THE ROLE OF OUTER MEMBRANE HOMEOSTASIS IN THE VIRULENCE OF GRAM-NEGATIVE BACTERIA

By

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ABSTRACT

This study investigated the underlying mechanisms of outer membrane homeostasis in Gram-negative bacteria. Using both the evolved laboratory strain *E. coli* K12 and the broad host range pathogen *Salmonella enterica* serovar Typhimurium, we have identified and characterised a series of non-essential genes responsible for the maintenance of the outer membrane barrier function. We have revealed their importance for bacterial pathogenesis suggesting their use as novel targets for drug development. This study has provided the first description of a pathway for phospholipid transport from the inner membrane to the outer membrane via the lipoprotein PlpA, a gene previously of unknown function. As several of the genes highlighted by our initial studies were associated with the biogenesis of virulence factors, we complemented our investigations by characterising the contributions of the *S.* Typhimurium Type V proteins to virulence in a murine model. These investigations have provided the first peer reviewed characterisation of a trimeric autotransporter from *Salmonella,* has identified a mechanism by which *Salmonella* can survive on tomatoes, and has highlighted the functional redundancy of these proteins in *Salmonella* infection. These findings have significantly advanced our understanding of the mechanisms mediating outer membrane homeostasis and the biogenesis and functions of virulence factors.

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CHAPTER 1

GENERAL INTRODUCTION

1.1. STRUCTURE OF THE GRAM NEGATIVE ENVELOPE

The structure of the Gram-negative cell envelope is strikingly different to that of Gram positive bacteria, which have a single cytoplasmic membrane surrounded by the peptidoglycan (1). In contrast, Gram negative organisms contain an additional outer membrane (OM) surrounding a thinner layer of peptidoglycan, enclosing this within a periplasmic space (1-3).

1.1.1. The structure and composition of the OM

The OM of Gram negative bacteria is selectively permeable, preventing entry of toxic and bactericidal compounds, including hydrophobic substances and anionic detergents (4, 5). The membrane forms an asymmetric bilayer, comprised of lipopolysaccharide (LPS) on the outer leaflet, transported and inserted by the lipopolysaccharide transport (Lpt) pathway and phospholipids on the inner leaflet (1, 2, 4). LPS is comprised of three sections, consisting of lipid A providing a membrane anchor for the attachment of core and O- polysaccharides (6). Lipid A is comprised of fatty acids with ester amine linkages to glucosamine disaccharides, containing six to seven acryl chains, attaching the core polysaccharide structures via ketodeoxyoctanoate (KDO) (1, 2). This structure induces a strong anionic charge, connected by cationic interactions, creating an impermeable barrier (1). The inner leaflet is comprised of phospholipids including phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) (2).

The membrane is further punctuated with an array of β-barrel proteins, inserted by the β-barrel assembly machinery (BAM) complex and lipoproteins that are attached to the inner leaflet, via the localisation of lipoproteins (Lol) pathway (1, 2). The integral β-barrel outer membrane proteins (OMPs) span the OM bilayer functioning as porins, membrane

stabilisation factors, efflux components or OM assembly complexes (1, 2). OMPs form monomers or trimers, comprised of between eight and 24 β-strands (dependant on the protein). The most common OMPs include the OmpF and OmpC trimers, with each monomer comprised of 16 β-strands. They are abundant with up to 250,000 copies per cell, allowing for the diffusion of small hydrophilic molecules <600 Da into the cell (1, 2). Other OMPs include LamB and OmpA, which are required for diffusion of maltose, maltodextrins and anions (1). In contrast, OmpA, can exist in a second non-porin form, which non-covalently binds peptidoglycan, stabilising the OM (1).

OM lipoproteins in *E. coli* can be either surface exposed on the outer leaflet or protrude into the periplasm from the N-terminal cysteine residue, anchoring them to the inner leaflet, as shown for Braun's lipoprotein Lpp (7). To date over 100 different lipoproteins have been identified in *E. coli* (1). In many instances the function has yet to be determined, though Pal and Lpp are required to maintain cell shape and stabilise the OM through covalent attachments to the peptidoglycan (specifically the diaminopimelic acid [DAP] residue in the case of Lpp)(1). Pal also interacts indirectly with the inner membrane (IM) protein TolA via interaction with the periplasmic TolB, which also directly interacts with TolA, providing an additional stabilising interaction with the IM, thought to be critical for correct invagination of the OM during daughter cell separation (8).

1.1.2. Periplasmic Space & Peptidoglycan

The periplasmic space is an oxidising environment, absent of ATP which enables Gram negative bacteria to sequester harmful compounds, restricting their entry to the cytoplasm (1). The periplasmic space encloses the rigidly structured peptidoglycan between the two membranes. Comprised of multiple layers of glycosaminoglycan, a few nanometres

thick, cross linking N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) through a 1-4 β link between DAP and D-alanine residues of the L-alanine, D-alanine, D-glutamic acid and DAP repeating structures, the peptidoglycan provides rigidity and cell shape. Preventing lysis due to changes in pressure (1, 9).

The biosynthesis of peptidoglycan has been relatively well characterised and shown to progress in a methodical manner as shown in figure 1.1. The first steps in the biosynthetic pathway are performed by cytoplasmic enzymes, commencing with MurA catalysing the transfer of a enolpyruvyl modiety to uridine diphosphate (UDP)-N-acteylglucosamine (GlcNAc) to generate enolpyruvyl UDP-GlcNAc, this is then converted to UDP-MurNAc through an NADPH dependant reaction, catalysed by the reductase MurB. MurC-F subsequently catalyse the ATP-dependant ligation of amino acid side chains, L-Ala, D-Glu, DAP and D-Ala-D-Ala, respectively to UDP-MurNAc, generating the UDP-MurNAc-L-Ala-D-Glu-DAP-D-Ala-D-Ala (MurNAc-pentapeptide)(10). Following the production of the MurNAc-pentapeptide, the IM localised MurY catalyses its transfer to the undecaprenyl phosphate carrier (C_{55}-P or UP) creating lipid I. This reaction is thought to be coupled to that of the MurG catalysed conversion of lipid I to lipid II, via the transfer of UDP-activated N-acteyl-D-glucosamine. Lipid II is then flipped across the IM by an as of yet unidentified flippase to the periplasmic face (10). The lipid II moiety is linked to the peptidoglycan strand by glycosyltransferase (GT) and transpeptidase (TP) enzymes, GT_{PGP} and TP_{PGP}, respectively, where the lipid II undergos initiation (combining two lipid II moieties) followed by elongation (adding a further two lipid moieties, ready for transpeptidation. Binding to the transpeptidase, converts the D-Ala-D-Ala to an acyl-enzyme intermediate, which can be then cross linked to the amino group of the peptidoglycan strands (10).

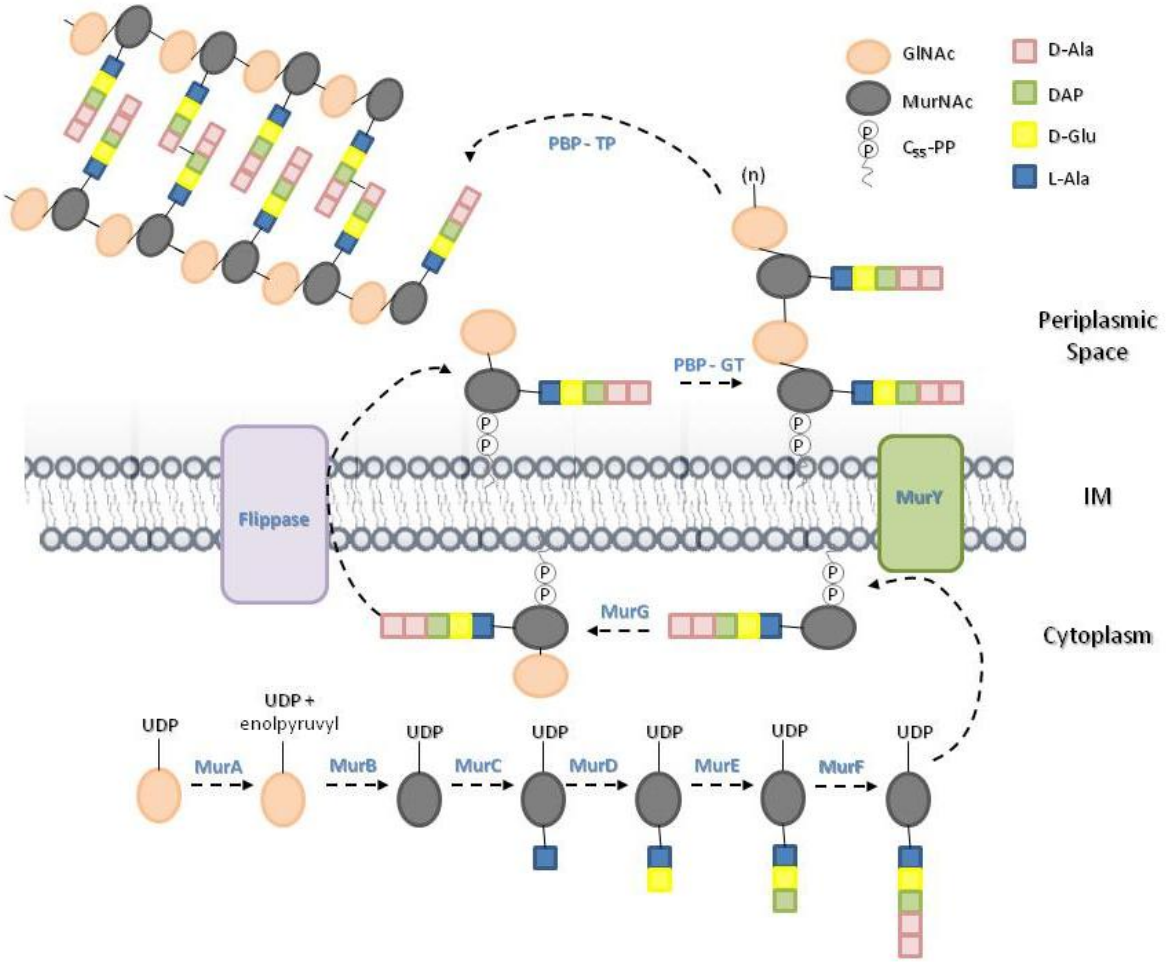


Figure 1.1. Peptidoglycan biosynthesis
Pathway of peptidoglycan biosynthesis, with individual components shown at each stage, and the enzymes catalysing each reaction shown in blue. Arrows depict the order of sequence, with the first six reactions catalysed in the cytoplasm of cell, before the MurNAc-pentapeptide is transferred to the inner leaflet of the IM membrane, where the addition of C_{55}-PP converts the molecule to lipid I, couple to MurG reaction converting lipid I to lipid II. The lipid moiety component is flipped across the membrane to the periplasmic face where it undergoes initiation and elongation reactions, combining multiple lipid II components, through the activity of PBP –GT (Glycosyltransferase) and TP (Transpeptidase).

Several OM components including, OMPs (OmpA), lipoproteins (MipA and Lpp) and cell envelope spanning complexes (Tol-Pal) can bind and associate directly with the peptidoglycan, acting as stabilisation factors, through tethering to both the OM and IM. In the case of OmpA and Pal, these interactions have been shown to be a result of non covalent interactions with the DAP residues of peptidoglycan (11, 12) . While the interaction with Lpp is mediated via a peptide bond, between the C-terminal Lys residue and DAP (13).

Numerous murine hydrolyase enzymes have also been identified for the degradation and hydrolysis of peptidoglycan, with variations in their specific target site. For example, amidases required for hydrolysis during daughter cell separation specifically target the N-MurNAc-L-Ala linkage, while muramidases such as MltA/MltB, cleave the linkages between MurNAc and GlcNAc components (13). The activity and expression of murine hydrolases is tightly regulated to prevent autolysis.

1.1.2.1. Periplasmic Chaperones.

Despite the absence of ATP, numerous proteins including chaperones, proteases, isomerases and disulfide bond catalysts function to deliver, degrade, fold and modify proteins destined for the OM. Chaperones including SurA, Skp and DegP (to name but a few), transport polypeptides from the Sec translocon at the IM to the BAM complex, catalysing their folding, and/or degradation as required (1, 2).

SurA, a peptidyl-prolyl isomerase (PPIase) forms the major chaperone pathway, with substrates including OmpA, OmpF, LamB and autotransporters (ATs) (14-16). The protein is composed of a 20 residue N-terminal region, two PPIase domains and a 35 residue C-terminal domain. Studies have shown the PPIase I domain is devoid of activity, and has more recently been suggested to form the binding site for OMP precursors during transit. In contrast, earlier

reports indicating the N-terminal region of the protein was required specifically for OMP precursor binding (17, 18). Deletion of *surA* induces serve membrane defects, resulting in elevated sensitivity to a range of compounds including vancomycin and SDS-EDTA (14, 15). In addition, abrogation of *surA* expression attenuates virulence in a range of pathogens, including *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), Uropathogenic *Escherichia coli* (UPEC) and *Shigella flexneri*, purportedly through disruption in the biogenesis of type 1 and P-fimbriae and the AT IcsA, respectively (18).

DegP and Skp comprise an alternative chaperone pathway, predicted to function in parallel to SurA, while double mutants of *degP* and *skp* show only minor defects in OMP biogenesis, double mutants of either *skp* or *degP* with *surA* are synthetically lethal (3, 19). Transcriptionally activated by the Cpx and Sigma E (σ^E) pathways, DegP has long been known to function in a temperature dependant manner, alternating between serine protease and chaperone activity when the temperatures drop below 28^{o}C (20-22). DegP provides cell envelope quality control, enabling the degradation or refolding of misfolded OMPs, preventing their accumulation in the periplasm and the induction of stress responses (21, 22). DegP normally exists as a hexamer ($DegP_6$), formed from two trimers, though the functional protein is a multimer, $DegP_{12}$ and $DegP_{24}$, the formation of which is stimulated by substrate interactions (20, 22). Crystal structures of DegP have shown the N-terminal protease contains two PDZ domains, possessing His_{105}, Asp_{135} and Ser_{210}, which are critical to both functions (21). With a molecular mass of 1.13MDa and diameter of 195Å, $DegP_{24}$ is capable of accommodating proteins up to 300 kDa during chaperone or protease activity, eight times larger than that observed for the cytoplasmic chaperone, GroEL (20, 22).

Skp, otherwise known as HlpA, comprises the alterative chaperone pathway, working with DegP, to collect OMP precursors that have fallen off the major SurA pathway (2, 19).

Characterisation of *a Δskp* mutant showed only minor defects in OMP assembly, though these mutants are defective for the release of OmpA precursors into the periplasm (1, 2, 23). Similar to *degP*, *skp* expression is also activated by σ^E and Skp is believed to be most critical during times of elevated cell envelope stress (24).

1.1.3. Inner Membrane Composition

The inner or cytoplasmic membrane is a phospholipid bilayer, decorated with α-helical proteins and protein complexes (25). The bilayer is comprised mainly of phosphatidyl ethanolamine and phosphatidylglycerol, with some cardiolipin phosphatidylserine and polyisoprenoid carriers for the translocation of sugar intermediates (1). Two main translocation systems exist for protein export across the IM: the Sec translocon and the Twin Arginine Translocation (TAT) system (1, 2).

1.1.3.1. Inner Membrane Translocation Pathways.

The SecYEG translocon is a heterotrimeric protein complex, accepting unfolded polypeptides. Proteins are delivered to the SecYEG translocon by two different routes: the post-translational chaperone dependent pathway and the co-translational signal recognition particle (SRP)-dependent pathway. Once engaged, polypeptide translocation is driven by proton motive force and the ATPase activity of SecA, an associated accessory protein. The precursors are translocated in an unfolded N- to C- terminal direction.

The majority of proteins exported by bacteria follow the post-translational chaperone-dependent pathway with substrates including OMPs and ATs (1-3). These proteins emerge from the ribosome and are maintained in a translocation competent conformation by a chaperone; in the case of *E. coli* this function is performed by SecB (1). Proteins exported in

this manner are characterised by the presence of an N-terminal Sec-dependent signal sequence that is cleaved by signal peptidase I after translocation through SecYEG. With the co-translational pathway, SRP binds the first transmembrane segment of the protein as it emerges from the ribosome and arrests translation (1). Subsequently, the ribosome-mRNA-nascent polypeptide complex is targeted to the IM by interaction of SRP with the IM receptor FtsY. This interaction aids the release of SRP and the transfer of the nascent polypeptide to the Sec translocon (26). Once SRP is released translation resumes such that export and translation occur contemporaneously. These substrate molecules can be released by the Sec translocon into the hydrophobic environment of the IM or into the hydrophilic periplasm (1, 26).

The Tat complex functions in parallel to the Sec system, transporting folded proteins across the IM. Substrates for the Tat system are distinguishable by the presence of two arginine residues within the N-terminal signal sequence (27-30). The complex is composed of five functional proteins TatA, B, C, D and E. TatA forms the translocator, which exists in two states, one as complex of TatABC, approximately 370 kDa in size and the second as a TatA homo-oligomeric complex, ranging between 50-500 kDa (31). TatB and C are required for substrate targeting and binding and form the basic components of the translocation complex with TatA (1, 31, 32). Until recently TatD was thought to be unrelated, though recent investigations have shown this soluble component, is required for 'quality control' of the translocated substrates. Although the protein is not thought to be required for translocation per se, once substrates have been targeted to the complex, TatD recognises those which are misfolded, targeting these for degradation (33). TatE is a paralogue of TatA, which can also form translocation complexes in the absence of TatA, though these are significantly smaller, ranging between 50-110 kDa in size. Recent investigations have proposed that TatE may interact with the TatABC complex, to form larger translocation complexes of over 400 kDa,

to accommodate substrates >90 kDa (31). Substrates of this complex range in size between 10-100 kDa and include the peptidoglycan amidases; AmiA and AmiC required for cleavage of NAM cross links during cell division and molybdoproteins TorA and DmsD (1, 28, 32, 33).

1.1.3.2. IM Sensory Pathways.

A number of IM associated protein complexes exist, sensing and responding to changes in cell envelope homeostasis, including EnvZ-OmpR responding to changes in osmotic pressure, σ^E and Cpx to cell envelope stress. The mechanism by which EnvZ (situated at the IM) senses osmotic pressure is largely unknown, though its autophosphorylation stimulates phosphorylation of the transcriptional regulator, OmpR, regulating the switching of expression between OmpF and OmpC (34-36).

The σ^E system, forms an extracytoplasmic stress response (ECR) system, sensing and responding to cell envelope stress in *E. coli*, through the regulation of 40 genes, including periplasmic chaperones, proteases, LPS biosynthesis and transcriptional regulators, including *fkpA, surA, htrA, rfaCDF* and *rpoE/D/H* (37, 38). The σ^E system comprises of the IM proteins DegS, RseA and RseP and the periplasmic protein RseB (39). Under non-stressed conditions σ^E is sequestered by the IM spanning RseA, whereby σ^E is bound to the cytoplasmic N-terminal of RseA. The interaction of σ^E and RseA is further stabilised by the binding of RseB to the periplasmic C-terminal of RseA, blocking the interaction of σ^E with RNA polymerase (39, 40). Under conditions of cell envelope stress, such as the accumulation of OMPs or high temperatures, a catalytic cascade is initiated resulting in the release of σ^E. The PDZ domains of DegS and RseP inhibit their catalytic functions under normal conditions, while the binding of unfolded OMPs to DegS results in activation of catalytic activity, promoting degradation of

periplasmic domain of RseA, thus releasing RseB into the periplasm. This activity simulates the catalytic response of RseP, resulting in the degradation of the transmembrane domain of RseA and subsequent release of σ^E into the cytoplasm, allowing for interactions with RNA polymerase, as shown in figure 1.2. (39, 40).

CpxA, R and P sense and respond to both extracellular and cell envelope stress, in a similar manner to the σ^E pathway (25, 38, 41). Originally described as a two component system, comprising of the 52 kDa histidine kinase, CpxA and 26 kDa response element, CpxR, the periplasmic sensory component CpxP was only later identified (25, 42). CpxA spans the IM with both periplasmic and cytoplasmic exposed domains, whereby binding of CpxP to the periplasmic sensory loop maintains the complex in an inactive form, with CpxR bound to the cytoplasmic face (25, 41). Under conditions of stress the complex becomes activated through a series of degradation and phosphorylation reactions; whereby CpxP is degraded by DegP, enabling ATP dependant autophosphorylation of the conserved histidine residues on CpxA, which in turn phosphorylates CpxR at a conserved aspartate residue, allowing for direct interactions between CpxR and DNA (25, 43).

The Cpx system responses to numerous cellular stimuli, including altered external pH, changes in membrane composition, the accumulation of misfolded pili subunits and the activation of NlpE by adhesion to hydrophobic surfaces (25, 42-45). The Cpx system functions across 34 operons, regulating and stimulating the expression of 50 genes, including periplasmic chaperones and proteases, peptidoglycan associated enzymes, IM associated proteins and cell envelope localised components (including *ppiA, degP, amiA, amiC, hlpX* and *yccA)* (43).

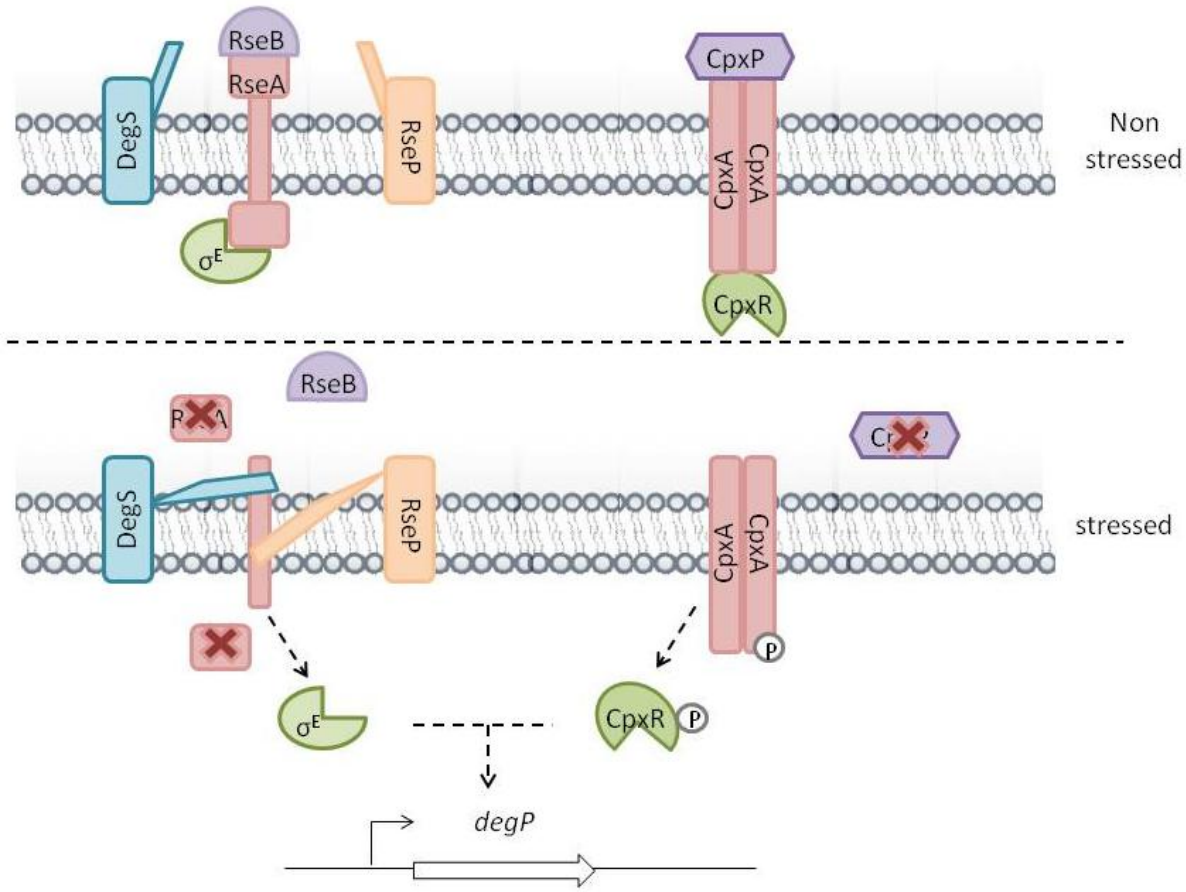


Figure 1.2. Membrane stress response pathways
Mechanisms for responding to extracellular and periplasmic stress in Gram–negative bacteria, showing the components of the Sigma (σ) E and Cpx systems under both non-stressed and stressed conditions. The σ^E pathway is comprised of RseA/B/P, DegS and σ^E. Under normal conditions σ^E is sequestered by RseA, stabilised by the binding of RseB. Under conditions of periplasmic stress and accumulation of unfolded OMP precursors, DegS becomes activated leading to the degradation of RseA on the periplasmic face, stimulating the release of RseB into the periplasm and the catalytic activity of RseP, which degrades RseA at a transmembrane site, subsequently releasing σ^E into the cytoplasm to allow interaction with RNA polymerase. The Cxp system comprises the periplasmic CpxP, IM CpxA and cytoplasmic CpxR. Under normal conditions CpxP is bound to CpxA, inhibiting histidine kinase activity, thus preventing phosphorylation of CpxR. Stimulation of Cpx system by cell envelop or extracellular stresses, causes the degradation of CpxP by DegP, allowing for autophosphorylation of CpxA, which in turn phosphorylates CpxR, leading to its release enabling interactions with DNA to promote transcription of specific genes. Example shown for both systems is the activation of *degP* transcription, though neither system is limited to this gene alone.

