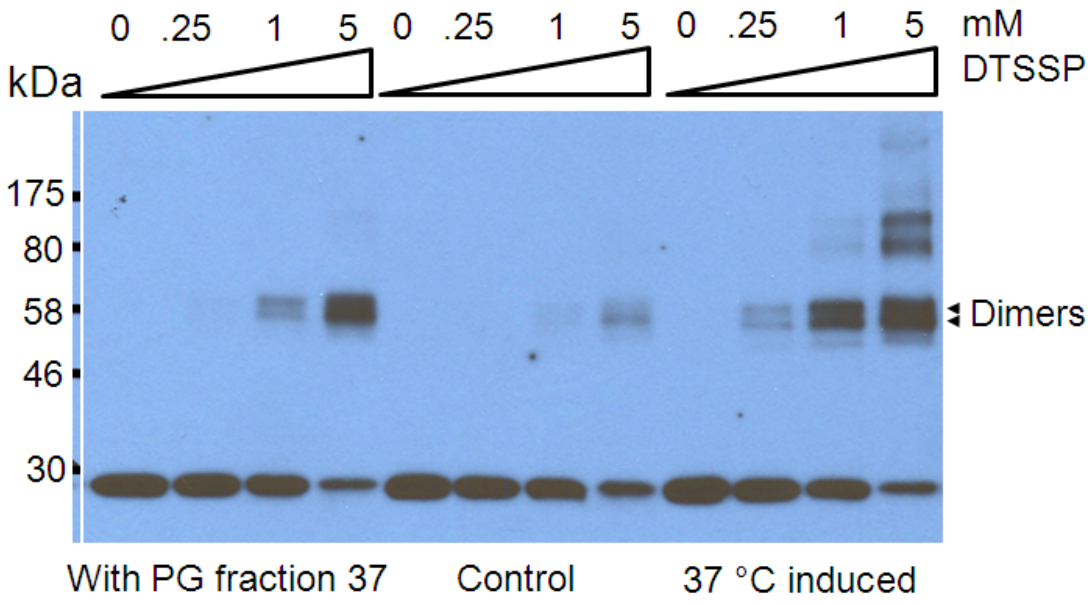


Fig. 27 Cross-linking analysis of pExeA in the presence of peptidoglycan fragments. N-His pExeA (80 nM) was incubated with *B. subtilis* mutanolysin-digested peptidoglycan gel filtration fraction 37 (Fig. 24, page 64) at 4 ºC for two hours and cross-linked with 0 to 5 mM DTSSP on ice for 30 min. A sample with no peptidoglycan added and an N-His pExeA multimer sample induced at 37 ºC were also cross-linked as controls. After cross-linking, the samples were applied to SDS-PAGE and immunoblotted with ExeA antiserum.

multimers. However, the quantity of N-His pExeA units in the multimers was difficult to estimate because of the low efficiency in cross-linking of the multimeric complexes.

3.5.3 Cross-linking analysis of the peptidoglycan gel filtration fractions

Since the cross-linking confirmed the interactions between pExeA and the *B. subtilis* peptidoglycan fraction 37, the whole set of mutanolysin-digested peptidoglycan gel filtration fractions were examined for their ability to interact with N-His pExeA by cross-linking analysis. The fractions were incubated with N-His pExeA at 4 ºC for two hours, followed by cross-linking with 1.2 mM DTSSP on ice for 30 min. To avoid quenching of DTSSP by the high concentrations of muropeptides that contain primary amines, fractions 40-44 were diluted to normalize the muramic acid concentration to 0.1 mM, approximately equivalent to its concentration across the rest of the column. Fig. 28 shows that the protein was cross-linked into the distinct double-band dimers in the presence of a wide range of peptidoglycan fractions 15-40, but not with fractions 41-44 which contained the muramic acid peak. The weak interactions with fractions 41-42 in the cosedimentation inhibition assays were not observed in the cross-linking assays, possibly because these samples were diluted for normalization of muramic acid concentration. As a control, the cross-linking was repeated with a mixture of fraction 36 and the diluted muramic acid peak fraction 43. The protein was cross-linked at the same level in the presence of the mixture as it was in the presence of fraction 36 alone, indicating that the lack of cross-linking with the muramic acid peak fractions was not because these fractions contained materials that inhibited the pExeA-peptidoglycan interactions or the cross-linking (Fig. 28). These results confirmed that pExeA did not interact with small muropeptides but with larger peptidoglycan fragments or special peptidoglycan structures that were not susceptible to mutanolysin hydrolysis.

3.5.4 Characterization of pExeA-interacting peptidoglycan fragments

To investigate the identity of the large peptidoglycan fragments or special peptidoglycan structures that interacted with pExeA in the above assays, the *B. subtilis* peptidoglycan fraction 36, which showed interactions with pExeA, was treated with 0.1 N HCl at 100 ºC for 15 min, which is known to cleave the lipid moiety of muropeptide lipid intermediates and probably also causes other modifications (van Heijenoort, 2007), and used

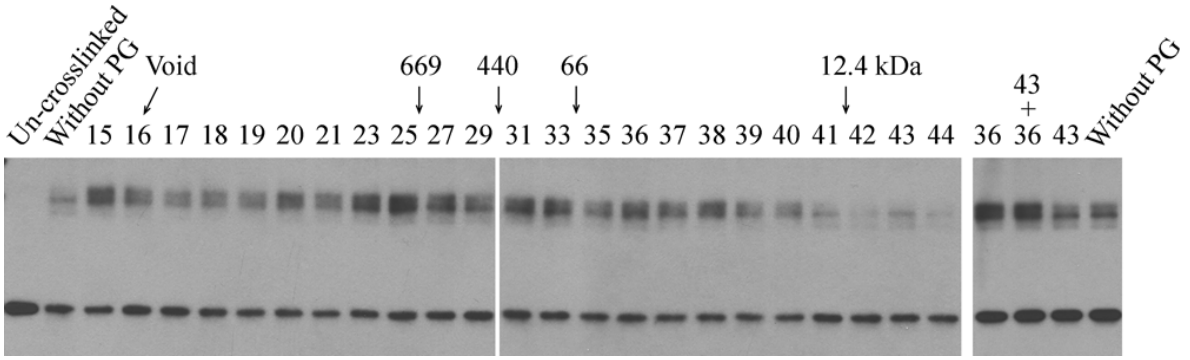


Fig. 28 Cross-linking analysis of mutanolysin-digested *B. subtilis* peptidoglycan gel filtration fractions. N-His pExeA (60 nM) was incubated with the mutanolysin-digested *B. subtilis* peptidoglycan fractions 15-44 (Fig. 24, page 64) for 2 hours at 4 ºC, followed by cross-linking with 1.2 mM DTSSP on ice for 30 min. The samples were examined by SDS-PAGE and anti-ExeA `immunoblot. Fractions 40-44 of the muramic acid peak were diluted to normalize the muramic acid concentration to 0.1 mM in order to prevent cross-linker quenching by muropeptides. The cross-linking was repeated with fraction 36 and the diluted fraction 43 as well as with a mixture containing both fraction 36 and the diluted fraction 43, as shown in the right panel. The locations of protein markers (Sigma) are also indicated at the top of the panel. 2000 kDa (void): blue dextran; 669 kDa: thyroglobulin (bovine); 440 kDa: apoferritin (horse spleen); 66 kDa: bovine serum albumin; 12.4 kDa: cytochrome c (horse heart).

in cross-linking analysis as described previously. Again, pExeA showed much increased cross-linking in the presence of fraction 36. However, the cross-linking was at the non-peptidoglycan control level when the fraction was treated with HCl (Fig. 29). This result suggests that certain modifying groups, which could be removed by HCl hydrolysis, were involved in the interactions with pExeA. The possible modifying groups will be discussed in detail in the discussion and future studies sections.

3.5.5 Gel filtration analysis of pExeA multimerization in the presence of peptidoglycan fragments

To better examine the multimerization of pExeA upon interactions with peptidoglycan, small peptidoglycan fragments were used in the following co-gel filtration studies. The mutanolysin-digested *B. subtilis* gel filtration fractions 38-40 (Fig. 24, page 64) were combined and incubated with N-His pExeA at 4 ºC for one hour, followed by gel filtration on a Superose 6 10/300 GL column. The resulting fractions were analyzed by immunoblot shown in Fig. 30. In the absence of peptidoglycan fragments, the 29 kDa N-His pExeA eluted with a peak in fraction 20 (1 ml fraction size) or an elution volume of 19.5 ml with an apparent molecular mass of only 18 kDa, possibly because of low protein concentration and weak interactions with the column matrix. Upon incubation with the peptidoglycan fractions 38-40, however, the protein was fractionated into much higher molecular weight fractions, peaking in fraction 15 or an elution volume of 14.5 ml with an apparent molecular mass of about 400 kDa. This result confirmed the interactions between N-His pExeA and the peptidoglycan fractions 38-40 in the cosedimentation inhibition and cross-linking studies (Fig. 25 and 28, page 65, 70). More interestingly, the protein-peptidoglycan complex apparent molecular weight of 400 kDa was much higher than the apparent molecular mass combination of N-His pExeA (18 kDa) and the peptidoglycan fractions 38-40 (20 kDa), suggesting that the protein formed large multimers comparable to the multimers induced at elevated temperature (Fig. 30).

3.5.6 Relationship between multimerization and interactions with peptidoglycan

Since the multimerization of N-His pExeA at low temperature required the presence of

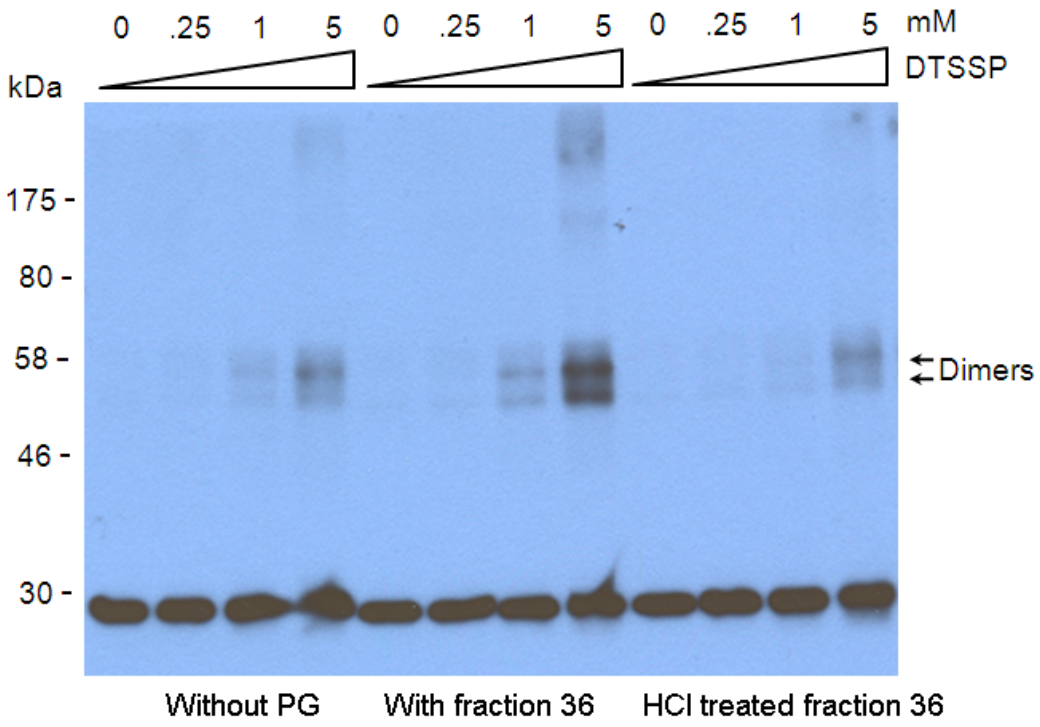


Fig. 29 Characterization of pExeA-interacting peptidoglycan fragments. The mutanolysin-digested *B. subtilis* peptidoglycan fraction 36 (Fig. 24, page 64) was hydrolyzed with 0.1 N HCl at 100 ºC for 15 min and neutralized with 0.1 N NaOH. The HCl treated and untreated samples as well as a non-peptidoglycan control were used in cross-linking assays with 40 nM N-His pExeA as described previously. After cross-linking, the samples were applied to SDS-PAGE and immunoblotted with ExeA antiserum.

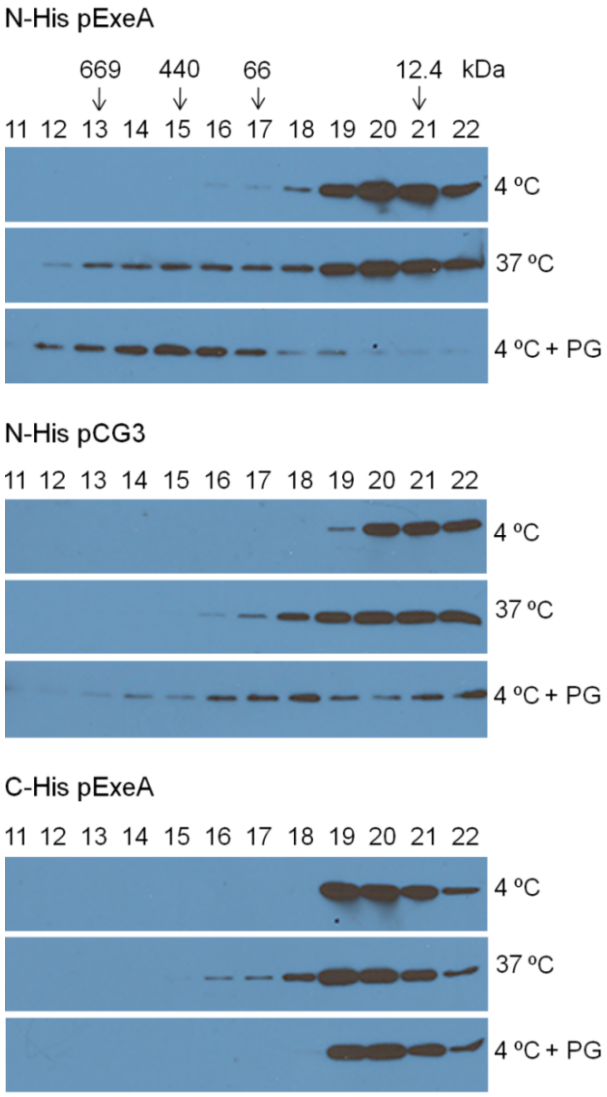


Fig. 30 Multimerization of pExeAs in the presence of mutanolysin-digested peptidoglycan fragments. N-His pExeA, N-His pCG3 and C-His pExeA (80 nM) were incubated with mutanolysin-digested *B. subtilis* peptidoglycan gel filtration fractions 38-40 (200 µl of each, total 650 µl, Fig. 24, page 64) in 40 mM sodium phosphate with 0.05% Tween 20 at 4 ºC for one hour before being applied to a Superose 6 10/300 GL column (GE Healthcare) in the same buffer for gel filtration at 4 ºC. pExeAs incubated at 4 ºC or 37 ºC in the absence of peptidoglycan fractions were also used as controls. Fractions 11-22 (1 ml fraction size) were analyzed by ExeA immunoblot for protein distribution. The positions of protein markers (Sigma) are indicated at the top of the figure. 669 kDa: thyroglobulin (bovine); 440 kDa: apoferritin (horse spleen); 66 kDa: bovine serum albumin; 12.4 kDa: cytochrome c (horse heart).

peptidoglycan fragments, it is apparent that the interactions with peptidoglycan induced the protein to multimerize. Two pExeA variants, N-His pCG3 and C-His pExeA, which showed decreased interactions with peptidoglycan (Fig. 16, page 51), were examined for multimerization in the presence of peptidoglycan fragments by co-gel filtration. Fig. 30 (page 73) shows that the mutant protein N-His pCG3 was still able to form multimers, however, they were smaller in size and the concentration was lower. Furthermore, C-His pExeA, which showed little binding in the cosedimentation assay, had almost no ability to form multimers. These data show that the multimerization was correlated to the interactions with peptidoglycan. In other words, the protein that showed the highest interactions with peptidoglycan also had the highest ability to form multimers (N-His pExeA); whereas those with less binding ability also displayed less ability to multimerize (N-His pCG3 and C-His pExeA). It is possible that the binding to peptidoglycan introduced conformational changes that favoured multimerization of the protein. The data also show that the two proteins, N-His pCG3 and C-His ExeA, had decreased ability to form multimers at 37 ºC, suggesting the involvement of the putative peptidoglycan binding domain in the temperature-induced multimerization. The data also imply that the interactions are complex and involve both binding and multimerization, which could not be distinguished in the cosedimentation and co-gel filtration assays.