Investigations into cell envelope stress responses mediated by σ^E and Cpx systems indicate significant overlap, with regards to the direct regulation of the same genes (i.e. *degP*), but also regulation of genes encoding functionally redundant proteins (i.e. isomerases such as FkpA and PpiA) (46). Though interestingly only components of σ^E pathway are essential for viability (46).

1.1.4. Cell envelope complexes required for the biogenesis of OM components

As discussed previously several complexes exist for the biogenesis and insertion of key OM components, including the Lol pathway for lipoprotein insertion into the OM, the Lpt pathway for LPS biogenesis and the BAM complex for the correct assembly of β-barrel OMPs (figure 1.3). Whilst no pathway, complex or individual proteins have been identified for the insertion of phospholipids into the inner leaflet of the OM, the Maintenance of Lipid Asymmetry (Mla) pathway has been shown to regulate OM phospholipid content.

1.1.4.1. Lol Pathway.

The Lol pathway distinguishes OM lipoproteins, ensuring correct cellular localisation and catalysing their insertion into the membrane (1). Lipoproteins, similar to other cell envelope destined proteins contain an N-terminal signal sequence facilitating transport across the IM by the Sec translocon. For lipoproteins destined for the OM an additional N-terminal consensus sequence, termed a 'lipobox', consisting of Leu-(Ala/Ser)-(Gly/Ala)-Cys, targets these to the Lol pathway (1, 30). In contrast, IM destined lipoproteins, contain an aspartate at position two of the mature protein, functioning as a Lol avoidance signal (30). In contradistinction to OMP precursors, lipoproteins are modified by phosphatidylglycerol/prolipoprotein diacylglyceryl transferase, Lgt, forming a thioesther

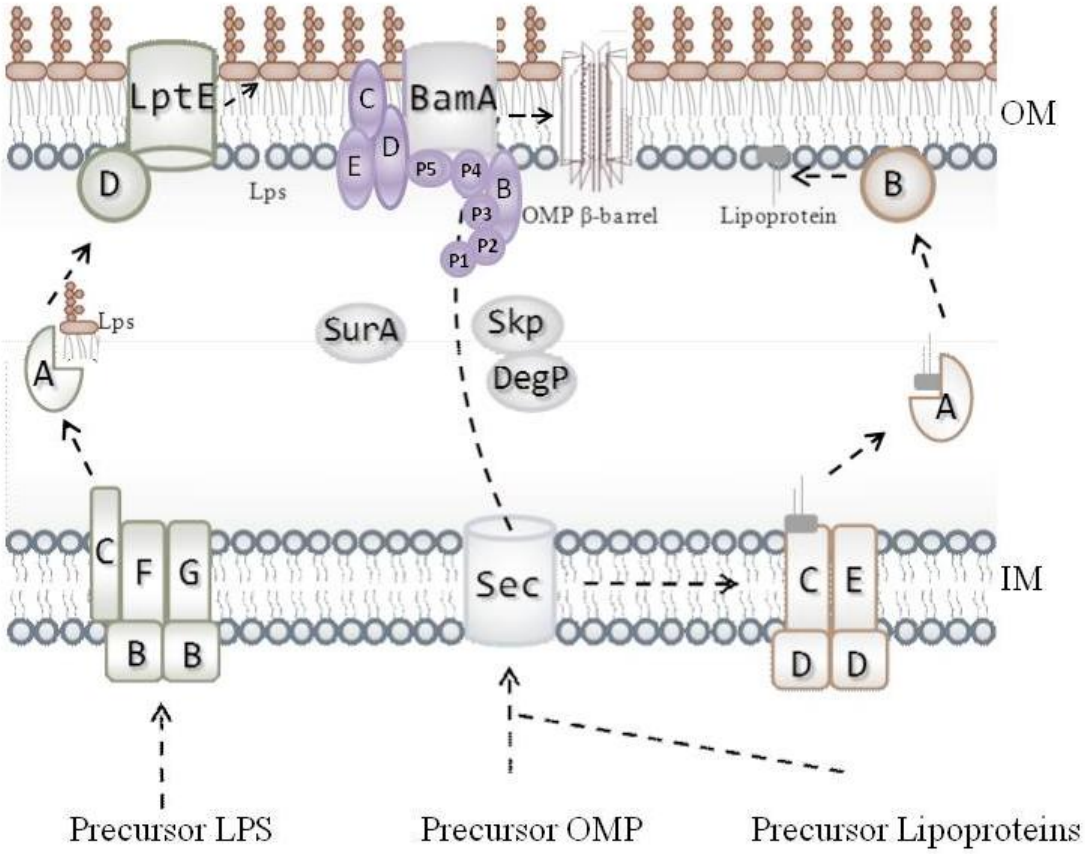


Figure 1.3. Cell envelope pathways for OM component transport and insertions
The components and pathways associated with LPS, OMP and Lipoprotein insertion into the OM, by the Lpt pathway, BAM complex and Lol pathway, respectively. All OM components are synthesised in the cytoplasm and transported by their dedicated pathways to the correct site. The Lpt pathway consists of LptB, C, F and G at the IM, whereby LptB energies the complex to allow release of LPS molecules to the periplasmic LptA, forming a bridge across the periplasm for the transport of LPS, where its transferred to the lipoprotein LptD and OMP LptE. Though the exact mechanism by which LPS becomes localised to the outer leaflet is unknown. The BAM complex folds and inserts OMP into the OM, OMP precursors are transported across the IM by Sec translocon and collected by periplasmic chaperones (including SurA, DegP and Skp) for transport across the periplasm to the BAM complex at the OM. SurA has been shown to directly interact with BamA POTRA (P) 1, transferring precursors to the complex. Lipoproteins BamB, C, D, E function in conjunction with the integral OM protein BamA to fold and insert β barrel OMPs into the membrane. Whereby BamB directly interacts with BamA through P2-5, BamD interacts with BamA independently of BamB, though P5, providing the means of interaction of BamC and BamE with BamA. The Lol pathway accepts OM destined lipoproteins from Sec machinery at the IM, transferring these to LolC (in complex with LolD and LolE at the IM), rearrangement of the complex allows for transfer of the lipoprotein to periplasmic LolA in an ATP dependant manner. LolA transports lipoproteins across the periplasm to LolB where they are transferred in an affinity dependant manner. Currently the mechanism by which LolB inserts lipoproteins into the OM is unknown.

diglyceride at the cysteine residue prior to signal sequence cleavage by signal peptidase II (1, 30). The N-terminal cysteine is then further modified by the phospholipid/apolipoprotein transacylase (Lnt), which adds a fatty acyl chain enabling the protein to be tethered to the membrane (1, 30).

The IM ABC transporter LolCDE (present in 1:2:1 stoichiometry) facilitates the transfer of the OM lipoprotein from the IM to the periplasmic LolA, in an ATP dependant manner, during which LolC acts as a scaffold for LolA, LolE reorganises the complex for transfer and LolD catalyses the binding and hydrolysis of ATP (30). LolA is responsible for the delivery and transfer of lipoproteins to the OM LolB lipoprotein, which is itself anchored to the inner leaflet of the OM. The subsequent transfer of lipoproteins from LolA to LolB is predicted to occur in a 'mouth to mouth' action between the respective hydrophobic cavities of each protein. Transfer from LolA to LolB is thought to be mediated simply by LolB having a greater affinity for the acylated portion of the molecule. The mechanism of transfer and insertion of lipoproteins from LolB to the OM currently remains ambiguous, though both the N-terminal LolB membrane anchor and Leu_{68} are thought to be critical (30).

1.1.4.2. Lpt Pathway.

All components of LPS, including the core polysaccharide and the O-antigen polysaccharide (where applicable) are synthesised in the cytoplasm or at the inner leaflet of the IM, and require transport across the IM to the periplasm and beyond to the OM (47). The biosynthesis of lipid A occurs at the inner leaflet of the IM, with the addition of two Kdo molecules, to which four fatty acid chains are attached, prior to the addition of two CMP-Kdo residues, via the CMP-Kdo transferase, creating the Kdo2-lipid IVA molecule. This molecule undergoes acylation to form the hexaacylated Kdo2-lipid A molecule, before the addition of

sugars, via a specific glycosyltransferase to create the oligosaccharide core (47). The core lipid A molecule is anchored to the IM, with the hydrophilic moiety exposed to the cytoplasm, for flipping by the ABC transporter, MsbA (47, 48).

O-antigen (where present) is also produced at the IM and attached to the lipid carrier undecaprenyl diphosphate. The precise O-antigen structure is highly variable amongst Gram-negative bacteria and is dependent on the nature, order and linkage of the different sugars within the polysaccharide. The core lipid A and O antigen molecules are ligated on the periplasmic side of the IM, by the ligase WaaL (47).

The hydrophobic nature of the LPS molecule presents a significant challenge to Gram-negative bacteria, since they must transport the molecule through the hydrophilic environment of the periplasm. To facilitate this transfer, Gram-negative bacteria have evolved the conserved and essential Lpt pathway. The Lpt pathway is a transenvelope complex formed by LptA-G (47, 49). LptB is cytosolic, forming a complex with LptC, F and G at the IM, while LptD and E comprise the OM β-barrel protein and lipoprotein, respectively (49). The ABC transporter LptB energises the complex, allowing for release of LPS from the IM to LptC. LptC mediates transfer of LPS to the periplasmic LptA protein, which forms a continuous bridge between the IM complex and the OM components. Transport of LPS across the periplasmic space to the OM components LptD and LptE is thought to occur in a continuous manner, much like a conveyor belt and although, the mechanism by which LPS is inserted into the OM still remains unclear(47, 49) two hypotheses exist: (1) LPS is inserted into the inner leaflet of the OM and flipped to the outer leaflet or (2) LPS is released into the membrane through lateral transfer from LptD (47).

1.1.4.3. BAM Complex.

The BAM complex is required for the correct insertion of folded β-barrel OMPs into the OM (50-53). The complex is made up of the essential OMP BamA (previously known as YaeT) and four lipoproteins, including BamB (YfgL), BamC (NlpB), BamD (YfiO) and BamE (SmpA), of which BamD is essential (50, 52, 54, 55).

BamA, is an integral OM-spanning β-barrel with an N-terminal extension of five polypeptide transport associated (POTRA1-5) domains extending into the periplasm (55, 56). The POTRA domains have been shown to mediate the interaction of BamA with BAM lipoproteins; BamB via POTRA2-5 and BamD via POTRA5, while POTRA1 interacts with the periplasmic chaperone SurA (55, 57). Despite having low sequence homology (<13% identity), the POTRA domains have been shown to demonstrate significant structural similarity. Each domain is comprised of three β sheets and two antiparallel α helices (55, 56, 58). However, POTRA3 displays key difference from the other POTRA domains; it possesses a longer loop between the two α helices and a β bulge located in β strand two, allowing for the binding of additional β strands (58). Subsequent NMR and SAXS analyses revealed each POTRA domain exists in a monomeric form, with rigid hydrogen bonding between POTRA1-2 and POTRA3-4 (58). Interestingly, the structures of POTRA2-3, solved by two separate groups, demonstrated significantly different degrees of conformation (30^{o} and 130^{o}) in the angle connecting these domains, suggesting this connection serves as a flexible hinge, to draw substrates closer to the BamA barrel (59, 60).

While the structures of all POTRA domains were determined some time ago, the structure of the BamA barrel was only published in September 2013 (61). The structure of the β barrel, indicates BamA contains a large periplasmic domain which is attached to the 16 stranded β barrel, with extracellular loops and periplasmic loops connecting the individual

strands. Loops 4, 6 and 7 have been shown to form a large 'dome like structure' across the top of the barrel, whereby loop 4 encodes an α helix predicted to be positioned horizontally to the membrane, allowing the formation of electrostatic interactions with loops 3 and 6 along the surface, sealing the barrel lumen from the extracellular environment (61). Uniquely the structure indicates the interactions between β strands 1 and 16 are sufficiently destabilised to promote lateral opening of the barrel into the OM, a mechanism previously thought to be biophysically unfavourable. Though consistent with this hypothesis, analyse of the local membrane environment surrounding the barrel, suggests the hydrophobic extracellular surface results in local destabilisation of membrane lipids, priming the membrane ready for OMP insertion (61). In light of this publication, several theories regarding the biogenesis of OM β barrel proteins may need to be reinvestigated, to reconcile previous hypotheses with this model for lateral transfer.

As described above the BAM complex includes four lipoproteins. BamB is highly conserved and interacts directly with BamA through POTRA2-5 domains mediating the transport of OMPs from chaperones (e.g. SurA) to the complex (52, 54, 56, 62, 63). Structural investigations have suggested BamB is capable of interacting with both BamA and unfolded OMPs simultaneously, by β augmentation, supporting the OMP precursor during folding (64). Deletion of *bamB* causes serve defects in OMP biogenesis, increased permeability and sensitivity towards antibiotics including vancomycin (54, 56, 57). However, the underlying molecular basis for this defect has yet to be elucidated.

BamC forms a surface exposed lipoprotein. It is predicted to maintain the stability and function of the BAM complex by enhancing the efficiency of interactions with OMPs. However, deletion of *bamC* results in only a slight membrane permeability defect, as determined by increased sensitivity to rifampicin (55, 56, 65).

BamD is the only other essential component of the BAM complex (52, 55). Interacting with BamA via POTRA5, it provides the scaffold connecting BamC and BamE to the complex (55). BamD is predicted to act as a substrate recognition protein, binding the C-terminus of β-barrel precursor molecules, functioning paradoxically with BamE to mediate the switching of BamA conformations (55, 58, 63, 66). In this model BamD mediates the conformational change of BamA, creating a protease sensitive form, where extracellular loop 6 becomes surface exposed (67). BamE restores BamA to the protease resistant form, in which loop 6 is predicted to reside in the barrel. The function of these morphological changes has get to be confirmed, though this change in conformation is proposed to represent the cycle of OMP biogenesis, whereby BamA is required to adopted an altered conformation, in order to complete the biogenesis, while BamE returns the complex to its native form upon completion (66).

BamE is also nonessential and was proposed to stabilise the complex through interactions with inner leaflet phosphatidylglycerol (68). Similar to that of other nonessential BAM components, deletion of *bamE* does not appreciably affect the biogenesis of ATs, although it does result in elevated sensitivity to markers for membrane permeability including vancomycin (69).

1.1.4.4. Phospholipid Transport.

To date no mechanistic pathway for the means which phospholipids are inserted into the inner leaflet of the OM has been identified, despite early investigations having shown the rates of phospholipid translocation from the IM to OM are not consistent with that of diffusion nor is this process coupled to protein or LPS biosynthesis (70). Though it has previously been suggested that lipoproteins may play a partial role, this hypothesis has yet to

be confirmed experimentally (30). Identification of the Mla pathway, provides significant insight into the means by which bacteria regulate, and prevent phospholipid accumulation in the outer leaflet of the OM (71), though this pathway functions solely for recycling and not insertion. The Mla pathway is comprised of MlaBDEF complex at the IM, the periplasmic component, MlaC and OM lipoprotein, MlaA (71). MlaA is predicted to remove phospholipids from the outer leaflet of the OM, transferring these to the soluble MlaC for trafficking back to the IM complex MlaBDEF (figure 1.4), after which the fate of the phospholipid is currently unknown (71).

This system differs from the already known mechanisms of PldA and PagP, which destroy outer leaflet phospholipids recycling them via the Fatty Acid Degradation (FAD) pathway and catalysing the transfer of palmitate from the sn-1 position on phospholipids to lipid A, respectively (72, 73). Consistent with these similar functions, over expression of *pldA,* but not *pagP,* can compensate for the loss of *mla* (71).

1.2. SECRETION SYSTEMS IN GRAM NEGATIVE BACTERIA

The ability of bacteria to secrete proteins is an essential facet of survival. Secreted proteins provide an array of beneficial functions for bacterial cells including acting as mediators of motility and nutrient acquisition, immunomodulators and adhesins. However, the impermeable nature of the OM poses a significant barrier to the secretion of proteins to the extracellular milieu since these proteins are larger than the diffusion limit of the OM. Gram-negative bacteria have evolved a limited number of specialised proteinaceous machineries to overcome this obstacle. To date seven OM secretion systems have been

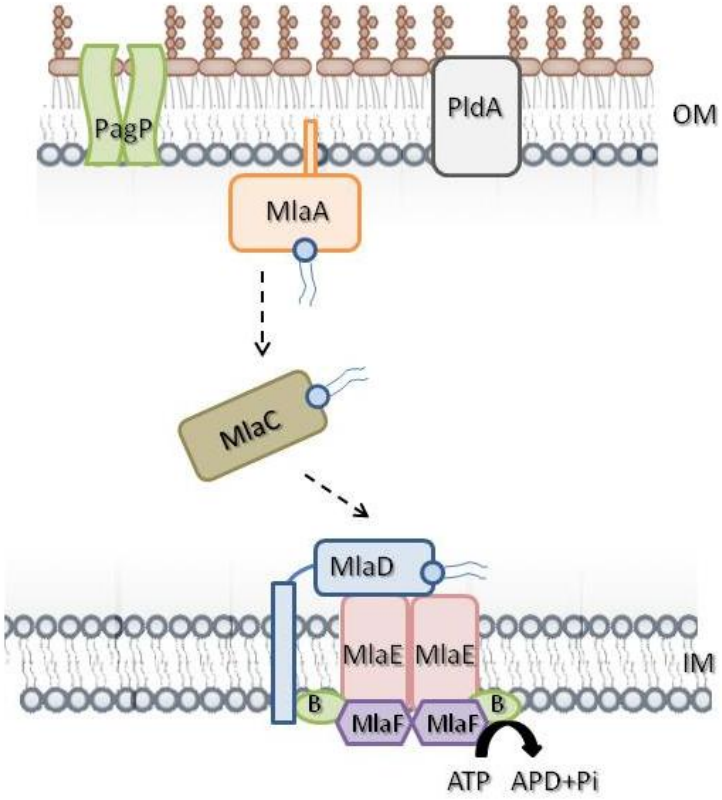


Figure 1.4. Mechanisms for maintaining OM asymmetry
The asymmetric nature of the OM, with LPS on the outer leaflet and phospholipids on the inner leaflet is maintained by three methods; the Mla pathway and the OM proteins PagP and PldA. The Mla pathway consists of the OM lipoprotein MlaA, which is predicted to remove phospholipids from the outer leaflet, transferring these to the periplasmic MlaC for transport back to the IM complex MlaB, D, E and F, where the fate of the phospholipids is unknown. PagP is localised to the OM with the active site exposed to the extracellular environment. The means by which PagP interacts with outer leaflet phospholipids is unknown, though predictions suggest the mislocalisation of phospholipids promotes access to PagP, which catalyses the transfer of palmitate from the sn-1 position to lipid A. PldA is found in two conformations, the inactive monomer or active dimer, binding of calcium promotes dimerisation, catalysing the removal of sn-1 and sn-2 fatty acid chains from the phospholipid backbone. Currently no pathway or mechanism has been identified for phospholipid transport to the OM.

identified, as shown in figure 1.5 and 1.6 (74, 75). Most of these utilize a series of protein complexes, comprised of numerous accessory proteins functioning in conjunction with chaperones and other factors. However, they can be largely subdivided into two categories: (1) the one step mechanism whereby substrates and effector molecules are transported directly from the cytosol to the either the extracellular environment or eukaryotic host cell or (2) the two step mechanism, whereby substrates require transport across the bacterial IM, prior to secretion (75).

1.2.1. One-step secretion systems across the OM

The Type I secretion system (T1SS), is composed of a tripartite system, delivering proteins of varying size (82- 8000 amino acids) directly from the bacterial cytosol to the extracellular environment. Substrates of the T1SS include toxins, proteases, lipases, S-layer proteins, bacteriocins and other proteins with as yet unknown functions. The best studied example is that of hemolysin found in UPEC and Enterohemorrhagic *E. coli* (EHEC) (75, 76). The T1SS comprises of the IM traffic-ATPase (ABC) or proton-antiporter, a periplasmic adaptor or membrane fusion protein (MFP) and an Outer membrane factor (OMF), which is normally TolC or a related protein (75). TolC forms a 12-stranded trimeric OMP with α-helical periplasmic extensions. The periplasmic domains of TolC interact with the IM ABC protein through interactions with the periplasmic MFP creating a continuous channel through the bacterial cell envelope (77). The system utilises ATP binding and hydrolysis to drive the transport of substrates (75).

A system with many analogies to the T1SS is the multidrug efflux pump formed by TolC, AcrB (the IM ABC) and AcrA (a periplasmic MFP), which mediates the interaction between AcrB and TolC. Together this complex confers resistance to compounds including

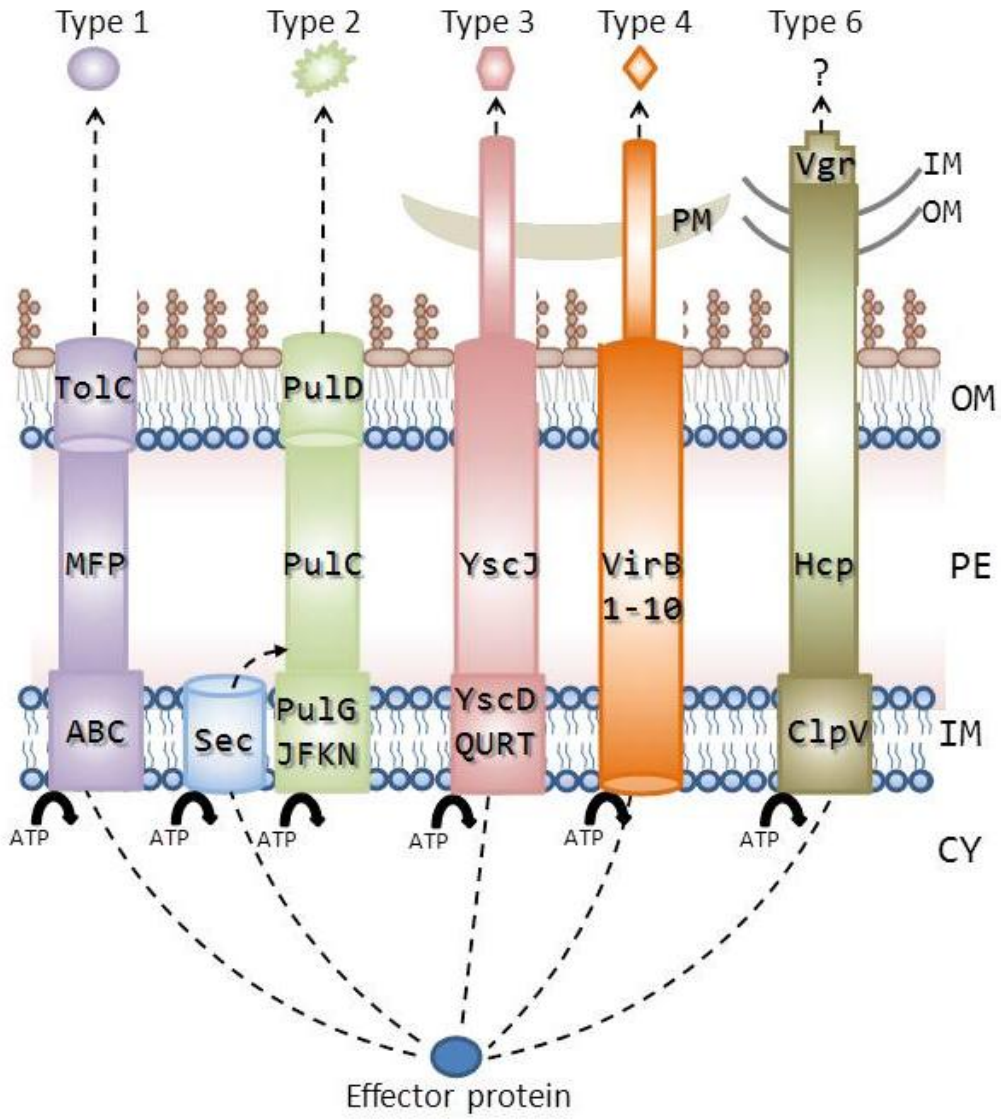


Figure 1.5. Type 1,2,3,4 and 6 secretion systems of Gram negative bacteria
Simplified schematic of secretion systems found in Gram-negative bacteria for the export of proteins and DNA to either the extracellular environment or the cytoplasm of host cells. Type1,3,4 and 6 provide one step secretion of their relevant substrates, while Type 2, requires the assistance of the Tat or Sec Translocon at the IM for transport of substrates to the periplasm in preparation for export. The Type 1 SS, is exemplified by the export of Hemolysin. Type 2 SS complex is envelope spanning, utilising ATP to drive secretion of the substrates to the extracellular environment. Type 3 secretion, also known as injectosome, transports effector molecules directly to the eukaryotic cell cytosol, with some of best studied examples being that of SPI1 and SPI2 encoded T3SSs in *Salmonella.* Type 4 secretion promotes conjugative transport of DNA, plasmids and other mobile genetic elements, directly to recipient cells. The Type 6 SS, is homologous to the that of bacteriophage secretion systems, enabling secretion of effector molecules directly into the host cell cytosol.

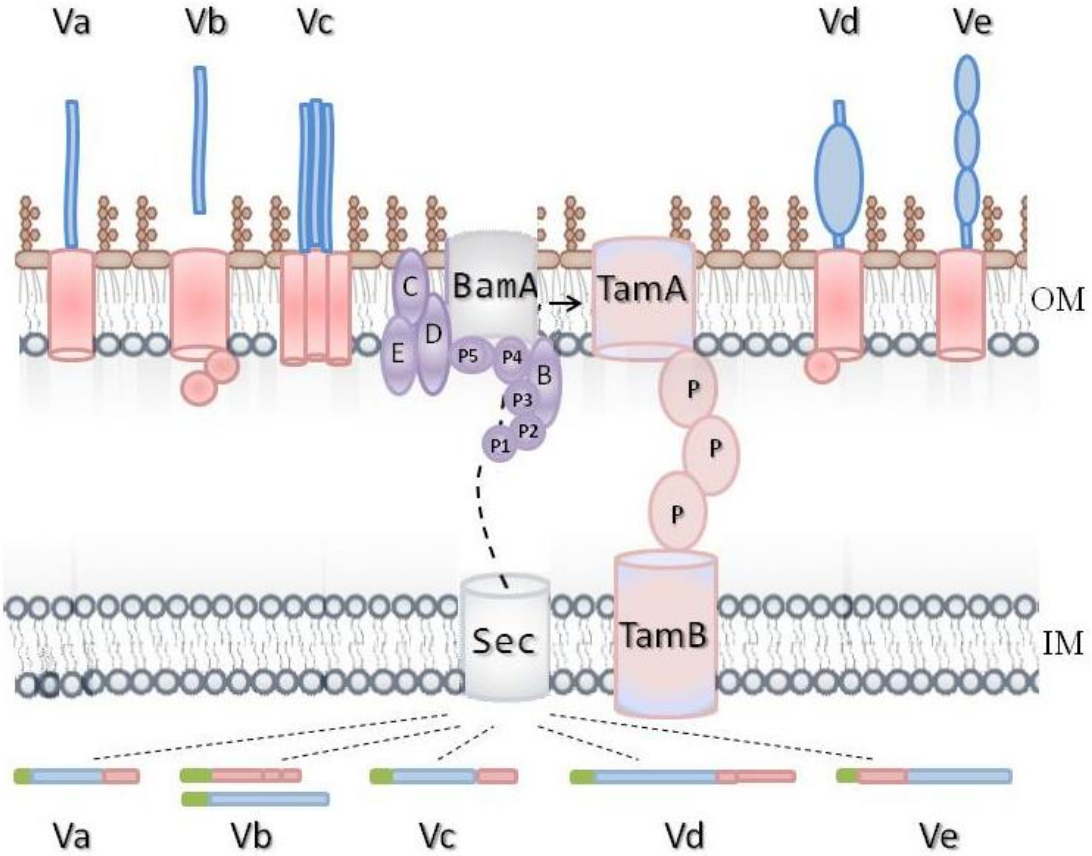


Figure 1.6. Type V secretion
The subclasses of the Type V SS, designated a-e. This two step system, uses the Sec translocon at the IM to transport AT precursors to the periplasmic space where they are collected by periplasmic chaperones (including SurA, DegP, FkpA) for transport to the BAM complex at the OM. BamA and BamD have been shown to be critical for the secretion of Va proteins, through the requirements for other subclasses have yet to be explored. The BAM complex folds and inserts the β-barrel domain into the OM, while the TAM complex is predicted to assist in passenger domain folding. Va proteins are encoded as a single polypeptide forming a 12 β-barrel in the membrane, with a secreted or surface exposed passenger domain, examples include ShdA and MisL from *S.* Typhimurium. In the case of Vb or two partner secretion, the β domain and passenger domain are encoded for by separate polypeptides. Where the β domains fold to form a 16 stranded barrel with two N-terminal POTRA domains, examples include FHA from *B. pertussis* and HMW1 from *H. influenza*. Vc comprise the TAAs, whereby three monomers conform to create the 12 β-barrel in the membrane with a trimerised passenger domain attached at the surface, including the prototypical example YadA from *Y. enterocolitica*. Vd, the fussed two partner secretion, is encoded by a single polypeptide with a POTRA domain encoded between the passenger and β-domains, with PlpD from *P. aeruginosa* being the main example. Ve are inverted ATs where the β-domains are encoded N-terminally, with C- terminal passenger domain, separated by a LysM domain. The main example is Intimin from *E. coli*.

antibiotics, dyes, organic solvents, detergents (78, 79) and natural metabolites including indole and siderophores (51, 77). Other drug efflux systems with differing specificities also exist within *E. coli* (80).

The Type III secretion system (T3SS), also known as the injectisome, mediates transport of effector molecules from the bacterial cytosol directly to the cytosol of eukaryotic cells. This system has been extensively studied in *Salmonella, Shigella, Yersinia* and various *E. coli* types, including Enteropathogenic *E. coli* (EPEC) and EHEC strains, to name but a few. The T3SS is composed of over 20 proteins, including highly conserved membrane proteins, cytoplasmic chaperones and several accessory proteins (75). The structure of the complex is significantly similar to that of flagella, with their biogenesis tightly controlled in a stepwise manner, with the initial formation of the basal body, preceding that of the needle (75). While the effector molecules secreted by these complexes have no obvious signal sequence, the N-terminal 20-50 residues commonly share an unusual enrichment of serine and there is evidence that the 5' mRNA plays a role in secretion. Suggesting a combination of co-transcriptional regulation of effectors with T3SS chaperones may aid in their targeting to the complex (81).

The Type IV secretion system (T4SS) is responsible for the conjugative transfer of DNA, plasmids and other mobile genetic elements (75). These systems are composed of 12 individual proteins, named VirB1-11 and VirD4, spanning the cell envelope. VirB4-B11 and VirD4 form the IM associated ATPase in conjunction with the IM complex formed by VirB6, B8 and B10, VirB7 and B9 comprise the OM complex, while VirB2 and B5 are required for surface contact with host cells and VirB1 functions as lytic transglycosylase, breaking down peptidoglycan to allow complex assembly. Additionally, VirB3 is IM associated, though its function is unknown (75). This secretion system has been extensively investigated in the

gastric pathogen *Helicobacter pylori* where it is encoded by the 40-kb *cag* pathogenicity island (*cag* PAI). The system translocates the cytotoxin associated gene A (CagA) into host epithelial cells, which stimulates the induction of NF-κB and pro-inflammatory immune responses (82).

The recently discovered Type VI secretion system (T6SS) was identified and named in 2006, after its initial identification in *Vibrio cholera* and *Pseudomonas aeruginosa.* Initially named as virulence associated secretion (VAS) due to its requirement for contact-dependent cytotoxicity in amoebae and macrophages (83). The T6SS is present in a variety of Gram negative organisms and its transcription (where examined) is linked to that of other virulence associated complexes, including T3SS, though is predicted to be tightly controlled through post translational modifications (84). T6SSs are commonly encoded for by 13-25 genes dependant of the species, forming a needle like structure similar to that observed with the T4SS (85, 86). Components of the T6SS are largely subdivided into three categories; membrane proteins (TssL and TssM) or lipoproteins (TssJ) forming a cell envelope complex with peptidoglycan, several proteins of unknown function (TssA, TssF, TssG and TssK) and bacteriophage tail like proteins (hemolysin coregulated protein [Hcp] and the valine–glycine repeat protein G [VgrG], TssB, TssC and TssE) (86). Of this latter group, TssB and TssC form sheath like tubular structures, enclosing the Hcp tubular domain, which is thought to holding the Hcp/VgrG complex in place at the cell envelope, until activation when release from TssB/C shealth provides propulsion to direct Hcp/VgrG through the cell envelope and into the membrane of the target cells (86).

1.2.2. Two-step secretion across the OM

The Type II secretion system (T2SS) is a two step secretion mechanism, with substrates requiring translocation to the periplasm, prior to secretion across the OM (75). Substrates for the T2SS can be translocated across the IM by the Sec or Tat translocons (75). The substrates then dock with the T2SS which is an envelope spanning complex comprised of 12-16 protein components. These are usually transcribed as a single operon and are highly conserved across both human and plant pathogens. In *E. coli* these proteins have been designated as GspA to GspO (75, 87). GspA and GspB form part of the IM complex, required for energy transduction from ATP hydrolysis, GspC selects the substrate, GspD oligomerizes forming the OM channel, GspE functions as cytoplasmic ATPase, GspF forms the inner transmembrane protein, while GspG to GspK form the major and minor pseudopilin (75, 87). Substrates of this system include a variety of toxins and enzymes, including lipase, elastase, alkaline phosphatase and phospholipases (87).

The chaperone usher (CU) pathway is responsible for the secretion of surface associated pili and fimbriae. With a dedicated periplasmic chaperone, and OM pore protein termed the usher component, directing assembly (75). The chaperone prevents polymerisation in the periplasm, and directs subunits to the usher platform where polymerisation is controlled in a sequential manner. (88). P and Type 1 pili are two of the most well studied examples in pathogenic *E. coli* strains, ranging in size between 12 and 20 kDa, dependant the number of major subunits. Both P and type 1 pili are relatively similar, though type 1 exhibit a shorter fibrillae tip (88). These multi-subunit structures are polymerized from the distal tip with PapG/FimH first, followed by an adaptor subunit PapF (P pilus only) and five to 10 subunits of PapE or one FimG. The adaptor subunits PapK/FimF connect the tip to the major rod PapA/FimA, of which potentially thousands of subunits maybe present, with the terminal

subunits PapD/FimC attached at the base, and in the case of P pilus, followed by the terminator subunit, PapH (88). These structures have long been associated with virulence, biofilm formation, adhesion and motility (75, 88).

1.2.3. The Type V Family

The Type V secretion system (T5SS) can be subdivided into classes Va-Ve, based on the genetic organisation and protein structure (figure 1.6). This system is the simplest and most wide spread protein secretion system in Gram negative bacteria (89). Proteins secreted by the T5SS are characterised by the presence of three key domains: (1) an N-terminal signal sequence, (2) a secreted or surface exposed passenger and (3) an integral outer membrane β-barrel to mediate translocation of the passenger domain to the cell surface (90-92).

The signal sequence is required for Sec-dependant targeting and translocation across the IM. Analyses of the signal sequences from several proteins secreted by the T5SS reveal the presence of two distinct groups: those containing a classical signal sequence of 20-30 amino acids, and those that encode a signal sequence of 50-60 residues (74, 90). When compared to the classical signal sequences, the larger signal sequences possess an N-terminal extension sequence of approximately 25 amino acids (74). Previous speculation regarding the function of the extended signal sequence, suggested it may be required for SRP targeting or to direct the polypeptide to an alternative translocator at the IM, such as the Tat system (93). However, more recent investigations have resolved this matter, having shown the extended sequence inhibits SRP binding, maintaining the protein in an unfolded manner whilst in the cytoplasm (74, 90). Interestingly, these extended signal sequences are confined to proteins larger than 100 kDa (90, 93). The various modes of passenger domain translocation across the OM are detailed in the sections below.

1.2.3.1.Type Va (Classical ATs).

The Type Va are also known as the 'classical ATs'. In the case of the classical ATs the protein is encoded by a single polypeptide containing a signal sequence, a C-terminally located translocator or β domain and an autochaperone (AC) domain located between the passenger and β domains (92). The translocator domain forms a 12 stranded β barrel in the OM, while the majority of the passenger domains (>97%) fold to form a right-handed β helical arrangement. In contrast, the remaining passenger domains (<3%) have been shown to form a globular structure, rich in α helices (94).

The term ATs was based on early hypotheses that the AT polypeptide contained all the elements required for its own secretion. Since their initial identification, with the isolation and characterisation of the IgA protease from *Neisseria gonorrhoeae* (95), significant amounts of research have shown this theory of self export is not strictly true, but requires numerous other cellular complexes and proteins to aid in their secretion and folding (90). Once the AT polypeptide emerges from the Sec translocon it must be trafficked to the OM, by periplasmic chaperones including SurA, DegP, Skp and FkpA which have been shown to be critical for translocation of EspP precursors (16, 96). Chaperone AT interactions prevent misfolding and the accumulation of aggregated precursors in the periplasm, though binding and degrading, respectively while en route (16).

As SurA is required for efficient AT biogenesis, and is known to dock with POTRA1 of the BAM complex, it was hypothesised that the BAM complex must be directly involved in the assembly of ATs. In support of this hypothesis, Jain *et al* (97) demonstrated that depletion of BamA inhibited the secretion of IcsA and SepA from *Shigella flexneri*. Since this investigation the involvement of the BAM complex in the assembly and biogenesis of ATs into the OM has been identified for several species (98, 99). As described above, chaperones

translocate AT precursors across the periplasmic space to the BAM complex, where the essential BAM components, BamA and BamD are required for biogenesis (16, 63, 90). Interestingly thoughly, the non-essential lipoprotiens BamB, C and E are not involved in AT secretion nor do they contribute to passsenger domain folding, in the case of Antigen 43 (Ag43) or Pet (100).

Whilst it is clear that the BAM complex plays a crucial role in the biogenesis of ATs, the mechanism by which the passenger domain is secreted to the exterior of the cell remains a subject of extensive research. Several models were proposed for the mechanism of passenger domain secretion: N-terminal threading, multimer, BamA, and hairpin. However, several investigations provided evidence that largely discounted two of these theories, with the most viable options remaining being that of the BamA and hairpin models (90).

The BamA model suggests the AT β barrel is maintained either in the pore of BamA or adjacent to BamA, in a semi-folded state, allowing for a larger pore size and thus secretion of the passenger domain in a hairpin like manner or alternatively the passenger domain is secreted via BamA first with the AT β domain being inserted second (90). Consistent with the proposed BamA model, studies in *N. gonorrhoeae* have shown BamA can form pores up to 2.5 nm in diameter, sufficient to accommodate a partially folded passenger domain (90). Additionally the recently described structural data for BamA does suggest lateral opening into the membrane is possible (61), which does give more premise to the theory of BamA maintaining the AT β barrel in a partially folded conformation to allow for passenger domain secretion in a hairpin like fashion. As investigations with ATs containing engineered passenger domains with large periplasmic secondary structures can block secretion when the secondary structure is above a certain size (101). Though closer inspection of these stalled intermediates indicates the C terminal region of the passenger domain is exposed on the

surface while the N terminal region is undetectable, in the barrel lumen and periplasm (101), consistent with the hairpin model of secretion. Thus the BamA model, promoting hairpin like secretion of the passenger domain, does additionally reconciling previous complications with the suggested idea of the AT β-barrel promoting hairpin like secretion of the passenger domain in the absence of other factors. Particularly regarding the issue that a folded AT β-barrel alone, is too narrow to accommodate anything more than two completely unfolded polypeptides (90), though the presence of linker region within the β barrel has been shown to be a requisite for passenger domain secretion in EspP (102). Providing further evidence towards the theory that BamA maintains the AT β-barrel in a semi-folded/incomplete conformation which does allow for the formation of linker region structures, to enable hairpin secretion and folding of the passenger domain, prior to AT protein release from BamA as a completed structure.

In addition to the above described model, the recently discovered TAM complex has been proposed to aid in passenger domain secretion after insertion of the β domain into the membrane by BamA (103). The TAM complex is comprised of the 16-stranded OM β barrel protein TamA, which contains three N-terminal POTRA domains extending into the periplasmic space, predicted to mediate interactions with the IM protein, TamB. Additionally, TamA is present across all classes of proteobacteria, and shows significant sequence homology to that of other Omp85 proteins, including BamA (103).

Investigations with *C. rodentium tamAB* double deletion mutants indicated the requirement of these proteins for OM localisation of the AT protein p1121 in this strain. While in *E. coli* K12, *tamAB* mutants expressing either Ag43 or EhaA are significantly reduced in their ability to autoaggregate indicating these proteins were not displayed on the bacterial cell surface in a functional manner (103-105). The TAM complex is predicted to

work in collaboration with the BAM complex, whereby the BAM inserts the AT β barrel into the OM, while TAM aids in the secretion of the AT passenger domain (103). However, this hypothesis requires to be reconciled with the recently published structural information for BamA and the current published models for AT passenger domain secretion.

Once secreted to the bacterial cell surface the passenger domain must be folded into its mature form. In the case of passenger domains that adopt a β-helix, the AC domain folds on extrusion to the bacterial cell surface and templates the folding of the β-helix. Several studies have suggested that the AC domain may drive passenger domain secretion with folding providing the impetus for movement of the passenger domain through the β-barrel lumen. However, this is controversial as heterologous constructs lacking the AC domain may be translocated to the bacterial cell surface and a small proportion of ATs, with surface exposed globular passenger domains, lack AC domains completely.

Once secreted to the bacterial cell surface the fate of the folded passenger domain can vary dramatically depending on the protein. The passenger domain may be cleaved releasing the passenger domain from the barrel e.g. the SPATE class of ATs, or it may remain as a single uncleaved polypeptide displaying the functional domain on the surface e.g. EstA (106-108). In a limited number of cases, the passenger domain may be cleaved but remain associated with the bacterial cell surface through a non-covalent interaction with the β domain e.g. Ag43.

1.2.3.2. Type Vb (Two Partner Secretion).

The Vb or Two-partner secretion (TPS) is similar to the classical ATs, with the distinct difference that the α and β domains are transcribed as two separate polypeptides each containing an N-terminal Sec dependant signal sequence, termed TpsA and TpsB,

respectively, which are commonly (but not always) transcribed from the same operon (109). Similar to other AT subclasses, once in the periplasm, the β domain is folded and inserted into the OM as a β barrel, allowing for the secretion of the passenger domain (91, 92).

In contrast to the Type Va subclass, the TSP β domains (TpsB) form a 16-stranded β barrel in the OM, containing two POTRA domains, extending into the periplasmic space. These β-domains belong to the Omp85 family of proteins and displays homology with both TamA and BamA. The POTRA domains are predicted to be involved in the recognition of a specific motif (termed the TPS domain) located in the N-terminus of the TpsA passenger protein. This recognition event initiates passenger domain secretion through the lumen of TpsB, facilitates folding of the passenger domain and is based on recruitment of TpsA β-strands by means of β-augmentation (110-113). The passenger domains of these proteins are often large, with many TpsA proteins >3000 amino acids. Despite the sequence diversity among these proteins, they usually fold into a right-handed β helical conformation similar to those observed for the classical ATs (109, 114).

1.2.3.3. Type Vc (Trimeric AT Adhesins).

The trimeric AT adhesins (TAA) are unique from the previous two classes. Whilst being transcribed as a single polypeptide, the β domains are considerably shorter than that observed for other ATs, composed of only 70-100 amino acids, in contrast to the ~300 amino acids observed for Va (115, 116). These shorter β domains contribute four β strands forming a homotrimer in the OM, generating the 12 stranded functional β-barrel in the OM (91, 92, 117, 118). The passenger domains also trimerise to form the functional moeity and normally consist of three individual subdomains; head, neck and stalk, as observable as Pfam:Hep_Hag (PF05658) motifs (head), a single Pfam:HIM (PF05662) motif (neck) and a series of small

unconserved repeats comprising the stalk (118). To date all characterised Vc proteins have been shown to function in adhesion, with the prototypical example being that of YadA from *Yersinia enterocolitica*, through several other characterised TAAs include UpaG from UPEC, Hia from *Haemophilus influenzae* and NadA from *Neisseria meningitidis*, to name but a few (75, 119).

1.2.3.4. Type Vd (One Piece TPS).

The recently described patatin-like protein, PlpD, identified in an environmental *Pseudomonas* strain lead to the annotation of a fourth type V subclass (120). Similar to the classical and trimeric ATs, Type Vd proteins are transcribed as a single polypeptide. However, the β domains are not homologus to the β-domains of these sytems but rather display homoly with the TpsB proteins of the Type Vb family of proteins. Like the TpsB proteins these proteins possess POTRA domains located between the passenger and β domains, indicative of a gene fusion event between TpsA and TpsB (92). To date no Vd proteins have been identified in *E. coli*, though further investigation may yield the identification of new proteins.

1.2.3.5. Type Ve – Inverted AT.

The Type Ve subfamily were only recently categorised as part of the T5SS family. These proteins are similar to the Type Va ATs, with the key difference being the inverted orientation of the passenger and β domains within the polypeptide (91, 92). Each protein is encoded as a single polypeptide, with an N-terminal Sec dependant signal sequence followed by the β domain, a LysM domain (associated with peptidoglycan binding) and the C-terminal passenger domain (121, 122). In contrast to the classical ATs, this inverted structure results in

passenger domain secretion occurring in an N to C terminal direction. However, like the classical ATs, this subfamily also produce 12-stranded β domains (91). Furthermore, the passenger domains of these proteins adopt an Ig-like fold which often possess significant disulphide bonding. Key examples of this subclass, include Intimin from *E. coli* and Invasin from enteropathogenic *Yersinia* spp. shown to be critical for adhesion and cell contact with host cells (91).

1.2.4. Functions of proteins secreted via the T5SS.

ATs characterised to date are associated with numerous pathogenic phenotypes, including binding extracellular matrix (ECM) proteins, adhesion and invasion of host cells, autoaggregation and biofilm formation, promoting serum resistance and apoptosis of specific cell types or functioning as toxins in host cells, to name but a few roles (75, 92). Though specific functions are often associated with particular protein subclasses and are commonly infection site and species specific, making functional predictions difficult. For example two of the three Type Va AT from *S.* Typhimurium, MisL and ShdA have been previously characterised. Consistent with the mode of *Salmonella* infection, these proteins have been shown to promote adhesion to ECM molecules, including fibronectin and collagen I (ShdA only), in addition to human epithelial cell lines (123, 124). While EhaB from EHEC, although also associated with ECM binding of collagen I, this protein additionally binds laminin and has been shown to be associated with phenotypes including biofilm formation and autoaggregation (125). While in stark contrast to these, the Type Va protein EstA from *Pseudomonas aeruginosa* encodes a GDSL passenger domain, with catalytic activity, preferentially hydrolysing of short chain fatty acids, particularly those ranging between four and six carbons (126, 127). All characterised TAAs (to date) have been associated with

adhesion to ECM molecules and various cell types. For example the prototypical example YadA from *Yersinia enterocolitica* binds a variety of extracellular matrix proteins, including collagen, laminin, and fibronectin (92). In addition to promoting serum resistance (92). By contrast the TAAs UpaG and EhaG from UPEC and EHEC, respectively, are more commonly associated with promoting biofilm formation and autoaggregation, though have been shown to bind bladder cells and colorectal epithelial cells, representative of the differing sites of infection associated with these strains (128). As general guide Table 1.1. details the phenotypes of a selection of proteins from various subclasses.