3.6 Computer analysis of the putative peptidoglycan binding domain

3.6.1 Analysis of the putative peptidoglycan binding domain family

A BLAST (Altschul *et al.*, 1990) search with InterProScan (Zdobnov and Apweiler, 2001) indicates that the C-terminal periplasmic region of ExeA contains a putative peptidoglycan binding domain (Pfam number PF01471), which is conserved in a variety of enzymes related to bacterial cell wall degradation (Ghuysen *et al.*, 1994), for example, spore cortex-lytic enzyme SleB in *Bacillus cereus* and hydrolase metallo (Zn) DD-peptidase in *Streptomyces albus* G (Moriyama *et al.*, 1996; Dideberg *et al.*, 1982). However, no convincing evidence that this domain does bind to peptidoglycan has been reported. Up to present, 2310 proteins have been shown to contain this domain, including eight proteins with crystal structures available (Pfam database). Seven of the eight crystal structures are eukaryotic (gelatinase A; gelatinase B; Prommp-1; Prommp-2-Timp-2 complex; human Type

IV collagenase precursor; human collagenase 3 and fibroblast stromelysin-1); one is prokaryotic (hydrolase metallo (Zn) DD-peptidase). All of the seven eukaryotic proteins are matrix metalloproteinases (MMP), which degrade extracellular matrix (Seiki, 1999). They contain an N-terminal domain that structurally resembles the peptidoglycan binding domain, but do not likely have a peptidoglycan binding function. It is thus interesting to compare the sequences and structures of the eukaryotic and prokaryotic proteins to identify and distinguish between the amino acid residues that are required for the folding of the domain and those that are required for the peptidoglycan binding function.

The putative peptidoglycan binding domain features a three α-helices structure, as illustrated by gelatinase A (PDB Protein data bank entry 1CK7) and hydrolase metallo (Zn) DD-peptidase (PDB entry 1LBU) (Fig. 31). The two structures are used as models for eukaryotic and prokaryotic, respectively, in this study.

The putative peptidoglycan binding domain family contains 2310 members, with 239 of them designated as seed proteins (4 protein sequences were withdrawn) (Pfam database for PF01471). The 235 seed proteins include 52 eukaryotic proteins and 183 prokaryotic proteins (1 archaea, 6 phages and 176 bacteria). The two groups of sequences were aligned separately by ClustalW2 (Larkin *et al.*, 2007) and viewed by Jalview (Clamp *et al.* 2004), as shown in Fig. 32. Both of the two groups show two conserved peptide regions linked by a non-conserved region of variable length. The consensus sequences of the two groups were also aligned for comparison of conserved amino acid residues (Fig. 33). In the figures, “conservation” measures the conservation of physicochemical properties of amino acid residues. “Consensus” gives the most frequent residues and their percentage shown by the height of the bars. “Quality” indicates the alignment quality based on BLOSUM62 score (Eddy, 2004) on observed substitutions. Although the prokaryotic group and eukaryotic group share some similarity in amino acid conservation profiles, they contain significant variations, suggesting their distinct functions. We propose that the common amino acid residues shared by both groups are involved in folding the three α-helical structure; the residues conserved only in one group might be more specific for their special functions, e.g. peptidoglycan binding in the prokaryotic group or extracellular matrix binding related function in the eukaryotic group. To identify the common residues and the specific residues, the following arbitrary standards were used. For common residues, the residues should have

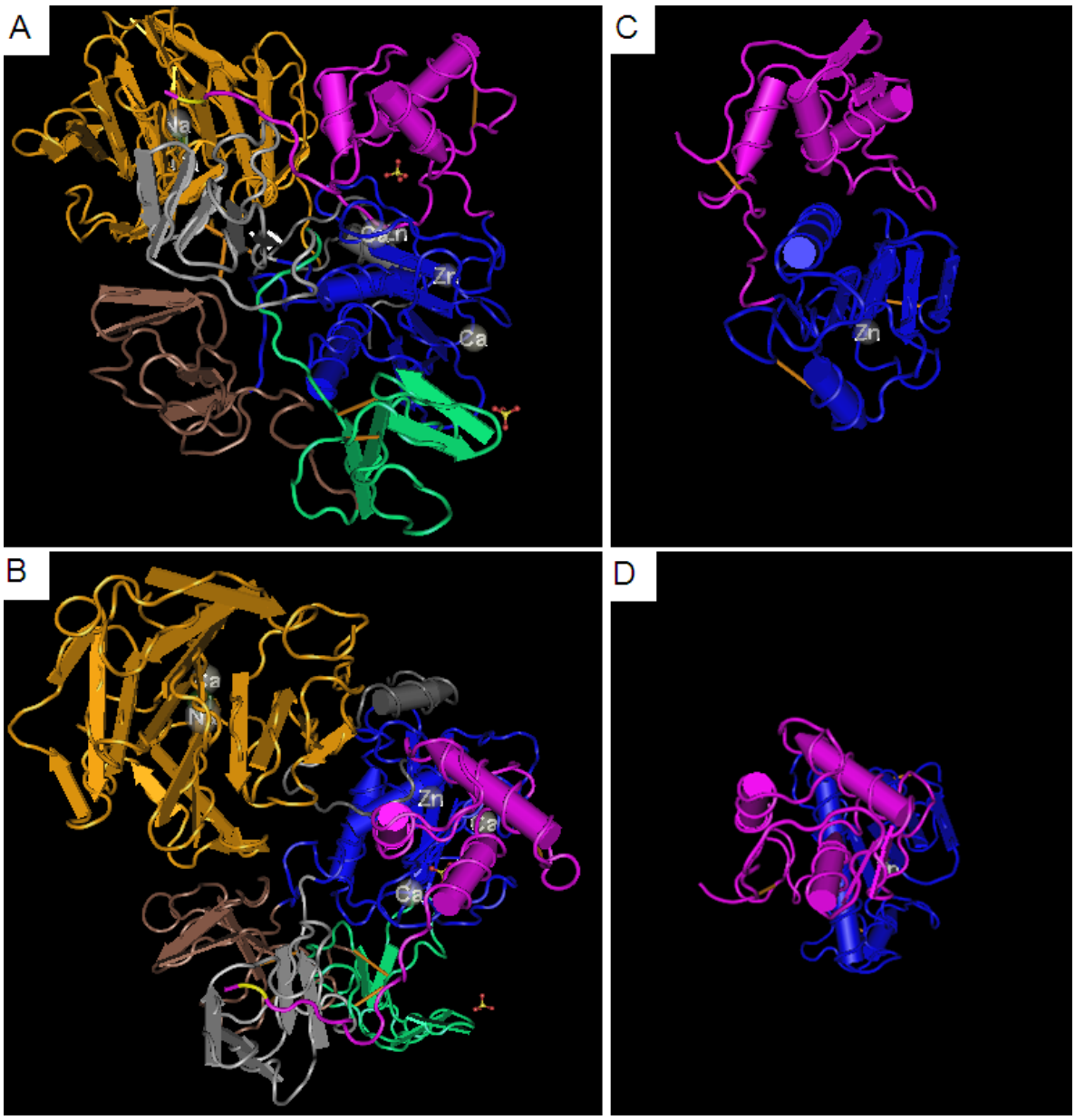


Fig. 31 Crystal structures of gelatinase A and hydrolase metallo (Zn) DD-peptidase. Gelatinase A (PDB entry 1CK7) and hydrolase metallo (Zn) DD-peptidase (PDB entry 1LBU) are used as model proteins for the eukaryotic structure and prokaryotic structure, respectively, in analysis of the putative peptidoglycan binding domain family (PF01471). The putative peptidoglycan binding domains, shown in pink, are comprised of three α-helices. A. Side view of 1CK7; B. Top view of 1CK7; C. Side view of 1LBU; D. Top view of 1LBU.