1.2.5. Outlook for T5SS research.

As ATs represent a family of large OM localised structures, there potential as possible vaccine candidates provides real promise as shown with the success of the acellular *Bordetella pertussis* vaccine against whooping cough, which includes purified Pertactin and Filamentous Hemagglutinin (FHA), Va and Vb proteins. While the recently developed Bexsero vaccine against Serogroup B meningococcal, contains the Vc protein NadA (129). Inducing protection without the risks associated with using attenuated bacterial strains, which have the capability of reverting to pathogenic forms (130). Investigations using other OM structures, including the OM porin OmpD from *S.* Typhimurium have shown similar promise, with the ability to induce T cell independent antibodies, which are protective (131). These results highlight the need for further investigation in this field.

Table 1.1. Type V Proteins – Classification and Functions

Name	V	Organism	Description	Reference
Antigen 43	a	*E. coli*	β helical, cleaved but remains covalently attached, autoaggregation and biofilm formation	(132, 133)
ApeE	a	*S.* Typhimurium	Globular α helical structure, Esterase, degrading C6-C16 fatty acid chains	(127)
EhaB	a	EHEC	β helical, ECM binding and biofilm formation	(125)
EstA	a	*P. aeruginosa*	Globular α helical structure, Esterase, degrading C4-C8 fatty acid chains, additional roles in motility and biofilm formation	(126, 127)
MisL	a	*S.* Typhimurium	β helical, ECM binding, required for intestinal colonisation and faecal shedding in mice	(123)
Pertactin	a	*B. pertussis*	β helical, Adhesion and resistance to neutrophil mediated clearance. Component of *B. pertussis* acellular vaccine	(92)
Pet	a	EAEC	β helical, cleaved passenger domain, SPATE, cytotoxin	(134)
ShdA	a	*S.* Typhimurium	β helical, ECM binding, required for intestinal colonisation and faecal shedding in mice	(124, 135-137)
FHA	b	*B. pertusis*	Binds C4 complement, component of the *B. pertussis* acellular vaccine	(138)
HMW1/2	b	*H. influenzae*	Adhesion to respiratory epithelial cells	(139)
ShlA	b	*S. marcescens*	Hemolysin	(140)
EhaG	c	EHEC	ECM binding and adhesion to colorectal epithelial cells	(128)
Hia	c	*H. influenzae*	Adhesion to epithelial cells	
NadA	c	*N. meningitidis*	Adhesion to epithelial cells, component of the Bexsero vaccine	(141)
UpaG	c	UPEC	ECM binding and adhesion to bladder cells	(119, 128)
YadA	c	*Y. enterocolitica*	ECM binding, adhesion to epithelial cells, serum resistance	(117, 142-144)
PlpD	d	*P. aeruginosa*	Lipase	(92)
Intimin	e	EPEC and some STEC	Adhesion and lesion formation	(92)

Additionally recent reports have also highlighted the potential of AT proteins as a means of recombinant protein production and surface display of antigens, whereby both the AT proteins, Pet and Hbp have been shown to tolerate the exchange of their native passenger domains, for that of other proteins (106, 145). Further enhancing the possibilities for which these proteins can be exploited.

1.3. SALMONELLA

Salmonella is a Gram-negative organism, which can be divided in two species, *Salmonella bongori* and *Salmonella enterica*. *S. bongori* is largely restricted to cold blooded animals, while *S. enterica* can be further subdivided (146). Within *S. enterica* numerous serovars exist, including those limited to human hosts, such as Typhi and Paratyphi or those capable of broad host range pathogenicity, including Typhimurium and Enteritidis (147).

S. enterica serovar Typhimurium demonstrates a broad host range, capable of pathogenesis in a number of species including humans, birds, livestock and poultry (148, 149). *S. enterica* infection commonly occurs via ingestion of the bacteria from contaminated food or water supplies (150). Due to the ability of *S.* Typhimurium to infect a variety of hosts, including those within the food chain, transmission between species can occur readily (123). Though in contrast to public perception, recent investigations into the source of numerous North American outbreaks have shown an ever increasing association with the consumption of contaminated fruit and vegetables. Indeed, consumption of contaminated vegetable produce is now the most prevalent source of non typhoidal *Salmonella* (NTS) infection in developed countries, rather than contaminated poultry (151-154).

1.3.1. Mode of *S. enterica* infection

S. Typhi and *S.* Typhimurium in humans and mice, respectively, causes systemic infection resulting in typhoid fever (147, 150, 155), while *S.* Typhimurium in humans usually results in a self-limiting non-typhoidal gastroenteritis and bacteremia in immunocompromised individuals or children (131).

As mentioned previously infection predominantly results from oral consumption, whereby bacteria require transit through the stomach to reach the small intestine. In order to survive the harsh environmental pH of the stomach, *Salmonella* encode acid tolerance response genes, including *fur* and *atp*, induced in conditions of low pH (156). Once at the small intestine, bacteria are capable of binding to epithelial and specialised M cells (localised to the Peyers patches, required for antigen uptake and presentation to phagocytes) by means of fimbriae and other surface structures, including AT proteins MisL and ShdA (123, 155, 157). After initial adherence, *Salmonella* induce cytoskeletal rearrangements within the host cell; these are manifested as membrane ruffling and subsequent engulfment of the bacteria into *Salmonella* containing vacuoles (SCV) (157).

S. enterica encode a variety of tools, to induce the changes in host cell morphology described above. The T3SSs (as discussed in Section 1.2.1), encoded on *Salmonella* Pathogenicity Island 1 (SPI-1), encodes a series of effector molecules required for invasion including, SopE and SptP which are associated with cytoskeleton rearrangements. Other effector molecules such as SipA, SopC, SopD and SopE induce signal cascades in the host cells leading to the recruitment of neutrophils and macrophages to the site of infection, in addition to the secretion of pro-inflammatory cytokines (147, 150, 155, 158).

Once the SCV matures its translocates through the epithelial cell, to the basal membrane, where it is able to interact with and enter macrophage and dendritic cells (147).

Ingestion by these cell types controls the spread of infection through the induction of reactive oxygen species and reactive nitrogen intermediates. However, these cells are often hijacked as a means of transport through the host lymphatic system and the blood (155, 159).

Once the SCV is formed, SPI-1 expression is strongly inhibited through the repression of HilA, by the PhoP/Q system. This results in the activation of an alternative T3SS encoded by SPI-2. SPI-2 expression promotes intracellular survival (158). The SPI-2 dependent secretion of the SpiC effector prevents SCV fusion with late endosomes and lysosomes, limiting bacterial killing by reactive oxygen and nitrogen (158). Other SPI-2 T3SS effector molecules associate with host cell microtubule bundles aiding the formation of *Salmonella*-induced filaments, which protrude from the SCV. These are thought to be required for intracellular replication of the bacterial within these vacuoles (147). Such effector molecules have long been associated with host cell cytotoxicity, enabling bacterial escape at the relevant time (155, 160). For example SpvB has been demonstrated to induce Toll-like receptor activation resulting pro-apoptotic responses, while SipB has been shown to induce activation of host caspase-1 (158). Once released from the macrophages, bacteria can survive extracellularly in the blood stream or rapidly colonise various sites, including the spleen, liver, gall bladder, mesenteric lymph (MEL) nodes and bone marrow, resulting in persistent infection and continual faecal shedding, as shown in figure 1.7 (155).

1.3.2. Investigating host responses to *Salmonella*

The mouse model provides an effective means of studying *S.* Typhimurium infections, both from the perspective of host pathogen interactions, but also for identifying and testing suitable vaccine candidates for efficacy. Investigations have highlighted a number of genetic factors affecting murine susceptibility to *S.* Typhimurium, including; *nramp1* which limits the

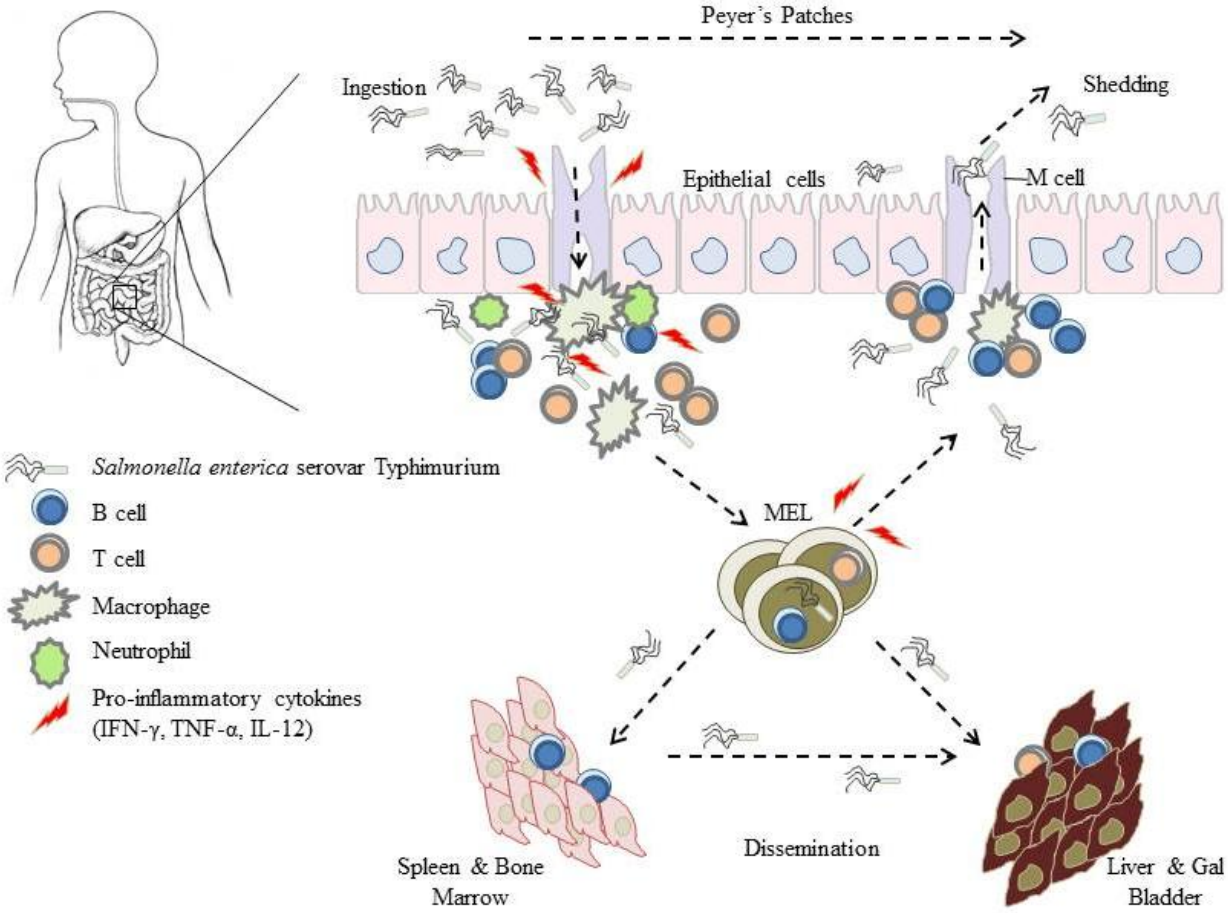


Figure 1.7. *Salmonella* Infection
The course of *Salmonella* infection following ingestion of contaminated food or water. Upon arrival in the small intestine, bacteria transverse the gut epithelia at the Peyers patches, using specialist M cells, associated with antigen presentation. Invasion at these sites stimulates the release of pro-inflammatory cytokines, promoting the recruitment of T cells, B cells, macrophages and neutrophils to the affected area. *Salmonella* can invade macrophages, residing intracellularly as required, providing a means for transport throughout the host lymphatic system, where bacteria can colonise sites including the Mesenteric lymph nodes (MEL), spleen, bone marrow, liver and gal bladder, allowing for bacterial reseeding back into the intestine and continued faecal shedding.

intracellular replication of bacteria in macrophages, by limiting the availability of divalent cations, providing key insight into host defences (155). However, within the same context the mouse model does provide a series of limitations, with a key defect being the lack of a satisfactory complement system (161).

Other immune factors including the major histocompatibility complex (MHC) class II and class III, $CD4^+$ and $CD8^+$ T lymphocytes are known to play key roles in bacterial clearance, while IFN–γ, IL-12 and IL-23 are hypothesised to control the intracellular replication of the bacteria, particularly in persistent infections (131, 155, 162). Importantly, resistance to re-infection is dependent on $CD4^+$ and $CD8^+$ T cells in addition to *S. enterica* specific antibodies (163).

Antibody responses are critical for limiting bacteremia, a major cause of mortality in infants between six and 24 months (131). The increased risk for *S. enterica* mediated septicaemia strongly correlates with the loss of maternal antibodies and activation of the humoral immune system (131). Therefore, the identification of bacterial factors that can induce a memory mediated antibody response is critical for the development of an effective vaccine.

1.3.3. *Salmonella* infection and vaccine development

Due to its prevalence across a broad range of species, *S.* Typhimurium infections are a significant cause for concern and are associated with almost 100 million cases of gastroenteritis each year (164). NTS infections are highly prevalent in African countries with sub-Saharan regions demonstrating some of the highest numbers where the infection predominantly affects children aged six months to five years and immune compromised individuals (131). Infection often presents as bacteraemia in the absence of other

gastrointestinal symptoms, making diagnosis difficult (131). Furthermore, HIV positive individuals demonstrate significantly higher risks of mortality, due to the loss of immune features such as Th17 from the gut mucosa, leading to reduced gut barrier and the dysregulation of critical cytokines, such as IL-12, which is responsible for the induction of IFN-γ (131, 165, 166).

Effective treatment of NTS infections has become increasingly difficult with the emergence of antibiotic resistant strains, including resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines. Requiring more routine use of other antibiotics including, cephalosporins and fluoroquinolones in order to treat the infection (167).

Currently no licensed vaccines exist for the treatment of *S.* Typhimurium in humans, and its significant prevalence in regions with poor healthcare and sanitation, particularly among infants and HIV positive individuals, makes this a global concern and therefore investigation into potential vaccine candidates should be a priority. To date OmpD from *S.* Typhimurium offers one of the most promising vaccine candidates (131). However, any potential vaccine candidates require significant investigation, to ensure bactericidal activity, in contrast to anti-LPS antibodies which have been shown to enhance the severity of the infection in some individuals by blocking, access to other bactericidal antibodies and complement (168).

1.4. AIMS

This study aims to investigate the mechanisms of membrane homeostasis and how this contributes to the virulence of the pathogenic Gram-negative organisms. By investigating the roles of non-essential genes, in the laboratory strain *E. coli* K12 and broad host range pathogen *S.* Typhimurium we aim to identify those required for the maintenance of membrane barrier function and normal homeostasis, characterise their specific functions, in the case of PlpA and assess their roles in virulence, using the murine model of *Salmonella* infection. Whilst determining the contribution of Type V proteins from *S.* Typhimurium to virulence, to ascertain their functional roles in and investigate their potential as future vaccine candidates.

CHAPTER 2

MATERIALS AND METHODS

2.1. SUPPLIERS

Unless otherwise indicated all culture reagents were purchased from Oxoid Limited and chemicals were purchased from Sigma Aldrich. *Thermus aquaticus* (Taq) polymerase was purchased from Thermo Scientific. Phusion High Fidelity DNA polymerase and alkaline phosphatase were purchased from New England Biolabs. T4 DNA Ligase was purchased from Invitrogen. Restriction Enzymes were purchased from Fermentus. Oligonucleotides were purchased from either Alta Biosciences (University of Birmingham) or Eurogentec (Hampshire, UK). Custom primary antibodies were generated by Eurogentec; secondary antibodies were purchased from Sigma Aldrich. Animals (C57BL6 mice) were obtained from Harlan, or directly from the Biomedical Services Unit (BMSU, University of Birmingham).

2.2. CULTURE MEDIA AND CONDITIONS

Bacterial strains and plasmids used in this study are detailed in table 2.1. and table 2.2., respectively. BW25113 is the parental strain for the KEIO library, therefore all single mutants and subsequent double mutants were constructed and assessed in this strain. *E. coli* BL21 strains were selected for protein expression studies, while *E. coli* M15 strains were selected for phenotypic assays, due to the levels of aggregation observed in *E. coli* BL21. *S.* Typhimurium SL1344 and the attenuated derivative SL3261 strains were selected for use in *Salmonella* studies due to the prior characterisation of these strains during both *in vitro* and *in vivo* experiments since their isolation in 1978 and 1981, respectively, while the availability of complete genome sequences provided an additional aid for molecular manipulations. Strains were routinely cultured in liquid and solid media, and stored as glycerol stocks at -85^{o}C, in the presence of 15% glycerol. Strains were cultured on Luria Bertani or M9 medium, as previously described (169). Unless otherwise stated, bacterial cultures were grown at 25, 30,

Table 2.1 - Bacterial strains used in this study

Strain	Description	Reference
	E. coli Cloning and Expression Strains	
BL21	*E. coli* DE3 T7 express, protein expression strain	Invitrogen
HB101	Non aggregating laboratory *E. coli* strain	(170)
M15 (pREP4)	*E. coli* strain for tightly regulated expression of pQE vectors, carrying pREP4	QIAGEN
NEB 5'alpha	Highly efficient compotent cells, derived from DH5α	New England Biolabs
Top10	*E. coli* strain for cloning and protein expression from pASK vectors	Invitrogen
	E. coli Strains	
BW25113	*rrnB3* DE*lacZ4787* DE*phoBR580 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568 galU95* DE*endA9*::*FRT* DE*uidA3*::*pir(wt) recA1 rph-1*	(171)
KEIO Library	Various gene disruption mutants in BW25113, disrupted between the second and seventh from last codon by a kanamycin resistance cassette	(171)
BW25113Δ*plpA*	BW25113 with *plpA* gene deleted	This Study
BW25113Δ*plpA gpmI::aph*	Derviative of BW25113 Δ*plpA* with the *gpmI* gene disrupted by kanamycin resistance caseette	This Study
BW25113Δ*plpA mlaA::aph*	Derivative of BW25113 Δ*plpA* with the *mlaA* gene disrupted by kanamycin resistance cassette	This Study
BW25113Δ*plpA mlaC::aph*	Derivative of BW25113 Δ*plpA* with the *mlaC* gene disrupted by kanamycin resistance caseette	This Study
BW25113Δ*plpA mlaD::aph*	Derviative of BW25113 Δ*plpA* with the *mlaD* gene disrupted by kanamycin resistance caseette	This Study
BW25113Δ*plpA mlaE::aph*	Derviative of BW25113 Δ*plpA* with the *mlaE* gene disrupted by kanamycin resistance caseette	This Study
BW25113Δ*plpA*	Derviative of BW25113 Δ*plpA* with the *mlaF*	This Study

mlaF::aph	gene disrupted by kanamycin resistance caseette	
BW25113Δ*plpA*	Derviative of BW25113 Δ*plpA* with the *pldA*	This Study
pldA::aph	gene disrupted by kanamycin resistance caseette	
BW25113Δ*plpA*	Derviative of BW25113 Δ*plpA* with the *pagP*	This Study
pagP::aph	gene disrupted by kanamycin resistance caseette	
	Salmonella Typhimurium Strains	
SL1344	*Salmonella enterica* serovar Typhimurium	(172)
SL3261	Derivative of SL1344 with Δ*aroA*	(173)
	Single Mutants	
SL1344	SL1344 with the *bamB* gene disrupted by a	This study
bamB::aph	kanamycin resistance cassette	
SL3261	SL1344 with the *bamB* gene disrupted by a	This study
bamB::aph	kanamycin resistance cassette	
SL1344	SL1344 with the *bamC* gene disrupted by a	This study
bamC::aph	kanamycin resistance cassette	
SL3261	SL1344 with the *bamC* gene disrupted by a	This study
bamC::aph	kanamycin resistance cassette	
SL1344	SL1344 with the *bamE* gene disrupted by a	This study
bamE::aph	kanamycin resistance cassette	
SL3261	SL1344 with the *bamE* gene disrupted by a	This study
bamE::aph	kanamycin resistance cassette	
SL1344	SL1344 with the *degP* gene disrupted by a	This study
degP::aph	kanamycin resistance cassette	
SL3261	SL1344 with the *degP* gene disrupted by a	This study
degP::aph	kanamycin resistance cassette	
SL1344	SL1344 with the *fkpA* gene disrupted by a	This study
fkpA::aph	kanamycin resistance cassette	
SL3261	SL1344 with the *fkpA* gene disrupted by a	This study
fkpA::aph	kanamycin resistance cassette	
SL1344	SL1344 with the *hlpA* gene disrupted by a	This study
hlpA::aph	kanamycin resistance cassette	

SL3261 *hlpA::aph*	SL1344 with the *hlpA* gene disrupted by a kanamycin resistance cassette	This study
SL1344 *plpA::aph*	SL1344 with the *plpA* gene disrupted by a kanamycin resistance cassette	This study
SL3261 *plpA::aph*	SL1344 with the *plpA* gene disrupted by a kanamycin resistance cassette	This study
SL1344 *surA::aph*	SL1344 with the *surA* gene disrupted by a kanamycin resistance cassette	This study
SL3261 *surA::aph*	SL3261 with the *surA* gene disrupted by a kanamycin resistance cassette	This study
SL1344 *tamA::aph*	SL1344 with the *tamA* gene disrupted by a kanamycin resistance cassette	This study
SL3261 *tamA::aph*	SL3261 with the *tamA* gene disrupted by a kanamycin resistance cassette	This study
SL1344 *tamB::aph*	SL1344 with the *tamB* gene disrupted by a kanamycin resistance cassette	This study
SL3261 *tamB::aph*	SL3261 with the *tamB* gene disrupted by a kanamycin resistance cassette	This study
SL1344 *ytfP::aph*	SL1344 with the *ytfP* gene disrupted by a kanamycin resistance cassette	This study
SL3261 *ytfP::aph*	SL3261 with the *ytfP* gene disrupted by a kanamycin resistance cassette	This study
	Autotransporter Single Mutants	
SL1344 *apeE::aph*	SL1344 with the *apeE* gene disrupted by a kanamycin resistance cassette	This study (Dhaarini Raghunathan)
SL1344Δ*apeE*	SL1344 *apeE::aph* with the kanamycin resistance cassette removed	This study
SL3261 *apeE::aph*	SL3261 with the *apeE* gene disrupted by a kanamycin resistance cassette	This study (Dhaarini Raghunathan)
SL1344 *misL::aph*	SL1344 with the *misL* gene disrupted by a kanamycin resistance cassette	This study (Dhaarini Raghunathan)
SL1344Δ*misL*	SL1344 *misL::aph* with the kanamycin resistance	This study

	cassette removed	
SL3261 *misL::aph*	SL3261 with the *misL* gene disrupted by a kanamycin resistance cassette	This study
SL1344 *sadA::aph*	SL1344 with the *sadA* gene disrupted by a kanamycin resistance cassette	(174)
SL1344Δ*sadA*	SL1344 *sadA::aph* with the kanamycin resistance cassette removed	(174)
SL3261 *sadA::aph*	SL3261 with the *sadA* gene disrupted by a kanamycin resistance cassette	(174)
SL1344 *sapP::aph*	SL1344 with the *sapP* gene disrupted by a kanamycin resistance cassette	This study
SL1344Δ*sapP*	SL1344 *sapP::aph* with the kanamycin resistance cassette removed	This study
SL3261 *sapP::aph*	SL3261 with the *sapP* gene disrupted by a kanamycin resistance cassette	This study
SL1344 *shdA::aph*	SL1344 with the *shdA* gene disrupted by a kanamycin resistance cassette	(124)
	Autotransporter Multiple Deletion Mutants	
SL1344 Δ*misL* Δ*sapP* *shdA::aph*	SL1344 with *misL* and *sapP* genes deleted and *shdA* gene disrupted by a kanamycin resistance cassette (3 in 1)	This study
SL3261 Δ*misL* Δ*sapP* *shdA::aph*	As described for SL1344 SL1344 Δ*misL* Δ*sapP* *shdA::aph*, in SL3261 background	This study
SL1344 Δ*misL* Δ*sapP* Δ*apeE* *shdA::aph*	SL1344 with *misL, sapP* and *apeE* genes deleted and *shdA* gene disrupted by a kanamycin resistance cassette (4 in 1 *apeE*)	This study
SL3261 Δ*misL* Δ*sapP* Δ*apeE* *shdA::aph*	As described for SL1344 Δ*misL* Δ*sapP* Δ*apeE* *shdA::aph*, in SL3261 background	This study
SL1344 Δ*misL*	SL1344 with *misL, sapP* and *sadA* genes deleted	This study

Δ*sapP* Δ*sadA* *shdA::aph*	and *shdA* gene disrupted by a kanamycin resistance cassette (4 in 1 s*adA*)	
SL3261 Δ*misL* Δ*sapP* Δ*sadA* *shdA::aph*	As described for SL1344 SL1344 Δ*misL* Δ*sapP* Δ*sadA shdA::aph*, in SL3261 background	This study
SL1344 Δ*misL* Δ*sapP* Δ*sadA* Δ*apeE* *shdA::aph*	SL1344 with *misL, sapP, sadA* and *apeE* genes deleted and *shdA* gene disrupted by a kanamycin resistance cassette (5 in 1)	This study
SL3261 Δ*misL* Δ*sapP* Δ*sadA* Δ*apeE* *shdA::aph*	As described for SL1344 Δ*misL* Δ*sapP* Δ*sadA* Δ*apeE shdA::aph*, in SL3261 background	This study

Table 2.2. – Plasmids used in this study

Plasmid	Characteristics	Reference
	Gene Disruption and Deletion	
pKD4	Template plasmid for the amplification of the Kanamycin resistance cassette, flanked by FLP sites, Kan^R	(175)
pKD46	Temperature sensitive, low copy number plasmid expressing the Red recombinase genes under the control of an arabinose inducible promoter, Amp^R	(175)
pCP20	Temperature sensitive plasmid encoding the FLP recombinase gene, Amp^R	(175)
pDOC-C	Medium copy number pre-recombination plasmid containing I-SceI sites for cloning fragments prior to recombination in vivo, Amp^R	(176)
pDOC-K	Template plasmid for amplification of the kanamycin resistance cassette, flanked by FLP recombinase sites, Kan^R	(176)
pACBSCE	Recombinase plasmid expressing I-SceI and the λ-Red genes, under an arabinose inducible promoter, Cat^R	(176)
pDOC-C *tamA*	Derivative of pDOC-C with kanamycin resistance cassette, flanked by 40 bases of homology to the STm *tamA* gene cloned between the I-SceI sites	This Study
	Various Expression Vectors	
pET17b	Constitively active T7 expression vector, Amp^R	Novagen
pET17b *plpA*	As described for pET17b with *E. coli plpA*	(177)

	cloned between NdeI and EcoRI	
pET17b *plpA* TM	As described for pET17b with *E. coli plpA* randomly disrupted by Transposon mutations throughout the gene, see Table 2.5 for details.	(177)
pET17b *plpA* STm	As described for pET17b with *S.* Typhimurium *plpA* cloned between NdeI and HindIIII	This Study
pET17b *plpA* H.i	As described for pET17b expressing codon optimised *Haemophilus influenza* homolog of *plpA*	Genscript
pET17b *plpA* P.m	As described for pET17b expressing codon optimised *Pasteurella multoclda* homolog of *plpA*	Genscript
pET17b *plpA* N.m	As described for pET17b expressing codon optimised *Neisseria meningitidis* homolog of *plpA*	Genscript
pET17b *plpA* V.c	As described for pET17b expressing codon optimised *Vibrio cholera* homolog of *plpA*	Genscript
pET17b *osmY*	As described for pET17b expressing codon optomised *E. coli* K12 *osmY*	Genscript
pET20b	T7 expression vector, Amp^R	Novagen
pET20b *plpA*	As described for pET20b with *E. coli plpA* gene cloned between NdeI and EcoRI	(177)
pET20b *plpA* PM	As described for pET20b *plpA*, with site directed point mutations at various sites, see Table 2.3 for details	(177)
pET20b *wbbL*	As described for pET20b with *wbbL* gene cloned between NdeI and HindIII	(178)
pET22b	IPTG Inducable expression vector, Amp^R	Novagen
pET22b *sapP*	As described for pET22b with *sapP* gene cloned between NdeI and EcoRI sites	This Study

pET22b *sadA*	As described for pET22b with *sadA* gene cloned between NdeI and EcoRI sites	(174)
pJH10	IncQ based expression vector, Tet^R	(179)
pJH10 *plpA*	Derivative of pJH104 with *E. coli* K12 *plpA* cloned between sites EcoRI and SacI	This Study
pREP4	Repressor plasmid, for controlled expression of pQE vectors, Kan^R	QIAGEN
pQE60 (Modified)	NdeI site at postion removed, NcoI site at position removed and replaced with an NdeI site, Amp^R	(174)
pQE60 *apeE*	As described for pQE60 with *S.* Typhimurium *apeE* gene cloned between NdeI and HindIII sites	This Study
pQE60 $apeE_{S10A}$	As described for pQE60 *apeE* with Serine at position 10 mutated to Alanine	This Study
pQE60 *sapA*	As described for pQE60 with *S.* Typhimurium *sapA* gene cloned between NdeI and HindIII sites	This study
pQE60 *sadA*	As described for pQE60 with *S.* Typhimurium *sadA* gene cloned between NdeI and HindIII sites	(174)
pQE60 *plpA*	As described for pQE60 with *S.* Typhimurium *plpA* gene cloned between NdeI and HindIII sites	This Study

37 or 42^{o}C with continuous agitation. Growth rates were determined by measuring optical density at OD_{600} using either an Ultraspec 2100 Pro (GE Healthcare Life Sciences, Buckinghamshire) or a FLUOstar Optima (BMG Labtech, Offenberg, Germany), as previously described (169). Mammalian cells including J774, RAW 264 macrophages, THP1 human monocytes and Caco-2 colorectal carcinoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) or RPMI at 37°C in a 5% CO_2 atmosphere (180, 181). THP1 cells were differentiated into macrophages 24 h prior to use, by the addition of 5 ng/mL of phorbol 12-myristate 13-acetate (PMA). Where appropriate antibiotics were added to the medium as the following concentrations: carbenicillin (100 μg/mL); chloramphenicol (30 μg/mL); kanamycin (50 μg/mL) and vancomycin (0-512 μg/mL). Stock solutions of antibiotics were prepared as previously described (169)

Suppressor mutants were generated by repeated culture on previously inhibitory concentrations of either vancomycin (200 μg/mL) or SDS (4.8%), in both liquid media and solid agar. In each instance six independent overnight cultures were applied to agar plates supplemented with the above and incubated at 37^{o}C until the appearance of colonies. Resultant colonies were cultured overnight in liquid media supplement as above, before being streaked onto agar containing the above compounds, to confirm the resistant phenotype.

2.3. DNA MANIPULATIONS

2.3.1. Preparation of genomic DNA, RNA and plasmid isolation

Genomic DNA was prepared from bacterial cultures using QIAGEN QIAamp DNA Mini Kit, in accordance with manufactures instructions. Cell lysate samples were prepared by pelleting bacterial cultures and resuspending in sterile deionised water (SDW), boiling for 15 min prior to pelleting the cell debris. Total RNA was isolated from both bacterial cultures and infected

mammalian tissues, using QIAGEN RNAprotect Bacterial Reagent (bacterial cultures) or QIAshredder (infected tissues) and QIAGEN RNeasy Mini Kit, in accordance with manufacturer's instructions. On the column DNase I digestion was performed using QIAGEN RNase-Free DNase Set. Plasmid isolation was performed using QIAGEN Miniprep Spin Kit. Samples were quantified either by Nanodrop or visual comparison with known standards.

2.3.2. PCR

PCR primers used in this study are described in appendix table 1. These were used at a final reaction concentration of 0.5 µM. MyTaq Red Mix 2x was used in accordance with manufacturer's instructions for cell lysate PCR reactions. The amplification of templates for cloning or mutation purposes Phusion High Fidelity Polymerase was used in accordance with manufacturer's instructors, using either genomic DNA or plasmid DNA as the template. When using Phusion High Fidelity Polymerase, dNTP's were added at a concentration of 200 µM (each). Reactions were run on Mastercycler epgradient S (Eppendorf, Stevenage).

2.3.3. Reverse transcription PCR

DNA free, total RNA was prepared as described previously. cDNA was prepared using SuperScript II Reverse Transcriptase and Random Hexamers and RT PCR using Taq Polymerase.

2.3.4. Cloning

DNA for cloning was purified using either a QIAGEN Gel Extraction Kit or QIAGEN PCR Purification Kit, in accordance with manufacturer's instructions. Template DNA and isolated plasmids were digested using Fermentus Fast Digest Enzymes, in accordance with

instructions. Digested plasmids were treated with alkaline phosphatase, prior to purification with the above described kits. Template and plasmid DNA were quantified, by comparison with known standards, and ligated using T4 DNA Ligase, prior to overnight incubation at 4^{o}C before transformation the following day. Resultant colonies were screened using either PCR (amplifying for the presence of the insert) or restriction digests (using enzymes specific to the insert) and confirmed by sequencing at the Function Genomics facility, University of Birmingham.

2.3.5. Preparation and transformation of competent cells

Competent cells were prepared as follows. Bacterial cultures were grown to an OD_{600nm} of 0.6 or 0.8 for chemically and electro competent cells, respectively. Chemically competent cells were chilled on ice for 30 min, prior to centrifuging (2880 x *g*, 4^{o}C, for 30 min). Cell pellets were resuspened in 0.1 M calcium chloride and stored on ice for 30 min, prior to centrifuging as above. The cells were washed and pelleted as described above, prior to re-suspending in 0.1 M calcium chloride and stored on ice for two hs, before centrifuging (as above). Pellets were resuspended in LBB containing 30% v/v glycerol and snap frozen, using ethanol and dry ice. Electro competent cells were incubated at 42^{o}C for 15 min, followed by 10 min incubation at 4^{o}C, with continuous agitation. Cultures were centrifuged as described previously. Pellets were resuspended in 20% v/v glycerol containing 1 mM MOPS pH7.4 and centrifuged. Cultures were washed as described above and resuspended in 20% glycerol containing 1 mM MOPS pH7.4, prior to snap freezing. Chemically competent cells were transformed by adding 5 µL of plasmid/ligation or exogenous DNA and incubated on ice for one h, prior to heat shock at 42^{o}C for 90 s followed by ice for 90 s. Electro-competent cells were transferred to a chilled electroporation cuvette 1mm prior to electroporation at 1800V

before the addition of 1 mL LBB. Transformants were recovered in LBB for approximately one h at an appropriate temperature, prior to plating on selective agar.

2.3.6. Construction of deletion mutant bacterial strains

2.3.6.1. DATSENKO AND WANNER METHOD FOR SINGLE GENE INACTIVATION.

Gene disruption mutants were generated using two techniques; Datsenko and Warner Method for single gene inactivation (175) and gene doctoring (176). Briefly, to generate mutants via the Datsenko and Warner method, the kanamycin resistance cassette was amplified from the template plasmid pKD4, to generate DNA fragment, containing 5' and 3' ends of 20 bases of homology to the flanking regions of the gene to be disrupted. The fragment was purified and quantified as described previously. The required bacterial strains were made electro-competent (as described previously) and transformed with pDK46. Resultant colonies were again made electro-competent, with minor alterations to the previously described method, incubations were performed at 30^{o}C, media was supplemented with 20 mM L-arabinose, pellets were washed with 10% glycerol and no 42^{o}C heat shock was performed. Electro-component pKD46 containing cells where electroporated with 200-500 ng the linear DNA fragments generated above. Cells were recovered at 37^{o}C and plated on selective agar, selecting for kanamycin resistance only. Resultant colonies were patch plated to confirm the loss of carbenicillin resistance (pKD46), prior to confirmation by colony PCR.

2.3.6.2. GENE DOCTORING.

Mutants generated via the gene doctoring method, were generated as described by Lee et al (176). Similar to the above described method, the kanamycin resistance cassette was

amplified from the plasmid pDOC-K, using primers containing 5' and 3' 40 bases of homology to the genomic DNA flanking the gene to be disrupted and 20 bases of homology to the kanamycin cassette, with distal I-SceI restriction sites. The resultant fragments were purified, quantified and digested with I-SceI. In parallel pDOC-C was also digested with I-SceI and treated with alkaline phosphatase (as previously described). The resultant PCR fragment was cloned into pDOC-C and the sequence confirmed by plasmid to profile sequencing (performed at functional genomics facility), to generate the plasmid pDOC-C *tamA*. The pDOC-C *tamA* vector and pACBSCE vector were co-transformed into electro-competent *S.* Typhimurium, selecting for resistance to chloramphenicol, carbenicillin and kanamycin. Resultant colonies were inoculated into LBB containing 0.5% L-arabinose, at 37^{o}C for approximately five hs. Samples were plated on LBA containing kanamycin and 5% sucrose and incubated overnight at 30^{o}C. Resultant colonies were patch plated onto chloramphenicol and carbenicillin, to confirm the loss of both pDOC-C and pACBSCE, prior to confirmation of recombination of the resistance cassette by PCR.

*2.3.6.3. REMOVAL OF ANTIBIOTIC RESISTANCE CASSETTE***.**

All confirmed colonies were phage transduced, prior to use. In some instances, the kanamycin resistance cassette was removed, using pCP20 and FLP recombinase system, as described by Datsenko and Warner (175). Briefly; cells were made electro-competent (as previously described) and pCP20 electroporated, colonies were selected for growth on carbenicillin at 30^{o}C. The following day, six colonies was selected and incubated at 42^{o}C for six h, prior to plating on LBA. Resultant colonies were then patch plated, onto LBA containing carbenicillin

or kanamycin to confirm loss of pCP20 and the resistance cassette, respectively, prior to PCR confirmation.

2.3.6.4. TRANSDUCTION USING P1 OR P22 PHAGE.

Mutations were transferred to a clean parental background using phage transduction (182). Briefly cultures harbouring the mutation to be transferred were incubated in the presence of varying concentration of phage stocks (P1 for *E. coli* strains, P22 for *S.* Typhimurium strains) for three to 20 h, for P1 and P22 respectively. The following day, phage lysates were harvested by the addition of chloroform, prior to centrifuging at 3000 x *g*, 4^{o}C for 15 min. Recipient bacterial strains, were incubated with varying amounts of phage lysate for 15 and 30 min, prior to the addition of 1 M sodium citrate and LB broth containing 5 mM calcium chloride and 10 mM magnesium sulfate before incubating for a further 45-90 min. Bacteria were pelleted and washed with 1x PBS three times, prior to plating on selective agar. All resultant colonies were screened by PCR to confirm the mutation.

2.3.7. Directed *Mutagenesis strategies.*

Mutations in protein-encoding DNA were introduced using Quikchange Lightning Kit (Agilent) and custom oligonucleotides or by random transposon mutagenesis using the Mutation Generation System Kit (Thermo) as described in manufacturers' instructions. All resultant colonies were sequenced to confirm the mutation/insertion site prior to use and are described in table 2.3.

Table 2.3 – Site direct and random sequence insertion mutants in *plpA*

Designation	Description
WT	As described for pET20b *plpA*, non mutated version for control and starting material
A88	Random linker sequence insertion after position A88,
D125	Random linker sequence insertion after position D125
L50	Random linker sequence insertion after position L50
D102	Random linker sequence insertion after position D102
G160	Random linker sequence insertion after position M159
W127	Random linker sequence insertion after position W127
V72	Random linker sequence insertion after position V72
Q113	Random linker sequence insertion after position Q113
N109	Random linker sequence insertion after position N109
Q76	Random linker sequence insertion after position Q76
V54	Random linker sequence insertion after position R53
V142	Random linker sequence insertion after position V142
N105	Random linker sequence insertion after position N105
I128A	Mutation at position I128 converting Isoleucine to Alanine
K131A	Mutation at position K131 converting Lysine to Alanine
R133A	Mutation at position R133 altering Arginine to Alanine
Q135A	Mutation at position Q135 Glutamine converted to Alanine
L137A	Mutation at position L137, Leucine converted to Alanine
S144A	Mutation at position S144A, altering Serine to Alanine
G83V	Mutation at position G83V, conserved Glycine to Valine
G160V	Mutation at position G160V, conserved Glycine at Valine
G83VG160V	Mutations at positions G83G160, mutating both Glycine residues to Valine
W127A	Mutation at position W127A, altering Tryptophan to Alanine
Y75A	Mutation at position Y75, alteration from Tyrosine to Alanine

2.3.8. DNA sequencing.

Illumina whole genome resequencing was done by Dr. David A. Rasko, University of Maryland, Baltimore, USA or Mr. Ashley Robinson, in the Henderson group at the University of Birmingham. Plasmid sequencing was done at the Functional Genomics Unit, University of Birmingham.

2.4. PROTEIN PURIFICATION AND ANALYSIS.

2.4.1. SDS-PAGE and Western immunoblotting

SDS-PAGE gels were prepared at 12% and 15% dependant on the protein of interest to be investigated. Reagents were prepared as previously described and gels were run in 1 x SDS Running buffer, between 80-140V for the required duration. Samples for SDS-PAGE analysis were prepared by mixing 1:1 protein sample with laemmli buffer (where not already directly resuspended) and boiling for 15 min. Western blots were performed as previously described (169). SDS-PAGE gels were transferred to Nitrocellulose Membrane (BioRad) using a traditional Western Sandwich with 1x Transfer Buffer containing 20% methanol. Membranes were blocked using 5% w/v skimmed milk powder prior to incubation with primary antibody (diluted 1:1000-1:10,000 dependant on the antibody), followed by secondary anti-rabbit or anti-mouse IgG (whole molecule) Alkaline Phosphatase Antibody Produced in Goats, 1:30,000 dilution. Western blots were exposed using BCIP/NBT Purple Liquid Substrate System for Membranes.

2.4.2. ELISA

Antibody (IgA, IgM and total IgG) responses were measured by coating Maxisorp 96 well plates (Nunc) with 5 µg/mL of purified protein in carbonate coating buffer. Plates were washed and blocked for 1 h at 37^{o}C with 1% BSA in PBS (blocking buffer). Test samples were added in blocking buffer and serially diluted and incubated for 1 h at 37^{o}C. Secondary anti-mouse IgA, IgG or IgM conjugated to alkaline-phosphatase (Southern Biotech) were diluted in blocking buffer and added for 1 h at 37^{o}C. Signal was detected using the SIGMAFAST p-Nitrophenyl phosphate system and plates were read at 405 nm.

2.4.3. Preparation of antibodies

Antibodies to specified proteins were raised in New Zealand White rabbits by Eurogentec. Antibodies were absorbed against the *E. coli* strain BL21 containing pET26b empty vector. Briefly, overnight cultures were pelleted by centrifugation at 2880 x *g*, 4^{o}C, 30 min. Pellets were washed using 1 mL PBS (Invitrogen) and freeze thawed three times using dry ice and ethanol. A 0.33 mL cell suspension was added to 1 mL of serum and rotated at 37^{o}C for 30 min. A further 0.33 mL of cell suspension was added and rotated as above. The remaining volume of cell suspension was added and rotated at 4^{o}C overnight. The following day the mixture was centrifuged 12000 x *g* for 35 min and the supernatant diluted 1:5 in PBS, prior to filtering using a 0.22 µM pore size filter. Antibodies were aliquoted and stored at -30^{o}C until required.

2.4.4. Preparation of cellular fractions

Bacterial cell cultures were fractionated to isolate whole cells, cell envelopes and outer membranes, with some minor alterations to the procedures described in Parham, *et al* (183).

Briefly cultures were grown to an OD_{600nm} 1, cells pelleted by centrifuging at 2880 x *g*, 4°C, for 30 min. Supernatant fractions were collected (as required) and secreted proteins precipitated as described below. Cell pellets were resuspended in 2x Laemmli buffer (Sigma) for whole cell samples or were resuspended in 10 mM Tris pH7.4, 2 mM PMSF prior to either sonication (5 x 30 s blast with 10 s intervals) or French press at 1500-2000 psi, unbroken cells were removed by centrifuging at 2880 x *g*, 4°C, for 30 min, supernatant fractions were transferred to clean vials and centrifuged at 50,000 x *g*, 4°C for one h 30 min. Resultant pellets were either resuspended in 10 mM Tris pH7.4, providing cell envelope fractions or processed further to obtain outer membrane fractions. Outer membranes were isolated from cell envelope fractions by resuspending the pellet in 10 mM Tris, 2% v/v Triton X 100 and incubated at room temperature for 30 min with gentle agitation, prior to centrifuging at 50,000 x *g*, 4°C for one h 30 min. Pellets were washed three times with 10 mM Tris pH7.4 and isolated by centrifuging as described above. In some instances prepared outer membrane fractions were washed with 5 M urea to remove aggregates or covalently attached material (184). Briefly the outer membrane fractions were prepared as described previously, the fractions resuspended in 1 x PBS with 5 M urea and placed on a rotary shaker for one h at 4°C. The membrane fractions were pelleted by centrifuging at 50,000 x *g*, 4°C for one h 30 min. Outer membranes were washed once with 1 x PBS prior to resuspending in fresh 1 x PBS.

Extracellular proteins were harvested by precipitation using 10% TCA (final concentration). Briefly, supernatant fractions were filtered through a 0.22 µm pore size filter, and stored in the presence of TCA at 4°C overnight. The following day samples were centrifuged at 24,000 x *g*, 4°C for 45 min to pellet precipitated material. Resulting material was washed with ice-cold methanol, and incubated at room temperature for 15 min, before

pelleting as described above. The resultant pellets were dried and resuspended in 2 x laemmli buffer.

2.4.5. Purification of integral membrane proteins

Proteins were expressed from BL21 DE3 pET22b induced with IPTG. Cultures were harvested at OD_{600nm}=1, and resuspended in 50 mM HEPES, 150 mM sodium chloride, 1 mM PMSF pH7.4, prior to sonication as described previously. The cell debris and inclusion bodies were pelleted by centrifugation at 20,000 × *g*. The pellet was the resuspended in 50 mM HEPES, 150 mM sodium chloride, 8 M urea, 1 mM PMSF pH7.4 and incubated at room temperature for 1 h. Insoluble inclusion bodies and cell debris were removed by centrifugation at 50,000 × *g* and the supernatant containing soluble protein was recovered. Fractions were run on SDS-PAGE gels, excised and gel slices homogenised in 0.5 ml of 50 mM HEPES, 0.1% SDS, pH7.4. Protein was electro-eluted in a Novagen elution system as per manufacturer's instructions. The eluted protein was diluted to 10 mL 50 mM HEPES, 0.1% SDS, pH7.4 and dialysed three times against 5 L of 50 mM HEPES, 150 mM sodium chloride, 0.5% Elugent detergent (Calbiochem). Refolding by dialysis was performed over three days. The folded state of the protein was assessed using tryptophan fluorescence at 280 nm excitation, emission scanned over 300 to 450 nm, 0.5 mm slit width, ~ 1 nm bandwidth, 1 s response time, 1 nm increments using a QuantaMaster instrument (Photon Technologies International).

2.5. PREPARATION OF LIPOPOLYSACCHARIDE

The production of long chain LPS was routinely examined in *S.* Typhimurium and some *E. coli* strains, using the method described by Browning *et al* (178). Briefly overnight cultures were harvested by centrifugation at 14,000 x *g* for 10 min. Cell pellets were resuspended in 2x laemmli loading buffer, before boiling and freezing in quick succession three times. The cell suspension was centrifuged at 14,000 x *g* for two min, before incubating in the presence of 5 mg/mL proteinase K at 56^{o}C for one h, before heat inactivating at 95^{o}C for five min. Samples were loaded on Invitrogen pre-cast 4-12% Bis-Tris SDS-PAGE gel and run at 200V for 35 min, before being stained with Invitrogen Silver Staining kit, in accordance with manufacturer's instructions.

2.6 DETERMINATION OF PROTEIN AND ENDOTOXIN CONCENTRATIONS

Protein concentrations were determined using either the tannin assay or Nanodrop, with BSA in the appropriate buffer, as the standard. Purified proteins were tested for the presence of endotoxin contamination, using a Sigma E-Toxate Kit (Sigma Aldrich) in accordance with manufacturer's instructions.

2.7. MICROSCOPY

2.7.1. Light microscopy.

Bacterial cultures were grown to an OD_{600nm} 0.6, harvested by centrifuging at 2880 x *g*, 4^{o}C, for 20 min, concentrated 20 fold in 1 x PBS, prior to applying to 1 mg/mL poly-L-lysine coated 13mm cover slips by centrifuging at 2600 x *g*, 15 min and mounted onto slides. In some cases bacteria were stained with fluorescent BODIPY-vancomycin 20 µg/mL (Invitrogen, UK) for 45 min at room temperature (protected from light), washed three times with PBS and mounted onto slides using 0.5 µL fluorescent mountant (Citifluor Ltd, UK),

prior to visualisation using phase contrast DIC, with an exposure time 0.2 s and green fluorescence filter, 1.5 s exposure time, and gain setting of 1, using either a Zeiss or Leica Microscope. Samples were visualised, by examining a minimum of five randomly selected fields with phase contrast Zeiss or Leica Microscope at 100 x magnification.