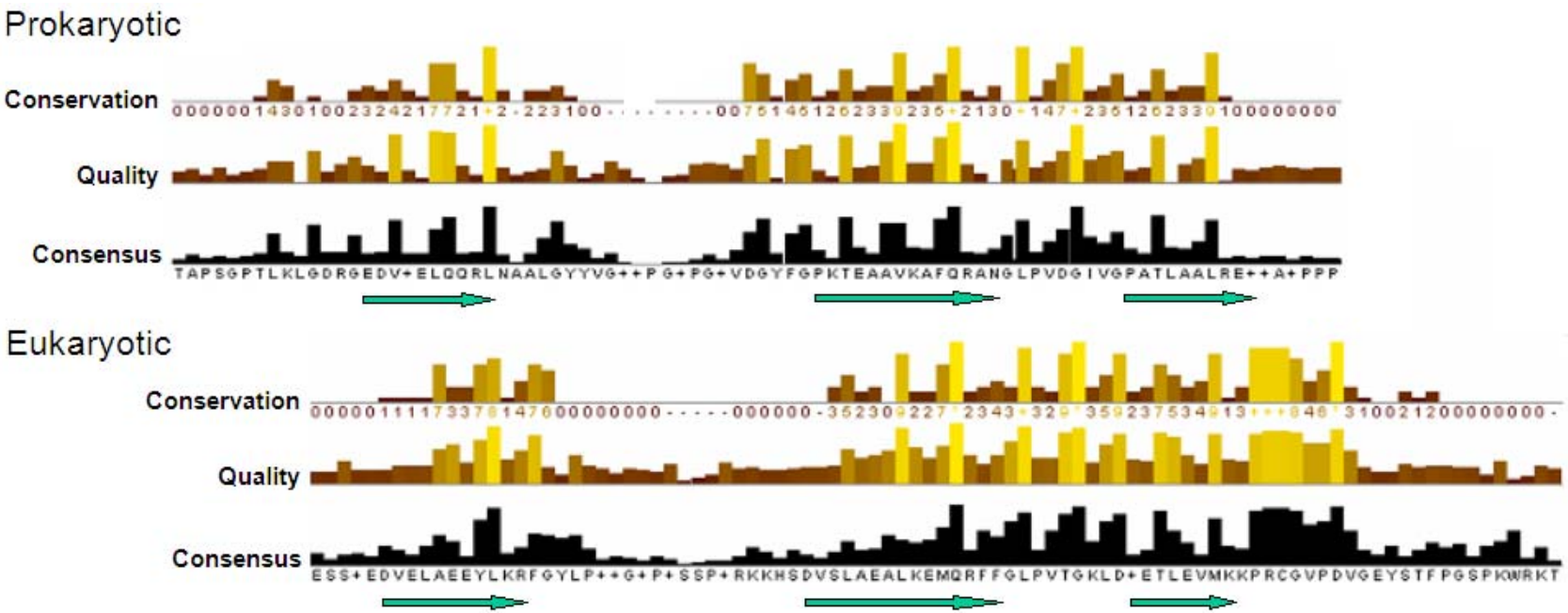


Fig. 32 Alignments of prokaryotic and eukaryotic members of the putative peptidoglycan binding domain family. The 183 prokaryotic seed proteins and 52 eukaryotic seed proteins of the putative peptidoglycan binding domain family (PF01471) were aligned separately by ClustalW2 (Larkin *et al.*, 2007) and viewed by Jalview (Clamp *et al.* 2004). Conservation measures the conservation of physicochemical properties of amino acid residues. Consensus gives the commonest residues and their percentage shown by the hight of the bars. Quality indicates the alignment quality based on BLOSUM62 score on observed substitutions. The prokaryotic consensus and the eukaryotic consensus are arranged so that the common conserved amino acid residues are in register (see more details in Fig. 33). The three α-helices are indicated with arrows.

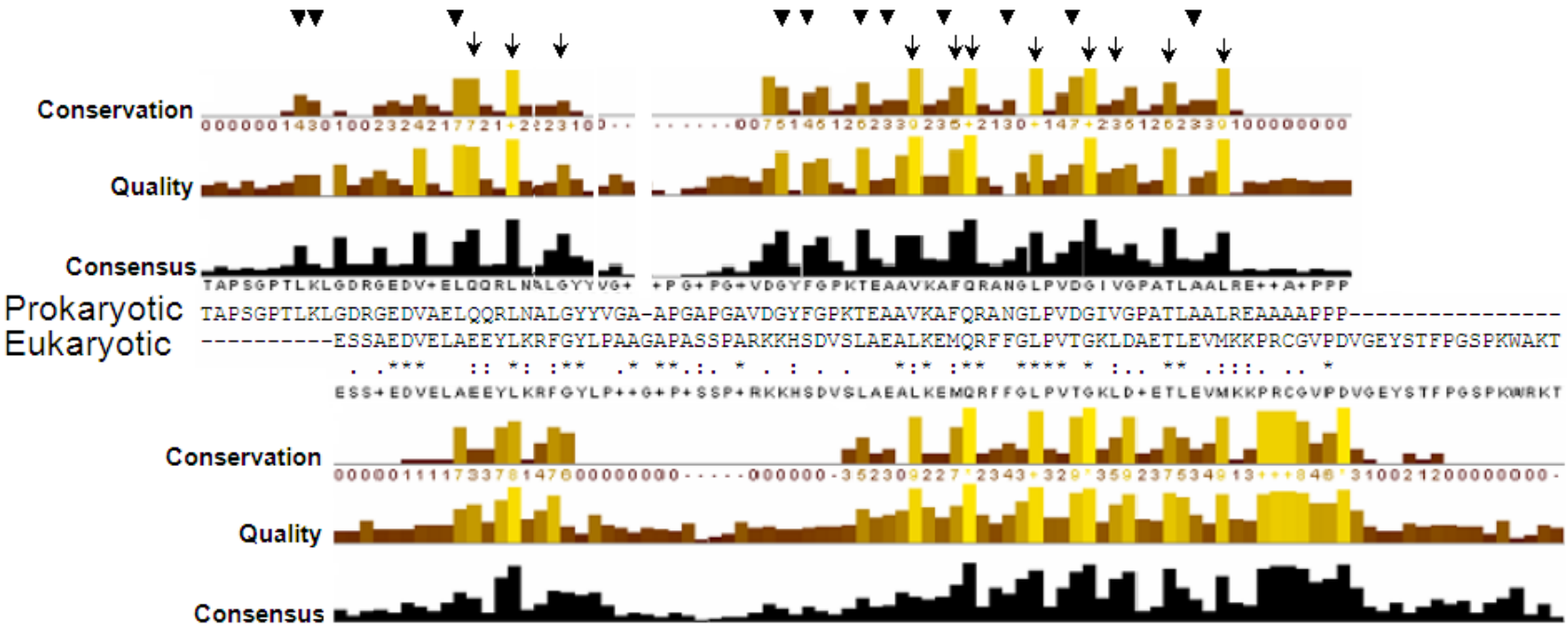


Fig. 33 Alignment of the prokaryotic consensus sequence and eukaryotic consensus sequence of the putative peptidoglycan binding domain family. The consensus sequences of prokaryotic and eukaryotic groups of the putative peptidoglycan binding domain family (PF01471) were aligned. The conservation profiles of each group are attached. The residues marked with ↓ are the common residues conserved in both groups with conservation indexes of 3 and above. The residues marked with ▼ are the putative peptidoglycan-specific residues conserved only in the prokaryotic group with conservation indexes of 3 and above. * indicates identical residues; : indicates conserved residues; . indicates semi-conserved residues.

conservation indexes of 3 and above and should be conserved in both groups. For peptidoglycan-specific residues, the residues should have conservation indexes of 3 and above in the prokaryotic group and should not be conserved in the eukaryotic group. The eukaryotic-specific residues were not analyzed in this study. The resulting residues are indicated in Fig. 33 (page 78), including 11 common residues and 11 peptidoglycan-specific residues. When these residues were marked on the 1LBU crystal structure, most of the common residues were buried in the structure, consistent with the hypothesis that they are required for folding; whereas the peptidoglycan-specific residues were located both on the surface and buried (data not shown). In addition, the peptidoglycan-specific residues did not strongly cluster. The peptidoglycan binding site was therefore not immediately evident using the analysis. It should be again noted that the nature of the interactions between the family members and peptidoglycan is unknown. Assigning a protein to this family is thus based solely on sequence similarity rather than on experimental evidence. This would include proteins that have similar structure but distinctive functions, e.g. the eukaryotic proteins, or non-functional proteins that contain mutations for important residues. The criteria for choosing peptidoglycan-specific residues were thus modified, including strengthening the weight of known peptidoglycan-binding proteins (1LBU and ExeA) in the alignment for conserved residues as described in more detail in the following section.