2.7.2. Scanning electron microscopy.

Bacterial cultures were grown, harvested and concentrated as described above, prior to applying to1 mg/mL poly-L-lysine coated 9mm cover slips by centrifuging at 2600 x *g*, 15 min and fixed with 2.5% Electron Microscopy (EM) grade glutaraldehyde (Polysciences, Northampton, UK) in 0.1 M phosphate buffer at 4˚C overnight. Samples for were serially dehydrated with aqueous ethanol (two x 15 min each) followed by 100% dried ethanol and dried with a critical point dryer prior to mounting and platinum coated for three min using a sputter coater. Five randomly selected fields were visualised, using 5,000 x to 90,000 x magnification.

2.8. DETERMINATION OF CELL HOMEOSTASIS

2.8.1. Chemical screen for OM integrity.

Bacterial isolates were screened using a 96 well single well inoculator to transfer 1 µL of culture, to LBA supplemented with antibiotics or other compounds (e.g.; vancomycin, SDS, sodium chloride, etc). Isolates showing a growth defect were rescreened using either streak plating or replica plating methods, as previously described (185) with a minimum of three biological replicates examined for each isolate. In some instances (i.e. screening of the KEIO Library) isolates were checked by PCR to confirm the genotype, prior to transducing into

parental strains. Single colony glycerol stocks were generated for all isolates tested. Figure 2.1. shows an example work flow of the screening technique.

2.8.2. Fluorescence-activated cell sorting (FACS) screen for cell viability

Propidium Iodide (PI) and bis-oxanol (BOX) staining was performed on exponential phase cultures (OD_{600nm}=0.6); harvested by centrifugation at 2880 x *g*, 4^{o}C, for 20 min, both prior to and post treatment with either vancomycin 100 µg/ml or SDS 4.8%. PI, 5 µg/ml and BOX, 10 µg/ml were prepared in PBS (containing 4 mM EDTA for BOX only) as required. Cell pellets were resuspended in PBS, prior to diluting 20 fold in FACSFlow Buffer (BD, UK), containing either PI and BOX singly or in combination, with immediate analysis using FACS ARIA II (BD, UK). Cells were illuminated with a 488 nm laser and data from 10,000 particles were collected. Forward- and side-scatter data were collected along with PI fluorescence (red, collected through an LP 565 mirror and BP 610/20 filter) and BOX fluorescence (green, collected through an LP 502 mirror and BP 530/30 filter). Particles smaller than bacteria were eliminated by adjusting the threshold values on FSC and SSC channels.

2.8.3. Assessment of efflux capability.

Hoescht accumulation assays were performed as described previously (186). Briefly, cultures were adjusted to OD_{600nm} 0.1 and the uptake of Bis-benzimide **(**Hoescht 33342) was determined in the presence and absence of efflux inhibitor, phenyl-arginine-β-naphthylamide, 100µg/mL (PAβN) using FLUOstar Optima plate reader. Significance was determined using Two –tailed student T-Test ($p=<0.05$).

2.9. MURINE MODEL OF SALMONELLA INFECTION

In-vivo investigations performed during this study were done so under the Personal Licence 30/9112 (Faye C. Morris), Project Licences 40/2904 and 30/2850 (Prof. A. F. Cunningham) at the Biomedical Services Unit, University of Birmingham Institution Licence 40/2404 or in collaboration with Prof. D. Maskell, University of Cambridge. All procedures and protocols were ethically approved and procedures performed under the appropriate supervision as required. Bacterial strains were grown in LBB, without antibiotics to an OD_{600nm}=1. Cultures were harvested by centrifuging at 9000 x *g*, washed twice with 1 x PBS, prior to diluting in PBS to density of either 5 x 10^5 (IP infection) or 1 x 10^9 (oral gavage). Infection doses were serially diluted and plated post infection to confirm the relevant bacterial dose.

Bacterial burdens were determined across a time course of infection, ranging from 24 h to 35 days. Animals were humanely sacrificed, blood, spleen, liver, MEL and payers patches collected. Organs were weighed and mashed in 1x PBS using 70 µm cell strainer. Samples were diluted in 1x PBS prior to plating on selective agar. Bacterial burdens were calculated to bacterial burden/g of organ. Remaining spleen samples were snap frozen in liquid nitrogen, and stored at -85^{o}C. Statistical significance of results was determined using a non-parametric Mann Whitney, two tailed test. Significance was determined as p= <0.05. Serum was isolated from blood samples by incubating at 37^{o}C for one h post collection and centrifuged at 16000 x *g*, 4^{o}C for 15 min. Sera was extracted and stored at -30^{o}C until required.

For challenge studies, purified protein samples were diluted in 1 x PBS or bound to Alum; to give a final concentration of 10 µg. Mice were immunised with protein and challenged with virulent bacteria at appropriate times.

2.10. *IN VITRO* ASSAYS FOR PATHOGENESIS

2.10.1. Adhesion and invasion assays

Cell lines were maintained as previously described. Bacterial cultures were washed twice in 1 x PBS and resuspended in serum-free culture medium (~10^8 CFU/ml). Tissue culture treated 24-well plates were seeded with 10^5 J774, RAW 264 or THP1 cells or 10^6 Caco-2 cells per well. Bacterial strains were added at a multiplicity of infection (MOI) of 1:100 and incubated at 37^{o}C for two h. Adherence was determined, by washing the infected cells with 1 mL PBS three times to remove the non-adherent bacteria prior to disruption with 1 x PBS, 1% (v/v) Triton-X 100, serial dilutions of this solution were plated on to LBA to enumerate CFUs. To investigate invasion, the cells were infected as described above, prior to incubation with DMEM or RPMI containing 100 µg/mL gentamycin for 2 h at 37°C and 5% CO_2 to kill the extracellular bacteria. Cells were washed three times with 1 x PBS, prior to solubilisation with with 1 x PBS, 1% (v/v) Triton-X 100. Solubilised samples were serially diluted and plated to calculate CFUs as above.

2.10.2. Binding to extracellular matrix (ECM) molecules.

ECM binding assays were performed as previously described (125). High capacity 96 well plates were coated with 10 µg/mL of the appropriate ECM molecule (Collagen I, III, IV, fibronectin, elastin and laminin, Sigma). Bacterial cultures were adjusted to 1x10^8 CFU/mL and 100 µL added per well. Plates were incubated at 37^{o}C for four h, prior to washing three times with 1x PBS. Samples were treated with 100 µL of 1 x PBS containing 0.05% Triton X 100, and serially diluted to 10^{-4}, prior to plating on LBA to determine CFU determinations.

2.10.3. Serum bactericidal assay.

The serum bactericidal assays were performed as described previously (187). Killing of various *S.* Typhimurium and *E. coli* strains were investigated using healthy human serum obtained from African and European individuals. Bacterial cultures were harvested at an OD_{600nm} 1 and resuspended in 1 x PBS, 10 µL of each sample was added to 90 µL of freshly-thawed, undiluted serum to give a final bacterial concentration of 10^6 CFU/ml. The mixtures were incubated at 37°C with gentle rocking (20 rpm) and 10 µL samples were withdrawn at 45, 90 and 180 min. Serial dilutions of the samples were plated onto LBA plates to determine the viable bacterial counts.

2.11. BIOINFORMATICS ANALYSIS

2.11.1. Gene Distribution

Gene distribution across other organisms and identification of homologues was assessed using NCBI BLASTP. All available Gram-negative bacterial genomes were probed using the genetic sequence of specific genes of interest, to identify species encoding homologs.

2.11.2. Protein domain architecture

Protein domain structures were analysed using Simple Modular Architecture Research Tool (SMART) software, to identify potential conserved domains and secondary structures, including the presence of signal sequences (and their respective cleavage site) and autotransporter β domains.

2.11.3. Protein Interactions

Potential interactions between different proteins were investigated using STRINGS 9.0 search tool database. This program highlights both known and predicted interactions based on genomic context, results from high throughput screening assays, co-expression assays and published literature. The database correlates information for over 5 million proteins from over 1100 organisms.

CHAPTER 3

THE ROLE OF NONESSENTIAL GENES IN THE MAINTENANCE OF THE OUTER MEMBRANE BARRIER FUNCTION

3.1 INTRODUCTION

The Gram-negative cell envelope is a dynamic structure consisting of the OM, periplasmic space (enclosing the peptidoglycan) and IM (1). The asymmetric OM, with phospholipids on the inner leaflet and LPS present on the outer leaflet creates a selectively permeable barrier, limiting entry to toxic antibacterial compounds, whilst allowing the diffusion of molecules <600 daltons (1, 4). This creates a significant problem for antibacterial treatment, as a number of antimicrobial compounds are unable to penetrate this selectively permeable barrier. Additionally, these compounds can also be substrates for efflux pumps, which are molecular "vacuum cleaners" that span the cell envelope to prevent solute accumulation. While research into new antibacterial compounds has significantly decreased over recent decades, the emergence of antibiotic resistance has increased alarmingly (188). Thus, the drive to identify new targets for drug development against Gram-negative bacterial infections is critical to maintaining everyday medical practice.

The cell envelope is a rich mosaic of components, including LPS, phospholipids, peptidoglycan, proteins and lipoproteins, encoded by both essential and non-essential genes (1, 2). Our current inventory of antibiotics includes drugs which target some of these components of the cell envelope e.g. the β-lactams that target peptidoglycan synthesis. On this basis, it seems logical that chemicals inhibiting the function of other essential pathways (OMP, LPS or Lipoprotein biogenesis) would also prove useful as antimicrobial agents. However, despite decades of research, which has significantly advanced our understanding of the various pathways involved in the biogenesis of key components, no new antimicrobials targeting these pathways have emerged (30, 56, 189, 190). Over the years, several studies have focused on the role of nonessential genes in maintaining cell envelope homeostasis, e.g. *bamB* encoding the BAM complex component, *ompA* encoding a porin, and *surA*, *degP* and

skp encoding various periplasmic chaperones. These mutations have been shown to induce elevated cell envelope stress and membrane defects (19, 50, 54, 191). However, the roles of nonessential genes, and the mechanisms by which they all converge to form a coherent response to support the maintenance of cell envelope function and to counteract different stresses, still remain elusive with many genes and pathways still functionally uncharacterised. Prior to commencing this project, no single investigation had sought to identify all the nonessential genes that contribute to maintaining the barrier function of the OM or sought to understand the mechanisms by which this is achieved. We hypothesised that chemicals which targeted the pathways encoded by these non-essential genes, whilst perhaps not bactericidal or bacteriostatic in nature, could serve to sufficiently weaken the bacterium such that it is incapable of living outside the confines of the laboratory test tube. Furthermore, these chemicals (potentiators) could be combined with currently available antibiotics to enhance the entry of the antibiotics into the bacterial cell, thereby increasing their effectiveness. This type of approach has previously been shown to be effective against methicillin resistant *Staphylococcus aureus* (MRSA), whereby the combination of tannic acid reduces the MIC of fusidic acid in this strain (192). While similar capabilities have been noted for the plant derived molecules ethyl gallate and epigallocatechin gallate from *Caesalpinia spinosa* and *Camellia sinesis*, respectively, both of which have been shown to enhance the activity of β-lactams (193). Although no potentiators are as of yet used in clinical settings, this remains an active avenue of research for combating a variety of bacterial infections (193, 194). Therefore, the overarching aim of this study was to identify the repertoire of genes required to maintain OM integrity. To achieve this aim we used the *E. coli* KEIO library.

The KEIO library is a series of in-frame single gene disruption mutants in all non-essential genes in the *E. coli* K12 strain BW25113 (171). The collection contains mutants for

3985 genes, with two independent isolates obtained for each, resulting in 7970 (171). The collection was created using the Datsenko and Wanner method for one step inactivation of chromosomal genes, replacing the gene of interest with a kanamycin resistance cassette, flanked by FLP Recognition Target sites (FRT), through the aid of the phage λ red recombinase system, encoded on the temperature sensitive pKD46 (175). This technique limits polar effects on other flanking genes by including the native initiation codon and six carboxyl terminal codons, the stop codon and 29 downstream bases, maintaining open reading frames (ORF), start codons and translation signals for overlapping genes (171). Since its creation, the collection has been rechecked whereby 96.1% (7428 isolates) were deemed to be correct and 98.3% (3800 ORF) of mutations were correct for at least one copy, with only 58 mutants incorrect due to a mixture or duplication (195).

Here we used chemical biology approaches to probe the KEIO library for mutations which conferred loss of OM barrier function. Gram-negative bacteria are normally resistant to antibiotics such as daptomycin and vancomycin since they are larger than the diffusion limit of the OM (191). Gram-negative bacteria are also normally resistant to many lipophilic compounds such as SDS. The influx of lipophilic compounds is limited by the OM barrier function and also by efflux pumps such as AcrAB-TolC (3). Thus, defects in OM integrity were determined by increased susceptibility of KEIO library mutants to two separate membrane permeability markers: the glycopeptide antibiotic, vancomycin and the anionic detergent SDS (196, 197). The genotype and phenotype for each mutant was confirmed and the impact of the mutation on growth rate, cellular morphology and efflux was assessed.

3.2. RESULTS

3.2.1. Screening of the KEIO library for loss of OM homeostasis

In order to select an appropriate screening concentration of vancomycin and SDS, we used the BSAC method (198) to determine the MIC for the parental *E. coli* strain, BW25113 against vancomycin and a series of concentrations increasing at regular intervals for SDS. Consistent with previous reports, we determined the MIC for vancomycin to be 256-512 μg/mL (199, 200). Unfortunately, due to the nature of SDS, we were unable to test concentrations higher than 8% w/v. However, we were able to demonstrate that this is insufficient to inhibit the growth of *E. coli* BW25113, as determined by growth of this strain on LBA supplemented with 8% w/v SDS. Therefore, we recorded for SDS an MIC >8%. Based on these data, and to limit the potential for false positives, we selected screening concentrations of 100 μg/mL vancomycin and 4.8% w/v for SDS, which were significantly lower than the recorded MIC values.

Using a multi-well inoculator, all 7970 isolates were screened for inhibition of growth on LB agar supplemented with either vancomycin or SDS at the concentrations described above. From this screening, 871 isolates were identified as defective for growth in the presence of at least one compound. Due to the variability associated with this technique and discrepancies between the duplicate mutations within the library, each of the 871 isolates was rescreened. For each isolate six individual colonies were selected from growth on LB agar plates and were restreaked onto LB agar supplemented with either vancomycin or SDS. Applying the strict criteria that only isolates exhibiting complete inhibition of growth were deemed defective, 201 isolates were selected for further investigation.

Despite the KEIO library being checked by PCR at the time of creation, Baba *et al* (171) only checked the junctions either side of the inserted cassette, but failed to check for

gene duplications, subsequently annotating the collection as containing a <1% error rate. Reanalysis by Yamamoto *et al* (195) indicated only 58 mutants contained duplications and they recorded 96.1% of the collection as genetically correct. Given the flaws observed by Yamamoto *et al* (195), we chose to confirm the validity of the mutations being investigated in this study. The 201 isolates identified were subjected to three separate PCR analyses, using a combination of gene specific flanking primers (positioned approximately 200 bases up and downstream of the gene, for which primer sequences are available in Appendix table 1) either together or in combination with internal forward and reverse primers designed to the kanamycin resistance cassette. In the case of our collection, ca. 30% of the isolates tested did not generate amplicons that would be associated with the correct insertion of the kanamycin resistance encoding *aph* gene and consequential ablation of the target gene. In total 72 genes were identified whose loss gave rise to a compromised OM barrier function as assessed by the inability to grow on LB agar containing vancomycin or SDS.

Closer inspection of the 72 genes within this list revealed a number of genes of unknown function, but also a number of instances where the known functions could not be attributed to membrane biogenesis or barrier function. Therefore, mutations were transferred back into *E. coli* BW25113 parental strain by P1 transduction. For each transductant the genetic lesion was confirmed using the three PCR methods described above. After confirming the genotype of the newly derived mutants, six individual colonies were selected, plated on LB agar containing vancomycin or SDS and assessed for loss of viability after overnight growth. Eighteen of the transductants failed to maintain the phenotype initially observed with the KEIO library mutant. This methodology (detailed in Fig. 3.1.) yielded a group of mutants harbouring mutations in 54 nonessential genes that could be confidently

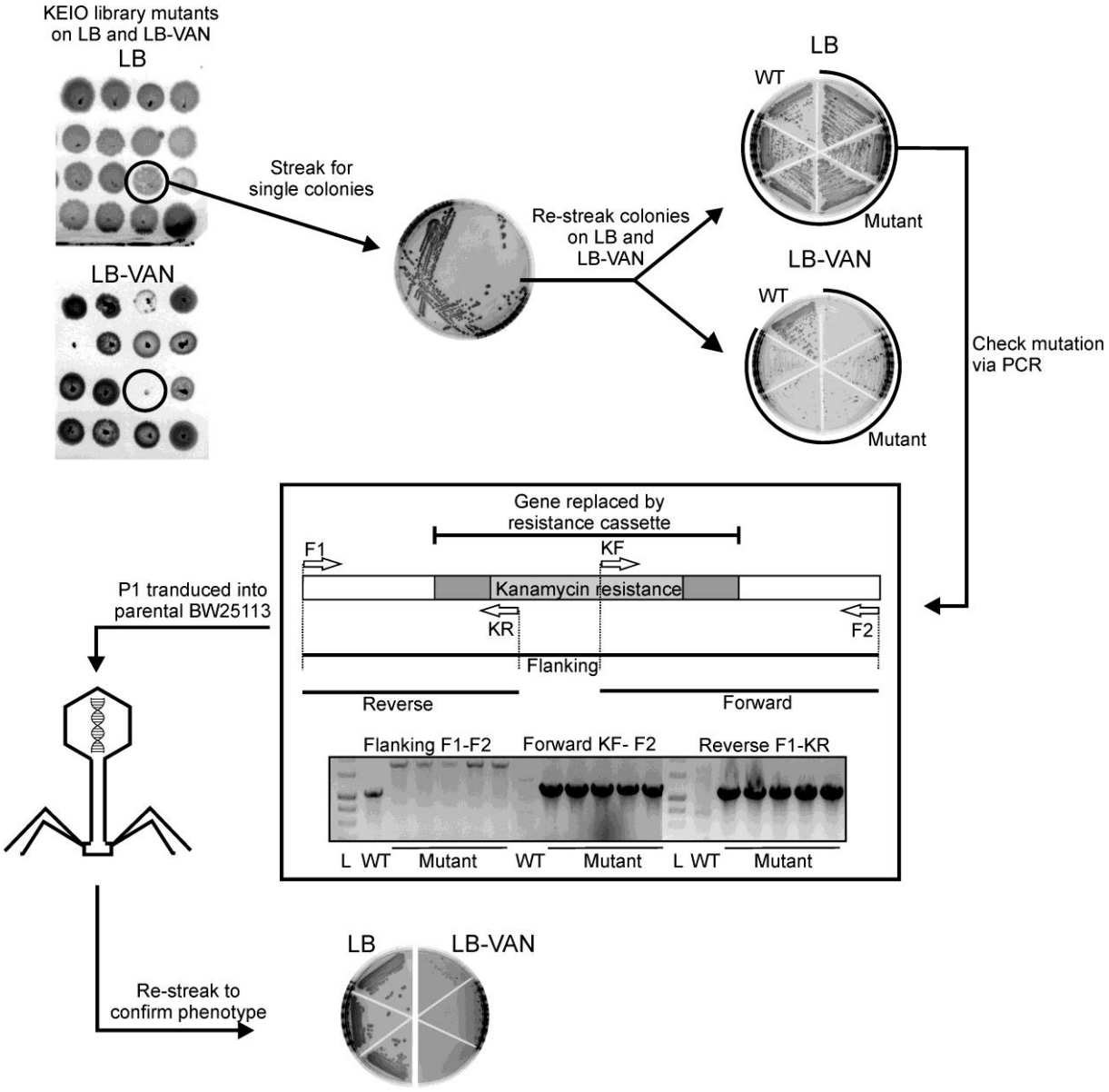


Fig. 3.1. Workflow diagram for screening of the KEIO library
The process of identifying *E. coli* BW25113 mutants with defective outer membrane homeostasis is depicted, along with the routes to confirmation of genotype and phenotype. The flowchart depicts the pathway for screening against vancomycin; the same pathway was applied to identify isolates with a loss of growth phenotype on SDS.

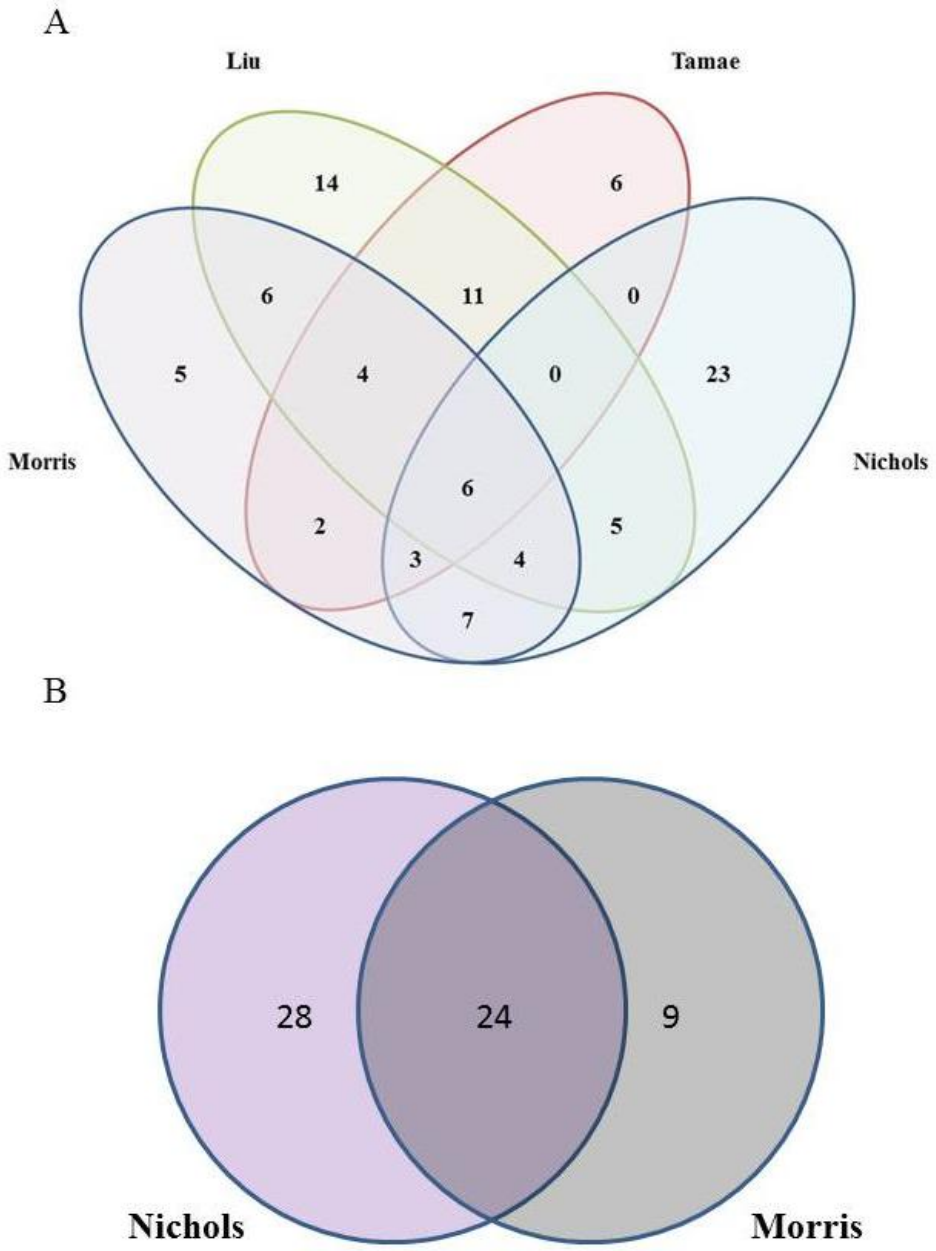


Figure 3.2. Comparison of datasets from screening the KEIO library for defective growth on vancomycin and SDS.
Panel A depicts the comparison of data from four separate studies that examined defects in the growth of KEIO library mutants in the presence of vancomycin. The integers represent the number of mutants which could not grow in the presence of vancomycin. Panel B depicts the number of genes found to be required for growth in the presence of SDS.

Table 3.1. Isolates Demonstrating Barrier Defects

Gene	Other Names	Vanc[a]	SDS	Function	Group[b]
aceE		✓	✓	Pyruvate dehydrogenase	1
aceF		✓	×	Pyruvate dehydrogenase	1
atpA	*papA*	✓	✓	Membrane-bound ATP synthase, α subunit	1
atpB	*papD*	✓	✓	Membrane-bound ATP synthase, subunit a	1
atpC	*papG*	✓	✓	Membrane-bound ATP synthase, ε subunit	1
atpD	*papB*	✓	✓	Membrane-bound ATP synthase, β subunit	1
atpF	*papF*	✓	Weak	Membrane-bound ATP synthase, subunit b	1
atpH	*papE*	✓	✓	Membrane-bound ATP synthase, δ subunit	1
fumA		✓	×	Fumarate hydratase class I	1
galU	*ychD*	×	✓	Glucose-1-phosphate uridylyltransferase	1
glnA		✓	✓	Glutamine Synthetase	1
gpmI	*gpmM*	×	✓	Phosphoglyceromutase	1
mipA	*yeaF*	×	✓	MltA-Interacting Protein	1
rpiA	*ygfC*	✓	×	Ribose 5-phosphate isomerase	1
acrA	*sipB*	×	✓	Component of AcrAB-TolC efflux system	2
acrB	*acrE*	×	✓	Component of AcrAB-TolC efflux system	2
bamB	*yfgL*	✓	✓	Component of the BAM complex	2
bamE	*smpA*	✓	×	Component of the BAM complex	2
fepB		✓	✓	Iron enterobactin transporter subunit	2
fepC		✓	×	Iron enterobactin Transporter Subunit	2
ompA	*tolG*	✓	×	Outer membrane Protein A	2
pal		✓	✓	Component of Tol-Pal complex	2
tatC	*mttB*	Weak	✓	TatABCE protein translocation system subunit	2
tolA		Weak	✓	Component of Tol-Pal complex	2
tolB		✓	✓	Component of Tol-Pal complex	2
tolC		×	✓	Component of AcrAB-TolC efflux system	2
tolQ		✓	✓	Component of Tol-Pal complex	2
tolR		Weak	✓	Component of Tol-Pal complex	2
skp	*hlpA*	✓	×	Periplasmic protein chaperone	3
surA		✓	✓	Periplasmic protein chaperone	3
cysB		✓	×	Transcriptional regulator for cysteine regulon	4
envZ	*tpo*	✓	×	Two component transcriptional regulator with OmpR	4
fur		✓	✓	Transcriptional regulator of ferric uptake associated genes	4
hfq		×	✓	RNA binding protein, stimulating RNA-RNA pairing	4
ompR		✓	Weak	Two component transcriptional regulator with EnvZ	4
rfaH	*sfrB*	×	✓	DNA-binding transcriptional antiterminator	4
rseA	*yfiJ*	×	✓	Negative regulator for sigma E	4
fabH		✓	×	3-oxoacyl-[acyl-carrier-protein] synthase III	5
htrB	*lpxL*	Weak	✓	Lauroyl acyltransferase	5
lipA		✓	Weak	Lipoate synthase	5
lpcA		×	✓	Sedoheptulose 7-phosphate isomerase	5

Table 3.1. Isolates Demonstrating Barrier Defects (continued)

Gene	Other Names	Vanc	SDS	Function	Group
msbB	*lpxM*	✓	×	Myristoyl-acyltransferase	5
rfaC	*waaC*	×	✓	LPS heptosyl transferase I	5
rfaD	*waaD*	×	✓	ADP-L-glycero-D-mannoheptose-6-epimerase,	5
rfaE	*waaE*	×	✓	Heptose 7-phosphate kinase and heptose 1-phosphate adenyltransferase	5
rfaF	*waaF*	×	✓	LPS heptosyltransferase II	5
rfaG	*waaG*	×	✓	LPSe glucosyltransferase I	5
rfaP	*waaP*	×	✓	Kinase p-hophorylating core heptose of LPS	5
gmhB	*yaeD*	×	✓	Heptose 1,7-bisphosphate phosphatase	5
plpA	*yraP*	✓	✓	Predicted protein	6
ybgT		×	✓	Predicted small membrane protein	6
yfgC		✓	×	Predicted Peptidase	6
yibP	*envC*	✓	✓	Predicted membrane protein	6
yciM		✓	✓	Predicted heat shock protein	6

[a]Defects in barrier function determined as the inhibition of growth: ✓ sensitivity observed as no growth; Weak, sensitivity observed as weak growth; × growth comparable with that of the parental strain *E. coli* BW25113.

[b]Functional Groups: 1. Metabolism; 2. Membranes and Transport; 3. Chaperones; 4 DNA and Transcription/Translation; 5. Fatty Acid/LPS and Macromolecule Biosynthesis; 6 Unknown Function and Predicted Proteins

considered to be essential for maintaining OM homeostasis under the conditions tested (Table 3.1.).

Since commencing this project, a number of other groups have also utilised the KEIO library for similar means. Three separate studies examined the sensitivity of the KEIO library mutants to vancomycin or SDS (199-201). Comparison of the published data, with that obtained during this study, highlights striking differences; Tamae *et al* (199), highlighted 32 genes required for resistance to vancomycin, while Liu *et al* (200) highlighted 51 and Nichols *et al* (201) identified 87 genes required for resistance to vancomycin, SDS or both reagents (Fig. 3.2.). Each mutant identified by the other studies, but which was not found in our investigation, was plated out and subjected to the same phenotypic and genotypic scrutiny described above. Of the 32 genes highlighted by Tamae *et al* only 15 were sensitive and genetically correct in our hands, of which four lost their phenotype on P1transduction (*dksA, rimK, ycbR* and *yhdP*), a further seven were incorrect by PCR and 10 were found to be correct by PCR but not sensitive, despite using the same concentrations of vancomycin. Similarly, Liu *et al* identified 50 genes critical for vancomycin resistance. However, only 20 of these mutants were found to be sensitive and genetically correct in our hands, seven of which were only weakly sensitive and were omitted from our list of 72. Two (*dnaK* and *rimK*) lost their phenotype on P1 transduction. A further four were incorrect by PCR, one was unconfirmed and 25 were correct but not sensitive. While Nichols *et al* (201) examined sensitivity to both vancomycin (50 μg/mL) and SDS (4%) these were at different concentrations to that used in our study. Of the 13 mutants highlighted as sensitive to both reagents in the Nichols *et al* study, only seven were consistent with our data, while five of these showed sensitivity to only one compound and the remaining mutant was not sensitive in our screen. Of the 35 isolates sensitive to only vancomycin, 11 were consistent with the current study. Finally, of the 39

isolates sensitive only to SDS, which were highlighted by Nichols *et al,* 15 mutants were consistent with our data; six were found to be genetically incorrect.

3.2.2. Characterisation of mutants with defective barrier function

To confirm the results were not distorted by artificially slow growth rates, we analysed the growth kinetics of the 54 isolates under standard conditions in LB broth at 37^{o}C (Figure 3.3). The majority of mutants showed similar growth kinetics to that observed for the parental strain, with a limited number showing reduced growth in the form of either slower growth rates, or earlier entry into stationary phase at lower optical densities, as seen for *bamB, fabH, glnA, surA, tatC* and *tolQ.* While several isolates displayed increased lag phase or slight reductions in growth rates, including *aceE, aceF, htrB, lipA, msbB* and several *atp* mutants, these reductions were not deemed to be sufficiently dramatic as to have affected the results of the earlier screening.

Having performed standard growth kinetics, we set about assessing the mutants using flow cytometry with PI and BOX staining, after standard growth conditions. PI and BOX staining is commonly used as means of discriminating between live, dead and stressed cells. PI stains the DNA of dead cells but is unable to penetrate the membranes of live cells, whilst BOX stains cells lacking IM potential (202-204). As shown in figure 3.4., the resultant plots for healthy and killed BW25113, demonstrates healthy cells localise predominantly to quadrant (Q) 3, due to minimal staining of either compound. In contrast killed cells localise to Q2 indicative of staining with both PI and BOX. Highly stress cells can stain for a loss of membrane potential (i.e. BOX only) with resultant Q1 localisation, though PI staining is only observed in dead cells, (i.e. Q4 localisation should not be possible in the presence of both dyes). Thus, screening by this method should reveal if significant portions of the

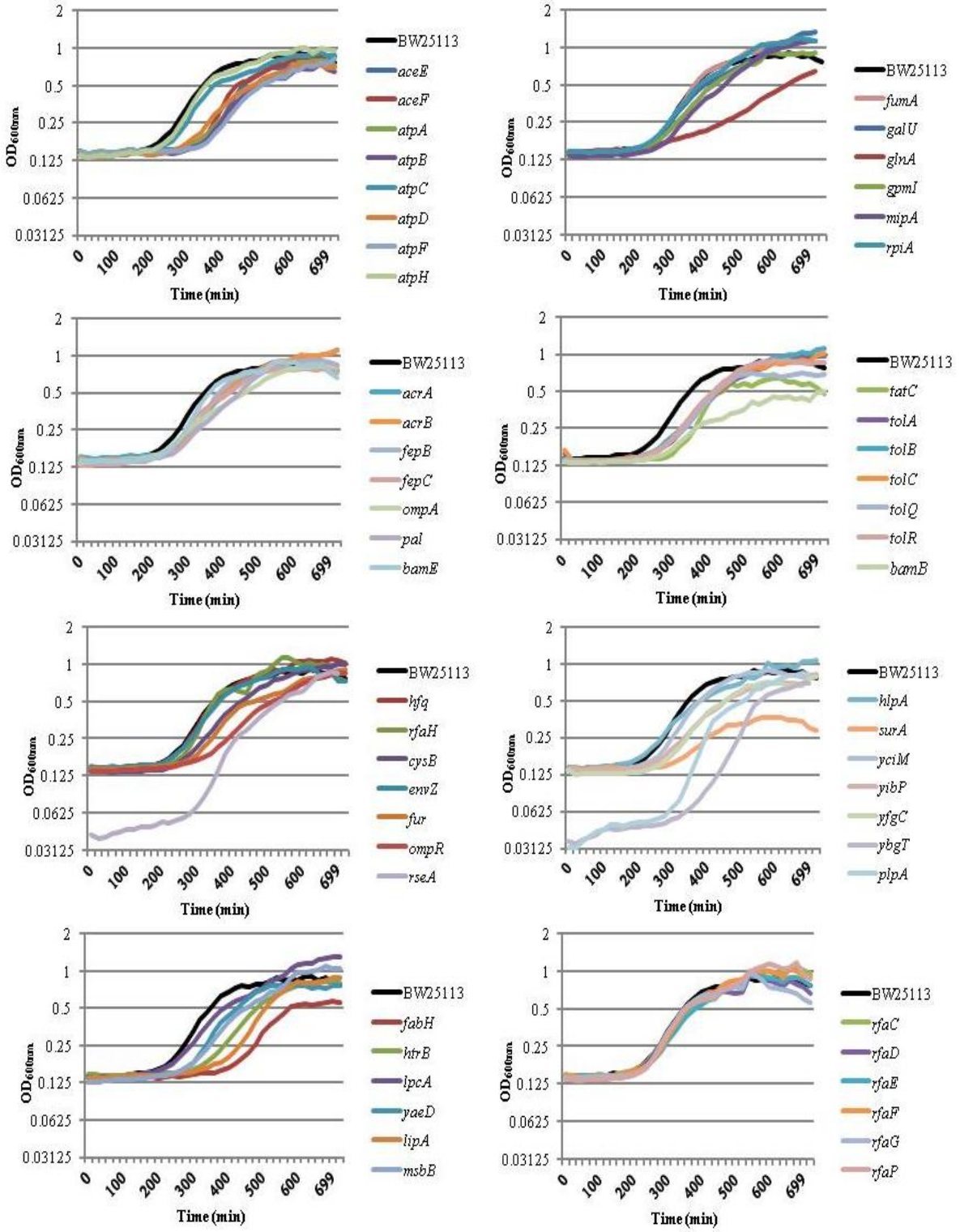
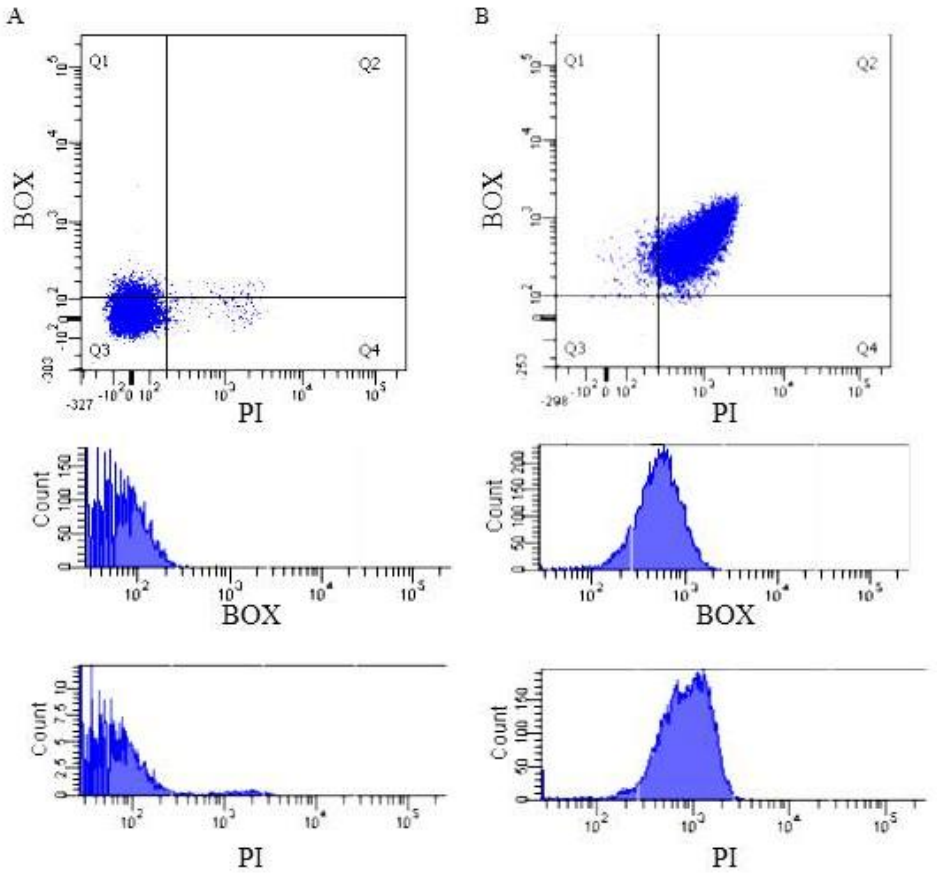


Figure 3.3. Growth kinetics for isolates with defective barrier function.
Growth kinetics for isolates sensitive to either vancomycin, SDS or both, grown under standard laboratory conditions in LB broth at 37^{o}C. Data represent the average of the three biological replicates with two technical replicates for each.



Q	Healthy BW25113	Killed BW25113
1	11.0 %	05.1 %
2	00.6 %	93.8 %
3	86.6 %	00.4 %
4	01.8 %	00.7 %

Figure 3.4.Flow cytometry Dot Plot and Histograms for PI and BOX staining
FACs dot plots, histograms and quadrant (Q) percentages for PI and BOX stained **(A)** healthy BW25113 and **(B)** ethanol killed samples, with combined PI and BOX Staining, showing under healthy conditions population localises to Q3, not staining with either compound, though when ethanol killed, the population substantially shifts from Q3 to Q2. Histograms show staining for BOX is detected by FITC channel, while PI is PE Texas Red.

population were stressed or losing viability during our analyses. We examined all isolates after growth in LB broth under standard conditions. The results are expressed as a percentage the population staing with the relevant dye (Table 3.2.).

For the majority of isolates, >95% of the population is present in Q3, with a few minor exceptions including *gpmI, skp, ompR, surA, tolR* and *yibP*. In these isolates >90% of the population is present in Q3 (with the exception of *skp* in which 89.9%), with the remaining population distributed across the other quadrants. Interestingly, some mutants show a larger proportion of the remaining population in Q4 (than in Q1 or 2), suggesting the resultant membrane defect may allow for PI penetration while the membrane remains largely intact Interestingly, the *skp* and *surA* mutants (both periplasmic chaperones) demonstrated the highest remaining proportion present in Q4.

3.2.3. Examination of cellular morphology

Changes in cell shape can significantly alter optical density readings obscuring changes that might occur in the number of viable cells. To complement the above studies, and to rule out the possibility that changes in cell size might be confounding our results, we chose to examine the cell morphology using SEM (Fig. 3.3.). SEM analysis was performed for the majority of mutants (48/54); we were unable to gain access to the SEM facility to complete the analysis. In many instances the mutants displayed normal morphology, with regards to cell size and shape. Two notable exceptions were strains harbouring mutations in *tatC* and *msbA*, which displayed an elongated rod shaped morphology. Furthermore, the *tatC* mutant showed a cell divison defect since individual rods did not appear to dissociate but rather formed chains. Interestingly, in many cases there appears to be a high level of vesicles present, including in the case of the *fabH, fumA, galU, hfq, lipA, ompA* and *pal* mutants.

Table 3.2. Population Statistics for Flow cytometric analyses of mutants

	PI and BOX Standard			
Gene	**Q1**	**Q2**	**Q3**	**Q4**
BW25113	0.1	0.2	99.4	0.3
aceE	0	0	99.9	0.1
aceF	0	0	99.2	0.8
acrA	0	0	99.8	0.2
acrB	0	0	99.6	0.4
atpA	0	0.1	99.7	0.2
atpB	0.1	0.3	99.1	0.5
atpC	0	0	100	0
atpD	0	0	100	0
atpF	0	0.1	99.8	0.1
atpH	0	0.2	99.2	0.6
bamB	0	0	99.7	0.3
bamE	0	0	99.9	0.1
cysB	0	0	99.8	0.2
envZ	0.3	0.5	98.5	0.7
fabH	0.2	0.2	99.3	0.4
fepB	0.1	0	99.8	0.1
fepC	0	0	99.8	0.2
fumA	0.1	0	99.8	0.1
fur	0.1	0	99.6	0.2
galU	0	0	99.8	0.2
glnA	0	0	99.9	0
gpmI	1.5	2.8	93.7	2
hfq	0.1	0	99.9	0
htrB	0	0	99.8	0.2
lipA	0	0	99.9	0
lpcA	0.1	0	99.5	0.5
mipA	0.1	0	99.8	0.1

	PI and BOX Standard			
Gene	**Q1**	**Q2**	**Q3**	**Q4**
msbB	0.1	0.1	99.7	0.1
ompA	0	0	99.6	0.3
ompR	0.9	1.9	92.5	4.7
pal	0.4	0.2	98.5	0.9
plpA	0.3	0.1	97.9	1.7
rfaC	0.1	0.3	99.5	0.1
rfaD	0.3	0.5	97.5	1.7
rfaE	0.1	0.2	97.4	2.3
rfaF	0.2	0.3	98.1	1.4
rfaG	0.2	0.1	98.4	1.2
rfaH	0.2	0.2	99.5	0.2
rfaP	0.1	0.1	99.6	0.2
rpiA	0.3	0.1	99.5	0.2
rseA	1	0.2	97.6	1.3
skp	1.7	0.7	89.9	7.6
surA	0.9	1.7	90.6	6.8
tatC	*1.2*	*0.4*	*97.7*	*0.7*
tolA	0.2	0.1	99.4	0.3
tolB	0.4	0.5	95.8	3.3
tolC	0	0	99.9	0.1
tolQ	0.3	0.2	98.7	0.9
tolR	1.7	1.3	94.9	2.1
yaeD	0.1	0	98.5	1.4
ybgT	0.2	0	98.4	1.4
yciM	0.2	0.1	99.1	0.6
yfgC	0	0	99.9	0.1
yibP	0.9	0.9	94.2	3.9

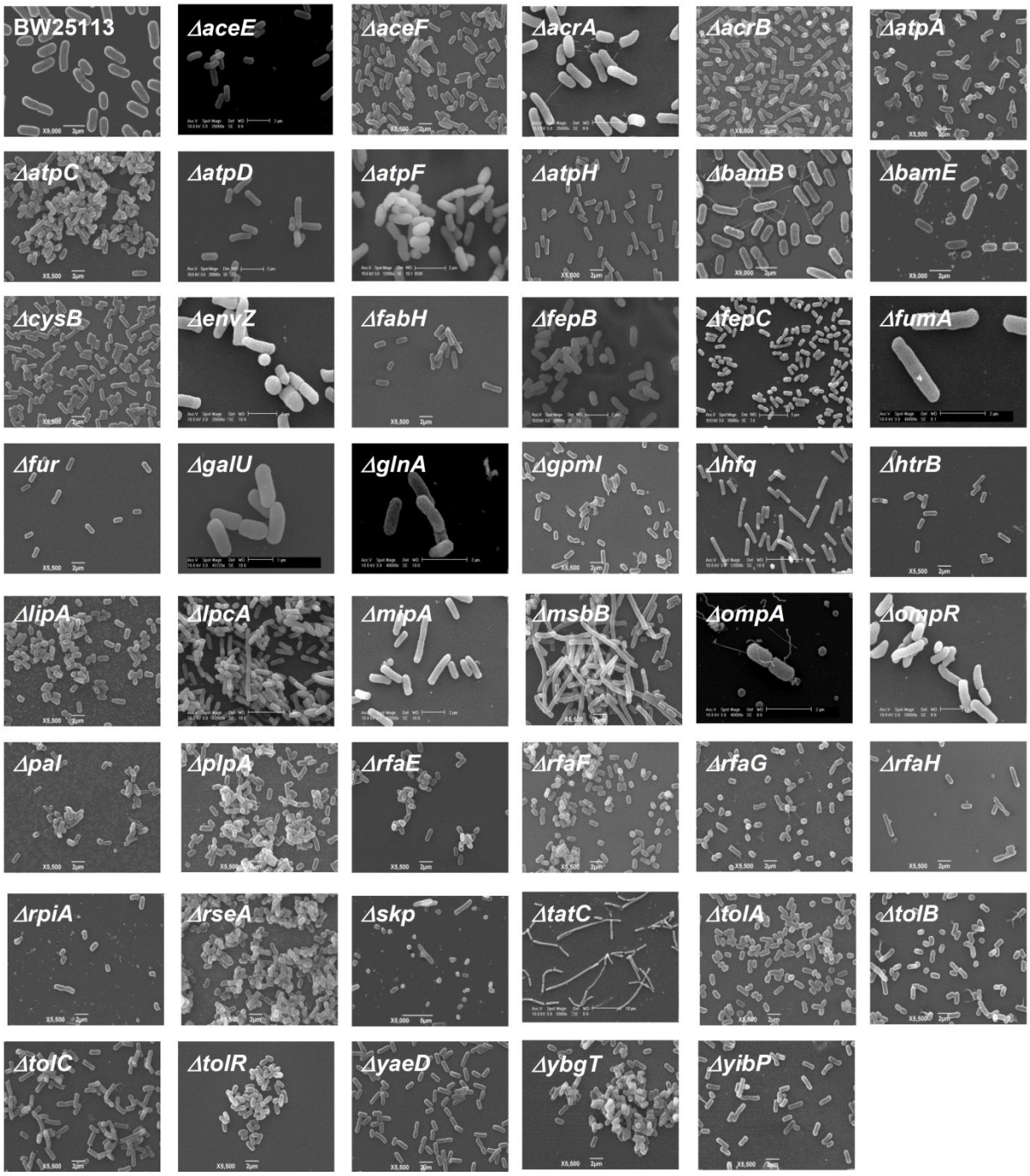


Figure 3.3. SEM for isolates with defects in OM homeostasis.
E. coli BW25113 mutants were imaged at different magnifications. The identity of each mutant is shown.

3.2.4. Investigation of the molecular basis for loss of OM homeostasis

As mentioned previously, defects in outer membrane homeostasis confer upon Gram-negative bacteria susceptibility to a variety of compounds. The molecular basis for loss of the barrier function is incompletely understood, however there are currently two major schools of thought. The first hypothesis suggests loss of the barrier function is related to structural instability in the OM resulting from uncoordinated assembly of OM components. The second school of thought suggests that defects in efflux activity leads to the accumulation of toxic compounds in the cell leading to cell death. Associated with this is the hypothesis that decreases in barrier function allow the ingress of toxic molecules to an extent that the efflux systems are overwhelmed, leading to accumulation of toxic substances and cell death. We hypothesised, if mutations led to significant structural instability then the periplasmic contents of the cells would leak out of the bacterial cell and if the mutation gave rise to defects in efflux on the ingress of toxic substance then this would manifest as accumulation of the substances within the cell.