3.6.2 Mapping and analysis of the putative peptidoglycan binding site

Streptomyces albus G hydrolase metallo (Zn) DD-peptidase (PDB entry 1LBU) is the only prokaryotic member of the putative peptidoglycan binding family for which the crystal structure has been solved. The enzyme is able to cleave the D-Ala-D-Ala peptide bond of muropeptide. The crystal structure shows that the protein is comprised of an N-terminal putative peptidoglycan binding domain (a.a. 1-90) and a C-terminal Zn-containing catalytic domain (a.a. 91-213) (Dideberg *et al.*, 1982; Ghuysen *et al.*, 1994), as shown in Fig. 31 (page 76).

The sequence of 1LBU was aligned with the consensus sequence of the prokaryotic group as well as ExeA (Fig. 34). The 11 common residues conserved in both the prokaryotic group and eukaryotic group are also conserved in 1LBU and ExeA. However, only 2 of the 11 putative peptidoglycan-specific residues are conserved in all of the three sequences,

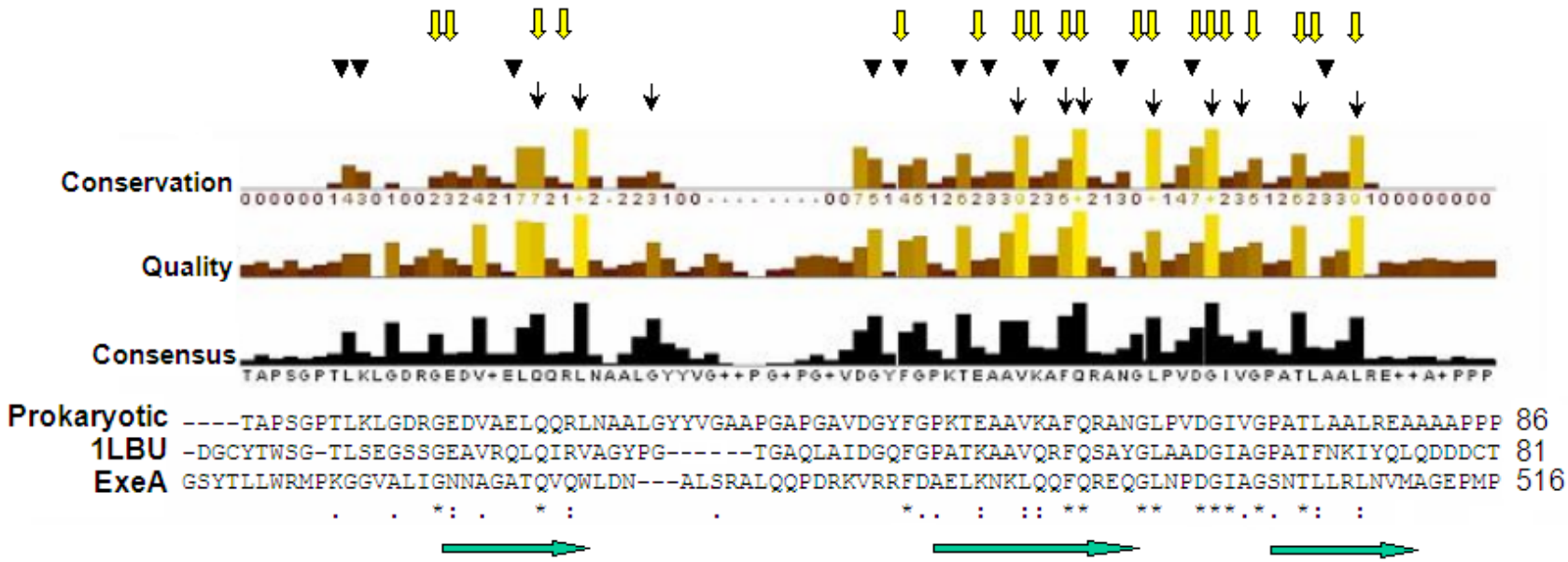
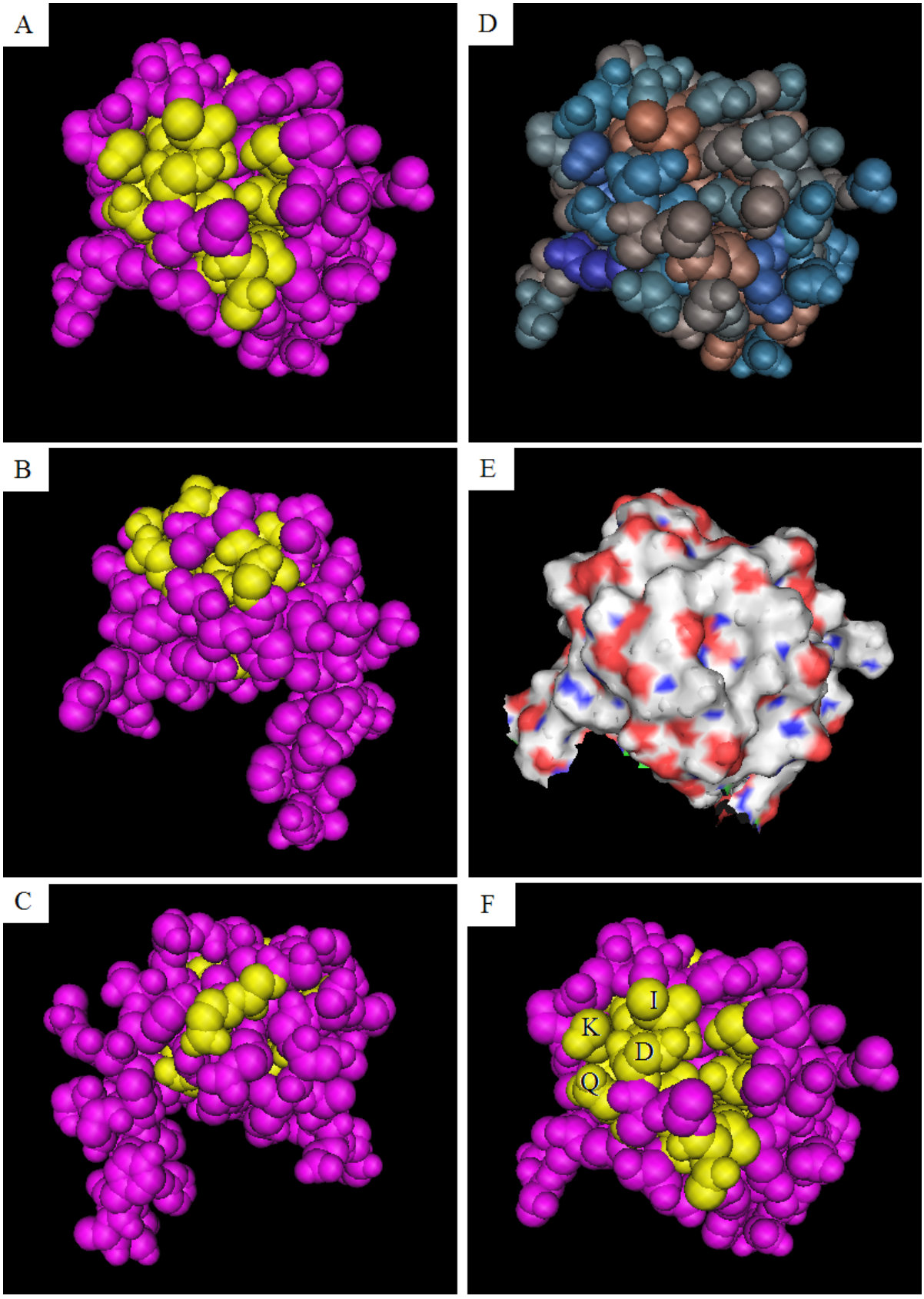


Fig. 34 Alignment of the putative peptidoglycan binding domain family prokaryotic consensus sequence, 1LBU and ExeA. The prokaryotic consensus sequence of the putative peptidoglycan binding domain family (PF01471) was aligned with 1LBU and ExeA. The conservation profile of the prokaryotic group is shown at the top with the 11 common residues conserved in both of the prokaryotic and eukaryotic groups and the 11 putative peptidoglycan-specific residues conserved only in the prokaryotic group indicated by ↓ and ▼, respectively. The 19 residues conserved in all three sequences are indicated by ⇩ . The three α-helices are indicated by ⟹. * indicates identical residues; : indicates conserved residues; . indicates semi-conserved residues.

suggesting that they are not essential for the peptidoglycan binding function. The alignment shows that the three sequences share 12 identical residues and 7 conserved residues. When the 19 residues were marked on the 1LBU structure, half of them were clustered at one location on the surface, including K47, Q51, D62, I64, Q54, G66, T69, L59 and G58 (Fig. 35). The site contains two hydrophobic residues (I64 and L59), one basic residue (K47) and one acidic residue (D62), as illustrated in Fig 35DE. We propose that this site is involved in the specific interactions with peptidoglycan. How these residues interact with peptidoglycan and what is the interacting ligand are not clear from the analysis.

Substitution mutations of three highly conserved amino acid residues in the putative peptidoglycan binding site of ExeA (F487S, L493S and G500D) have been constructed prior to the structural analysis of this study. They abrogated the normal function of ExeA in secretion and its interactions with peptidoglycan (Howard *et al*., 2006; this study). The three amino acid residues correspond to F53, L59 and G66 of 1LBU, which are buried below the surface in the crystal structure. The three mutations may thus disrupt the normal folding of ExeA. Mutant ExeAs containing F487S and L493S did exhibit greatly reduced stability *in vivo* and *in vitro* (data not shown). To better understand the putative peptidoglycan binding site, four residues exposed on the surface have been chosen for mutagenesis analysis. They are K481, Q485, D496 and I498, corresponding to K47, Q51, D62 and I64 of 1LBU, respectively, as shown in Fig. 35F. Alicia Miller, a technician in Dr. Howard's laboratory, is performing the mutagenesis experiments. The mutant ExeAs will be used in *in vivo* functional studies and *in vitro* binding studies to provide further understanding of the putative peptidoglycan binding site. Preliminary data have shown that this putative peptidoglycan binding site is required for the normal functions of ExeA in ExeD secretin assembly and secretion *in vivo* and in binding with peptidoglycan *in vitro* (unpublished results).

Fig. 35 Mapping and analysis of the putative peptidoglycan binding site. The 19 highly conserved residues in the prokaryotic consensus sequence, 1LBU and ExeA of the putative peptidoglycan binding domain family in Fig. 34 (page 80) are marked in yellow on the crystal structure of the putative peptidoglycan binding domain of 1LBU. K47, Q51, D62, I64, Q54, G66, T69, L59 and G58 cluster at one site on the surface and form the putative peptidoglycan binding site. A. Top view of the putative peptidoglycan binding domain. B. Front view. C. Back view. D. Top view for hydrophobicity distribution (hydrophobic residues in red and hydrophilic residues in blue). E. Top view for charge distribution on surface (oxygen in red and nitrogen in blue). F. Residues chosen for mutagenesis in ExeA (K47, Q51, D62 and I64).


A
D
B
E
C
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I
K
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Q

4. DISCUSSION

4.1 ExeA interacts with peptidoglycan

ExeAB have been shown to form a complex in the inner membrane and function to locate and/or assemble the ExeD secretin into the outer membrane (Ast *et al.*, 2002). Since a putative peptidoglycan binding domain is present in the periplasmic region of ExeA, it is proposed that the role of ExeAB is to generate gaps in peptidoglycan, a barrier in trans-envelope assembly, to allow ExeD to traverse into the outer membrane (Howard *et al.*, 2006). In support of this hypothesis, disruption of this domain by mutagenesis abrogated the normal function of ExeA. The putative peptidoglycan binding domain (PF01471) is conserved in many peptidoglycan-associated proteins; however, no convincing evidence has been reported for interactions between the domain and peptidoglycan. It was therefore the objective of this thesis to examine ExeA-peptidoglycan interactions to provide further understanding for the function of the putative peptidoglycan binding domain and the role of ExeA in the T2SS of *A. hydrophila*.

In this study, the interactions between ExeA and peptidoglycan were first shown by cross-linking *in vivo*. Wild type ExeA, but not the mutant proteins for the putative peptidoglycan binding domain, was found to cross-link to peptidoglycan, suggesting the involvement of the domain in ExeA-peptidoglycan interactions (Fig. 9, page 39). However, we could not exclude the possibility that the cross-linking was actually mediated through other proteins that formed an ExeA-containing complex with peptidoglycan.

More convincing evidence of ExeA-peptidoglycan interactions was provided by the *in vitro* binding studies, in which the purified C-terminal periplasmic region of ExeA (pExeA) was able to cosediment with peptidoglycan preparations treated with boiling SDS and pronase (Fig. 15, page 49). This indicates that pExeA interacted directly with peptidoglycan, but not with proteins, lipids or other impurities attached to peptidoglycan. However, it must be noted that only a very small amount of pExeA cosedimented with peptidoglycan. In these assays, 100 μM or more muramic acid units of peptidoglycan could bind less than 10 nM pExeA (Fig. 14, 15, page 47, 49). The possibility that the cosedimentation was caused by non-specific interactions needs to be addressed. Strong evidence in support of specific interactions was provided by a mutant pExeA, which contained a substitution mutation in the putative peptidoglycan binding domain and showed

greatly reduced ability to cosediment with peptidoglycan (Fig. 16, page 51). Another pExeA construct, in which the His-tag was added at the C-terminal end of wild type pExeA, almost totally lost the ability to interact with peptidoglycan (Fig. 16, page 51). This may be explained on the basis that the putative peptidoglycan binding domain is located in the C-terminal half of pExeA; the closely attached C-His tag could therefore impair the normal structure of this domain or block the binding site for peptidoglycan access. In addition, 40 mM sodium phosphate, 0.05% Tween 20 and 10 μg/ml bovine albumin were used in the binding buffer to overcome weak and non-specific interactions. The low binding capacity might therefore indicate the low amount of ExeA-binding sites in the peptidoglycan preparations. Alternatively, high affinity may require the N-terminal domain of ExeA or ExeB, which were absent in these assays.

The interactions between pExeA and peptidoglycan were also demonstrated in the cosedimentation inhibition, co-gel filtration and *in vitro* cross-linking assays (Fig. 23, 25, 27, 28, 29, 30, page 62, 65, 68, 70, 72, 73). In these studies, pExeA was able to interact with peptidoglycan fragments, suggesting that intact peptidoglycan or large fragments are not required.

The pExeA-peptidoglycan interactions were characterized in different buffer conditions. The cosedimentation was highest at pH 6.0 and almost abrogated at pH above 7.4 (Fig. 17, page 52). The periplasm is slightly acidic (pH 6.5), because protons diffuse into the periplasmic space from the environment due to Donnan equilibrium across the outer membrane (Dhungana *et al.*, 2003). In addition, bacteria release protons into the periplasm to establish protonmotive force (Mitchell, 1966). The observed pH requirement for the cosedimentation thus fits with the *in vivo* conditions. Ionic strength was also found to affect the pExeA-peptidoglycan interactions. Ionic concentrations above 120 mM (50 mM NaCl plus 40 mM sodium phosphate, pH 6.5) greatly interfered with the cosedimentation (Fig. 18, page 53). The binding affinity of pExeA and peptidoglycan is not determined, since several trials with a series of different pExeA concentrations in cosedimentation assays failed to generate a saturable binding curve (data not shown). This may have been partially caused by the rigid net-like structure of peptidoglycan that could non-specifically trap proteins. It might also be caused by the complicated pExeA-

peptidoglycan interactions, which have been shown to involve multimerization of the protein (discussed in the next section).

During the preparation of this thesis, a report of physical interactions between peptidoglycan and the putative peptidoglycan binding domain was published (Briers *et al.*, 2007). The endolysins KZ181 and EL183 of *P. aeruginosa* bacteriophages ΦKZ and EL both contain an N-terminal putative peptidoglycan binding domain and a C-terminal catalytic domain and are able to locally hydrolyze peptidoglycan to facilitate phage infection. In those studies, the two putative peptidoglycan binding domains were fused to green fluorescent protein (GFP), separately. Both of the fusion proteins were able to stain the cell surface of outer membrane-permeabilized *P. aeruginosa* homogeneously. It was also shown that the fusion proteins were able to stain *P. aeruginosa* peptidoglycan sacculi prepared with SDS treatment. The binding peaked at pH 8 and 8.5, much higher than the optimum pH (6.2 and 7.3) for the peptidoglycan lytic activities of the two proteins and the pH requirement (below 7.4) in our studies. Although the discrepancy could be caused by different characteristics of KZ181/EL183 and ExeA, other explanations should be addressed. Firstly, they did not use mild detergents to control non-specific interactions. In our studies, peptidoglycan samples exhibited a considerable hydrophobicity that could cause non-specific cosedimentation of both wild type and mutant pExeAs, ~1000 times more than that when 0.05% Tween 20 was added (data not shown). Secondly, a necessary control, mutant proteins that disrupted the putative peptidoglycan binding domain, was not included in the GFP fusion studies. Although a control of GFP without the putative peptidoglycan binding domain attached was used in their *in vivo* and *in vitro* experiments, the possibility of non-specific interactions caused by the domains was not excluded.

The nature of ExeA-peptidoglycan interactions is not known. In peptidoglycan hydrolysis assays, the purified pExeA failed to decrease the turbidity of peptidoglycan suspensions of different bacteria (*A. hydrophila*, *E. coli* and *Micrococcus*) in all conditions tested (data not shown). The question is therefore raised: how does the protein generate gaps on peptidoglycan if it does not have hydrolytic activity? It should be noted that the full function of ExeA may require full length of ExeA or its associating protein ExeB. Besides direct hydrolysis, gaps could be achieved by disruption of the normal biosynthesis of peptidoglycan, for example, inhibition of peptidoglycan synthetic enzymes and activation

of peptidoglycan lytic enzymes. The peptidoglycan could also be locally modified by ExeA and thus exhibit abnormal susceptibility to these peptidoglycan-specific enzymes. In future studies, one direction could be to examine the possible interactions with ExeA and peptidoglycan-specific enzymes.

Another interesting question is how ExeD finds the gaps and traverses through the peptidoglycan barrier, if ExeA does generate them. Connections are thus required to relate the ExeAB complex to the rest of the T2SS machinery. One connection was found between ExeAB and ExeC in the *in vivo* cross-linking studies. ExeB was co-crosslinked to peptidoglycan in the presence of wild type ExeA, but not in the presence of mutant ExeA for the putative peptidoglycan binding domain (Fig. 10B, page 41). This confirmed the ExeAB complex reported previously (Schoenhofen *et al.*, 1998). More interestingly, ExeC was also co-crosslinked to peptidoglycan in the presence of ExeA (Fig. 10A, page 41). These data suggest a complex of ExeA-B-C that is associated with peptidoglycan through the putative peptidoglycan binding domain of ExeA. GspC has been reported to interact with GspD (Gérard-Vincent *et al.*, 2002; Lee *et al.*, 2004; Possot *et al.*, 1999). In addition, direct ExeA-ExeD interactions were also demonstrated in other studies in Dr. Howard's laboratory (unpublished data). These interactions may thus lead GspD to the assembly site created by GspAB.

4.2 ExeA forms multimers on peptidoglycan

Multimerization of pExeA at elevated temperature is intriguing. After purification, the protein was kept at 4 ºC for storage. At this temperature, the majority of the protein was in the form of monomers and dimers; however, the protein started to multimerize as the temperature was raised above 25 ºC, as shown in the DLS, gel filtration and native PAGE studies (Fig. 19, 20, 21, 22, page 55, 56, 57, 60). The multimers were not likely caused by denaturation and aggregation at high temperature, since they ran as distinctive bands in the native PAGE and thus had defined structures. The physiological significance of pExeA multimers was better addressed by the co-gel filtration and cross-linking experiments. After incubation with small peptidoglycan fragments at 4 ºC, the protein fractionated as large multimers of 400 kDa or higher (Fig. 30, page 73). Interactions with peptidoglycan were apparently required for the multimerization. Two pExeA constructs, N-His pCG3 and C-

His pExeA, showed greatly reduced multimerization, corresponding to their reduced cosedimentation with peptidoglycan (Fig. 16, 30, page 51, 73). *In vitro* cross-linking showed that after incubation with peptidoglycan fragments, pExeA was cross-linked into distinctive closely spaced double bands similar to these of the temperature induced multimers, suggesting they shared a similar structure (Fig. 27, page 68). The binding with peptidoglycan perhaps introduced conformational changes of the protein or lowered the energy barrier for multimerization, which could be alternatively achieved by elevated temperature.

The finding that pExeA formed multimers on peptidoglycan is novel but not surprising. Two components of the T2SS apparatus, GspE and GspD, have been shown to form a hexametric ring in the inner membrane platform and a 12-14 member multimeric ring in the outer membrane, respectively (Robien *et al.*, 2003; Nouwen *et al.*, 1999 and 2000; Chami *et al*., 2005). In fact ring-like structures are prevalent in many trans-envelope machineries, for example, the type IV pilus, flagella and type III secretion systems (Craig and Li, 2008; Cornelis, 2006). In particular, the PulD secretin in *K. oxytoca* has been found to contain two rings. One protrudes in the periplasmic space, comprised of the N-terminal domain. One inserts in the outer membrane and forms the channel for secretion, comprised of the C-terminal domain (Chami *et al.*, 2005). It was found that the N-terminal domain of ExeD was able to interact with pExeA in yeast two-hybrid assays (unpublished data in Dr. Howard's laboratory). The predominant pExeA multimers were about 400 kDa (Fig. 30, page 73), calculated to contain 12-14 subunits of the 29 kDa protein, consistent with a secretin multimer containing 12-14 GspD subunits (Chami *et al.*, 2005; Johnson *et al.*, 2006).

It is possible that ExeA facilitates T2SS assembly by forming a multimeric ring on peptidoglycan. This may generate a gap large enough to accommodate the apparatus. Alternatively, the ExeA ring may act as a scaffold to direct the assembly of other components through ExeA-B, ExeA-C, ExeA-D and ExeC-D interactions.

4.3 An unsolved puzzle: what special peptidoglycan structure does ExeA bind to?

The identity of the peptidoglycan fragment interacting with pExeA or the putative peptidoglycan binding domain is not clear. It must be a common component conserved in

different bacteria, because pExeA was able to interact with peptidoglycan samples from *A. hydrophila*, *B. subtilis*, and *E. coli* (Fig. 15, 25, 27, 28, 29, 30, page 49, 65, 68, 70, 72, 73). This is also suggested by the wide distribution of the putative peptidoglycan binding domain among both Gram-positive and Gram-negative bacteria species (Pfam database). However, it also must be an unusual peptidoglycan structure. In cosedimentation assays, 100 μM muramic acid units of peptidoglycan could only bind less than 10 nM pExeA (Fig. 14, 15, page 47, 49). After *B. subtilis* peptidoglycan fragments were fractionated by gel filtration, the muramic acid peak fractions failed to interact with pExeA in cosedimentation inhibition and cross-linking assays (Fig. 25, 28, page 65, 70). Instead, the protein could interact with a wide range of fractions of apparent molecular weights from 20 kDa to over 600 kDa with no detectable muramic acid contents. It is possible that large peptidoglycan pieces were required for the interactions; however, an incomplete digestion of peptidoglycan, which greatly moved the muramic acid peak towards high molecular weight fractions and thus contained more large peptidoglycan fragments, did not exhibit more interactions with pExeA in cross-linking analysis (data not shown). It is more likely that the large peptidoglycan fragments, which interacted with pExeA, contained special structures that were not susceptible to mutanolysin hydrolysis. The special structures may include chemical modifications, for example, N-deacetylation, O-acetylation and N-glycolylation, uncommon peptide length, for example, two and three amino acid residues, or unusual arrangement, for example, tri-muropeptides and tetra-muropeptides (Vollmer *et al.*, 2008). The peptidoglycan binding domain may therefore not have a general peptidoglycan binding function, but a role in binding a special peptidoglycan structure during peptidoglycan biogenesis or at certain cell locations.

Another possible reason for the large peptidoglycan fragments in gel filtration is that they are the artifactual result of hydrophobic aggregation. Although mature peptidoglycan is hydrophilic upon removal of hydrophobic lipoproteins, it is actually linked with a number of hydrophobic Lipid II moieties. Boiling SDS and pronase treatments are not able to cleave the undecaprenyl moiety. The hydrophobic moieties might clump to form particles of different sizes in gel filtration. It is therefore possible that pExeA-peptidoglycan interactions require the Lipid II intermediate. This could explain the apparently sparse nature of the pExeA-binding site in peptidoglycan preparations, since the Lipid II

intermediates only link to peptidoglycan during glycan synthesis. One possible effect of these interactions is interruption of normal peptidoglycan biogenesis and thus the creation of gaps in the peptidoglycan for T2SS apparatus assembly. This possibility was first examined by treatment of intact peptidoglycan with 0.1 N HCl at 100 ºC for 0 to 30 min to remove the lipid moiety (van Heijenoort, 2007). The sample showed at first increased and then decreased ability to cosediment pExeA (data not shown). This result is difficult to explain. It may be caused by removal of hydrophobic moieties and increased solubility allowing better interactions with pExeA. Or, perhaps the peptidoglycan sacculi were hydrolyzed to smaller pieces and this increased the accessibility of the protein to its binding sites.

To resolve the above possibilities, the acidic treatment was repeated with the *B. subtilis* peptidoglycan gel filtration fraction 36. The treated sample lost the ability to cause pExeA multimerization in the cross-linking analysis (Fig. 29, page 72). This suggests that the undecaprenyl moiety was involved in the interactions with pExeA. However, we cannot exclude the possibility that the HCl treatment could also modify peptidoglycan by deacetylation or removal of other residues. It should be noted that the methods used to purify peptidoglycan in this thesis involved unbuffered SDS solutions and hydrofluoric acid (for *B. subtilis* peptidoglycan), which should release the O-acetyl groups (Vollmer 2008). If the O-acetyl groups were involved in the interactions and the methods for peptidoglycan purification caused partial deacetylation, they would explain the apparently sparse nature of the pExeA-binding sites. However, if the O-acetyl groups were fully hydrolyzed during peptidoglycan purification, it would exclude the possibility of involvement of the O-acetylation in these interactions. In future studies, special care should be taken to preserve the labile O-acetyl groups in peptidoglycan purification, as described by Bera *et al.* (2005) or Weadge and Clarke (2007).

Other efforts to identify the ExeA component that interacts with peptidoglycan included the computer analysis of the putative peptidoglycan binding domain. When the highly conserved amino acid residues were located in the putative peptidoglycan binding domain of the 1LBU crystal structure, many of them were clustered at one surface location, suggesting that this site is the putative binding site for peptidoglycan (Fig. 35, page 82). The folding of this site (three α-helices) is quite different than the peptidoglycan binding

sites in known crystal structures of transpeptidases (Macheboeuf *et al.,* 2006), transglycosylases (Lovering *et al.*, 2008), lytic transglycosylases (Scheurwater *et al.*, 2008) and lysozymes (Strynadka and James, 1996).

5. CONCLUSION

The studies in this thesis provide evidence that demonstrates the interactions between ExeA and peptidoglycan both *in vivo* and *in vitro*. The interactions caused ExeA to multimerize, favouring a model in which ExeA and ExeB form a multimeric ring on peptidoglycan to generate a gap large enough to accommodate the type II apparatus and/or to act as a scaffold for assembly of the system (Fig. 36). It is unfortunate, however, that the peptidoglycan ligand for the putative peptidoglycan binding domain has not yet been identified. This difficulty perhaps reflects the nature of peptidoglycan, which is not simple or homogenous, but rather a complicated and heterogeneous structure.

6. FUTURE STUDIES

6.1 Identification of the peptidoglycan ligand

The studies in this thesis have shown that pExeA binds to an unusual peptidoglycan structure that is not susceptible to mutanolysin hydrolysis. This fact poses a difficulty for the identification of the peptidoglycan ligand by HPLC and MS, which generally require uniform molecules. The finding that HCl treatment of peptidoglycan could remove modifying groups that are required for interactions with pExeA has implied a solution for this problem.

The possible peptidoglycan modifications could be analyzed by HCl treatment of the peptidoglycan fractions that can interact with pExeA followed by HPLC and MS to analyze the hydrolysis products, particularly small molecules that may be released from peptidoglycan. In addition, peptidoglycan samples from different bacteria with known modifications, for example, N-deacetylation, muramic δ-lactam and O-acetylation as listed by Vollmer (2008), may be used in interaction studies with pExeA. O-Acetylated peptidoglycan preparation and analysis has been documented by Pfeffer *et al.* (2006). Alternatively, *in vitro* modifications of peptidoglycan, such as O-acetylation and N-acetylation with acetic anhydride as described by Strating and Clarke (2001) and Briers *et*

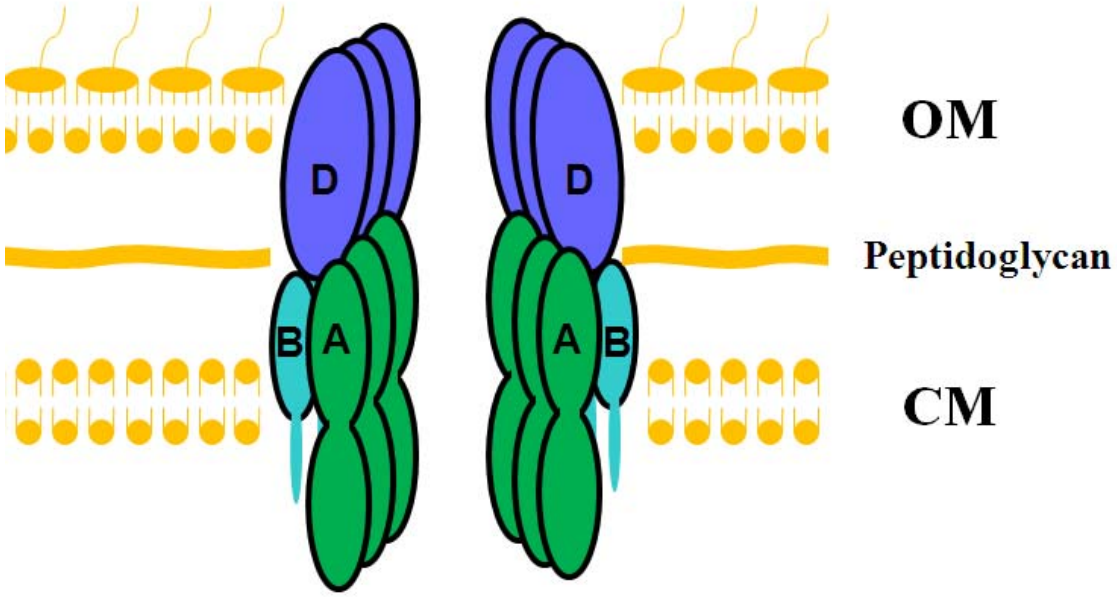

Fig. 36 Model of the ExeAB ring on peptidoglycan. ExeAB forms a multimeric ring-like structure on peptidoglycan to generate a gap for assembly of the ExeD secretin.

al. (2007), might assist the identification. To examine the involvement of the undecaprenyl moiety in the interactions, Lipid II intermediate may be extracted using the method described by Higashi *et al.* (1967).

6.2 *In vitro* assembly of T2SS subcomplex

Our studies have suggested that ExeAB may create an assembly site and/or act as a scaffold for assembly of the rest of the T2SS components. In particular, ExeA was required for co-crosslinking of ExeC to peptidoglycan. ExeA-ExeD and ExeB-ExeD interactions have been shown in yeast two-hybrid assays (unpublished data in Dr. Howard's laboratory). However, *in vitro* binding studies failed to show the ExeA-ExeD interactions. In these assays, pExeA or the N-terminal domain of ExeD was immobilized to a matrix via His-tag affinity or amine coupling. These techniques have two disadvantages, e.g. prevention of protein multimerization and requirement for pH higher than the physiological pH in the periplasm (His-tag), which have been shown to be important in pExeA-peptidoglycan interaction in this thesis.

In future studies, the interactions among other T2SS components could be analyzed using an *in vitro* cosedimentation system that includes peptidoglycan, pExeA and other Exe proteins. Since pExeA binds and cosediments with peptidoglycan, interactions between pExeA and other proteins may cause them to cosediment with the peptidoglycan as well. In such assays, immobilization of the proteins is not required, and this system may thus better mimic the *in vivo* environment of the periplasm, where ExeA multimer formation at the assembly site may be required to recruit other T2SS components. In addition, by presenting different Exe proteins in the assays, the order of T2SS assembly could also be investigated.

The interactions could also be examined by *in vitro* cross-linking as described in this thesis, in which multimerization of pExeA has been demonstrated. By comparison of the cross-linked samples immunoblotted with different antibodies, we may be able to show hetero-complex and homo-multimerization in these interactions.

6.3 Observation of pExeA ring-like structure

In this thesis pExeA has been shown to form multimers upon interactions with peptidoglycan or at elevated temperature. It is thus proposed that the protein forms a ring-

like structure. The possibility of ring-like structure formation could be examined by electron microscopy as described by Chami *et al*. (2005), in which the authors demonstrated that PulD, a homologue of ExeD in *K. oxytoca*, forms a 12-multimer ring structure. Alternatively, the multimers could be examined by atomic force microscopy (Andersen *et al.*, 2009).

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