To test the first hypothesis, we examined the culture supernatant fractions of each mutant after overnight growth in LB broth using standard conditions. The supernatant fraction was separated from cells and precipitated using TCA. Resultant fractions were compared by SDS-PAGE to supernatant fractions of chemically lysed and untreated *E. coli* BW25113 cultures. Similar to other *E. coli* K12 strains, BW25113 does not naturally secrete proteins into the supernatant fraction (106). A number of isolates (21 of 54) accumulated proteins in the supernatant fraction, including *aceE, aceF* (weakly), *gpmI, hfq, mipA, msbB, ompA, ompR, pal, rfaD rfaF, rfaG, rfaH, rfaP, surA, tatC, tolA, tolB, tolQ, tolR* and *yibP* (Fig. 3.4.). Interestingly, this correlated with production of vesicles observed by SEM (Fig. 3.3.) These fractions were subjected to immunoblotting using antibodies raised against the periplasmic

chaperone DegP, confirming the proteins observed were of periplasmic origin (data not shown). In parallel to examining the supernatant fractions the cell envelope fractions of each mutant was harvested and analysed by SDS-PAGE (Fig. 3.5). With the exception of *ompA* and *ompR* mutants, where the absence of OmpA and OmpF/C are observed, respectively, no major differences were noted between the parent and mutants.

To determine whether the elevated sensitivity was due to defective efflux, we investigated the efflux activity of all 54 isolates, using the Hoescht accumulation assay as described by Coldham *et al.* (186). The substrate, Bis-benzimide **(**Hoescht 33342), naturally diffuses into the cell and emits a fluorescent signal upon binding to the DNA. This compound is a substrate for the RND efflux pumps, which in healthy cells results in a standard base line of fluorescence (based on the balance between diffusion into the cell and efflux out). Inhibition of efflux activity in these cells, through the addition of the broad spectrum efflux pump inhibitor, phenyl-arginine-β-naphthylamide (PAβN), functioning through competitive inhibition of RND pumps (205, 206), provides a suitable control for comparison with potential efflux mutants; these mutants display significantly increased fluorescence due to increasing intracellular concentrations of Hoescht 33342. The majority of the isolates demonstrated comparable efflux activities when compared to the wild-type *E. coli* BW25113 (Fig. 3.6.). Notable exceptions were the isolates harbouring mutations in the genes encoding the known efflux system AcrAB-TolC. In addition, the *hfq, envZ, skp, yciM, yibP* and the *rfa* mutants showed increased accumulation of Hoescht 33342 (Fig. 3.6.). To investigate whether the increase in fluorescence was due to impaired efflux we added PAβN to the assay. Only in the case of the *rfa* mutants did PAβN addition fail to increase Hoescht 33342 accumulation. Surprisingly, a number of isolates demonstrated a significant increase in efflux activity

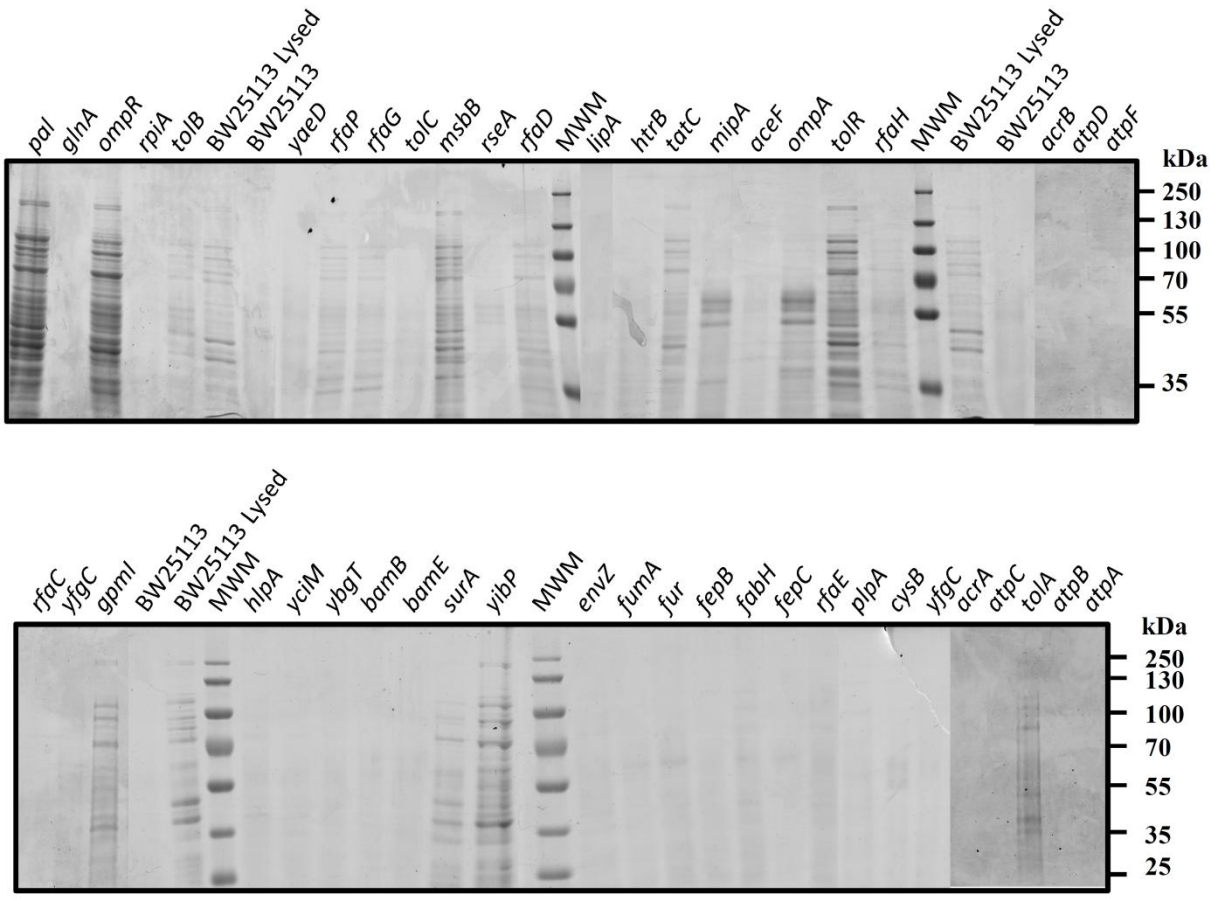


Figure 3.4. SDS-PAGE analysis of culture supernatant fractions
TCA precipitated supernatant fractions from isolates with membrane barrier defects were analysed by SDS-PAGE. The size of the molecular weight markers (MWM) are depicted to the right of each gel.

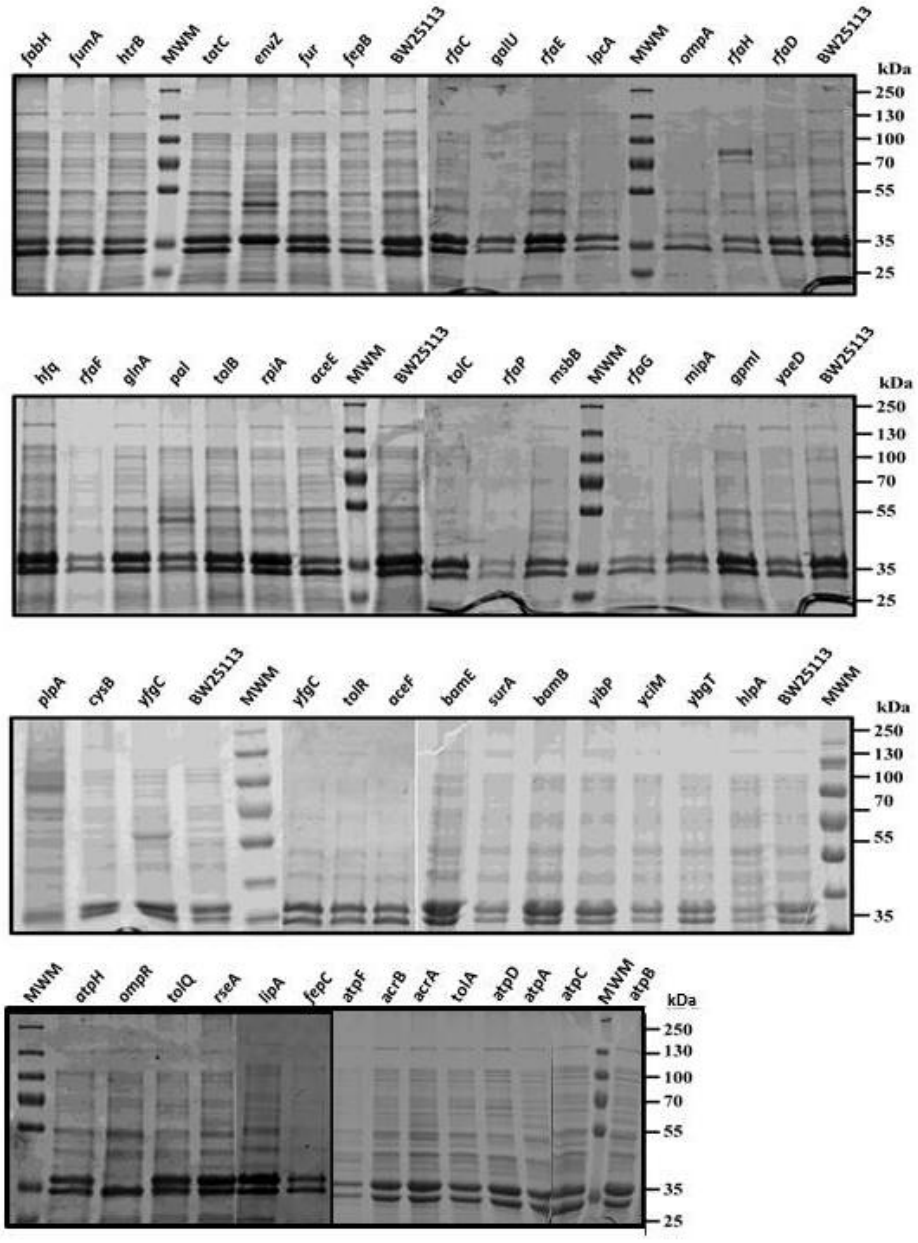
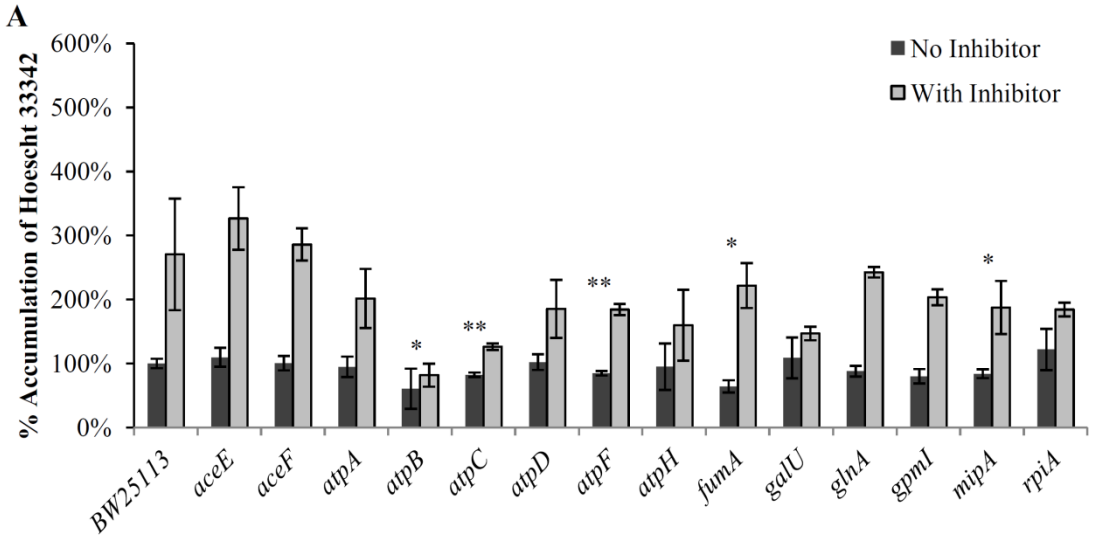
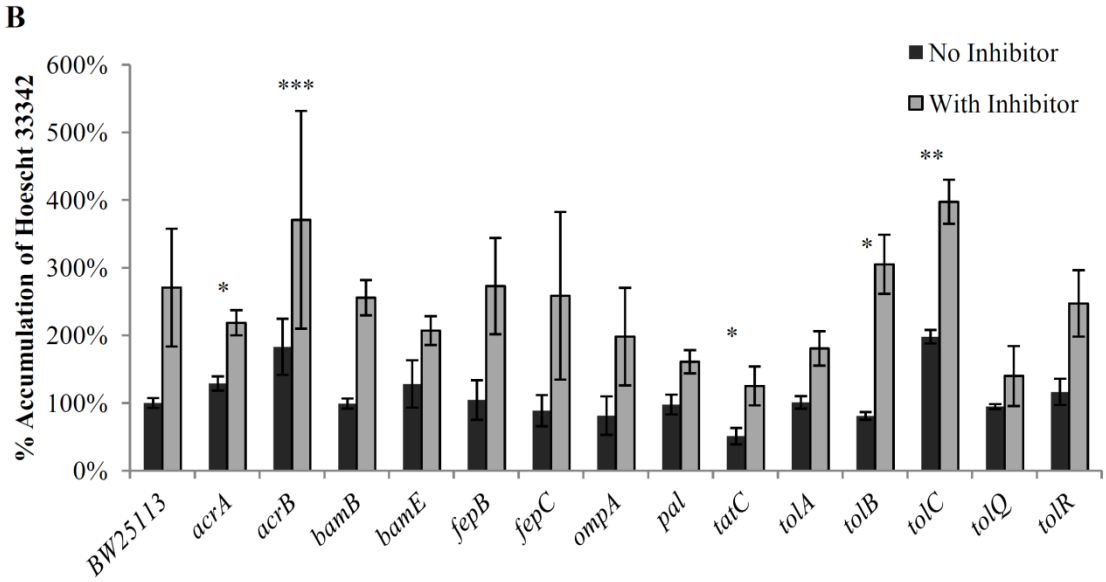
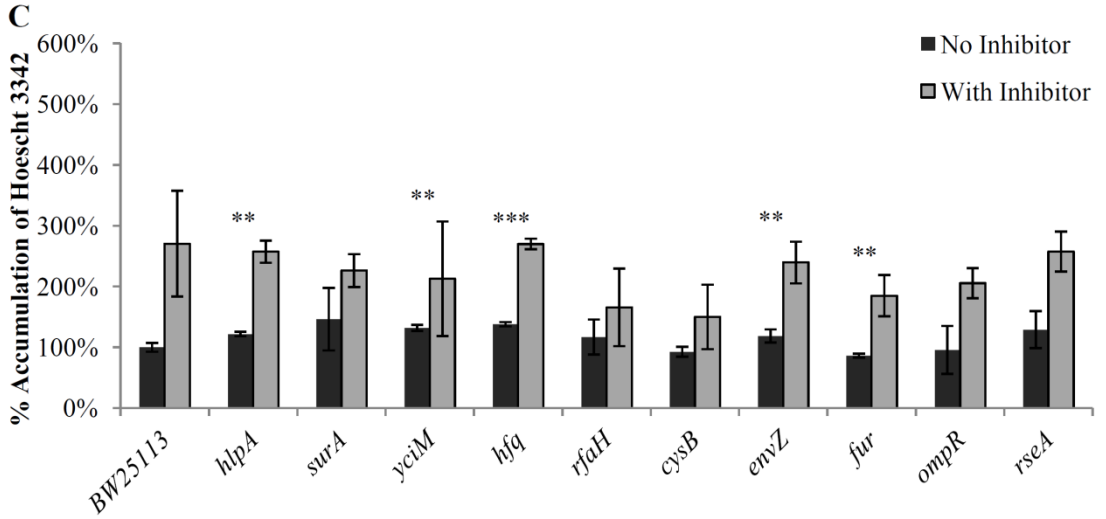


Figure 3.5. Cell envelope preparations
Cell envelope preparations from isolates with membrane barrier defects were analysed by SDS-PAGE. The size of the molecular weight markers (MWM) are depicted to the right of each gel.


A
% Accumulation of Hoescht 33342
600%
500%
400%
300%
200%
100%
0%
No Inhibitor
With Inhibitor
BW25113
aceE
aceF
atpA
atpB
atpC
atpD
atpF
atpH
fumA
galU
glnA
gpmI
mipA
rpiA


B
% Accumulation of Hoescht 33342
600%
500%
400%
300%
200%
100%
0%
No Inhibitor
With Inhibitor
BW25113
acrA
acrB
bamB
bamE
fepB
fepC
ompA
pal
tatC
tolA
tolB
tolC
tolQ
tolR


C
% Accumulation of Hoescht 3342
600%
500%
400%
300%
200%
100%
0%
No Inhibitor
With Inhibitor
BW25113
hlpA
surA
yciM
hfq
rfaH
cysB
envZ
fur
ompR
rseA

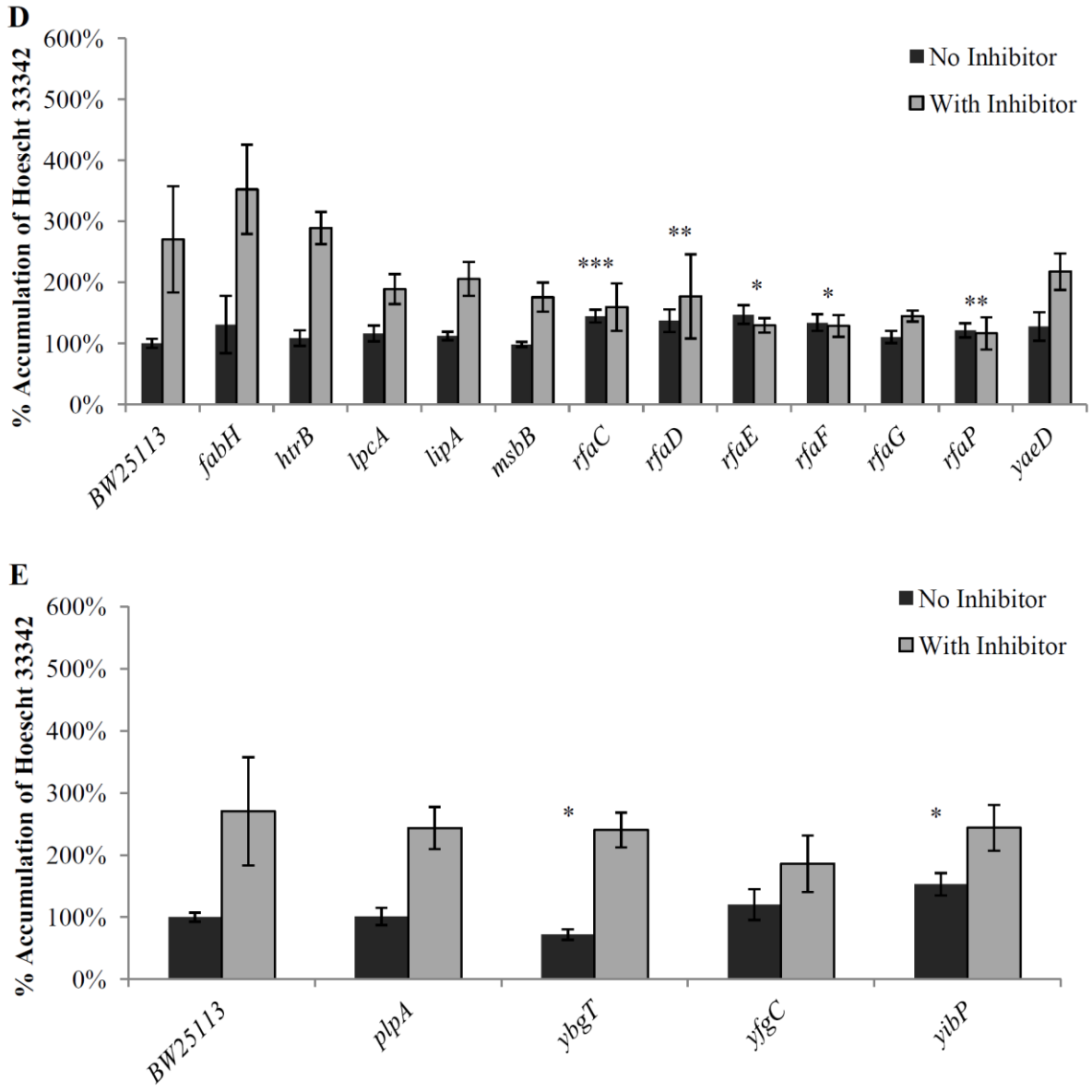


Figure 3.6. Efflux activity of all isolates

The efflux activity of all isolates with compromised OM homeostasis was determined using the Hoescht 33342 accumulation assay. Fluorescence levels set to 100% for BW25113 without an inhibitor. All mutants were compared against BW25113 to generate of a percentage of fluorescence by comparison both in the presence and absence of the broad range efflux inhibitor, PAβN. Significance was determined using a student T test and P-values were scored <0.05 (*), <0.009 (**) or <0.0009 (***).

compared to that of the parental strain e.g. *atpB, atpC, atpF, fumA, mipA, tatC, tolB, fur* and *ybgT*.

3.2.5. Can Flow cytometry distinguish cells with compromised barrier function?

As we had established that flow cytometry could distinguish between healthy, dead and stressed cells we hypothesised that this assay might yield a high-throughput assay to distinguish mutants which would succumb to the presence of vancomycin, SDS or other toxic compounds to which wild-type *E. coli* are normally resistant. To test this hypothesis, we chose a selection of isolates we had previously shown to be either sensitive or resistant to either vancomycin or SDS. As all mutants displayed normal viability when analysed by flow cytometry with PI and BOX staining. Cultures were grown to mid-log phase (OD_{600}=0.6) in LB broth at 37^{o}C under standard conditions, after which either vancomycin or SDS was added to the culture at final concentrations of 100 μg/mL or 4.8% w/v, respectively. Incubation was continued for two hours before the addition of PI and BOX, and fluorescence measurements were taken as normal. A large portion of the population of the *E. coli* BW25113 parent strain remained viable after treatment with both compounds (Table 3.3.). Most mutants behaved in a manner that might be expected. Those that demonstrated susceptibility to vancomycin, SDS or both compounds in the plate assay also demonstrated a shift from live (Q3) to dead (Q2) or stressed (Q1) cells in the flow cytometry assays. However, several of the mutants gave contradictory results. For example, *aceF, fepB* and *glnA*, incubated with vancomycin continue to show >90% residing in Q3, despite previous sensitivity to this compound in the plate screening assay. All isolates incubated with SDS (except *yciM*, which displays 85.7%) show <80% of the population residing in Q3. In some instances, this is more significant, with mutants such as *rfaF* and *rseA* displaying <2% of the population in this quadrant.

Table 3.3. PI and BOX staining of isolates with defective barrier function

	PI and BOX Post Vancomycin				PI and BOX Post SDS				Phenotype in Agar (Sensitivity)[a]	
Gene	Q1	Q2	Q3	Q4	Q1	Q2	Q3	Q4	Vanc	SDS
BW25113	0.3	0	98.5	1.2	3.7	1.6	92.3	2.4	×	×
aceF	1.9	0.7	93.1	4.3					✓	×
bamB	3.4	14.3	13.9	68.5	1.2	1	12.6	85.3	✓	✓
fabH	8.8	18.4	30.5	42.3					✓	×
fepB	0.6	0.4	96.9	2.2	3.3	31.8	61.6	3.3	✓	✓
glnA	2.4	1.9	91.3	4.4					✓	✓
gpmI					1.4	40.9	44.9	12.8	×	✓
mipA					0.1	0.3	78.6	21.0	×	✓
ompR	7.6	24.9	23.6	43.9					✓	Weak
pal	4.4	4.6	66.7	24.4	6.6	55.2	27.8	10.5	✓	✓
plpA	29.3	15.2	50.2	5.3	0.4	1.0	32.3	66.3	✓	✓
rfaF					0.5	1.2	1.2	97.1	×	✓
rpiA	12	0.8	85	2.2					✓	×
rseA					0.1	1.8	1.3	96.8	×	✓
surA	18.3	54.4	10.5	16.9	8.1	9.8	51.1	31	✓	✓
tolC					4.3	4.7	44.1	46.9	×	✓
yciM	7	8.2	67.2	17.7	0.8	0.5	85.7	13	✓	✓

[a] Phenotype as determined previously in solid agar supplemented with either compound, ✓ sensitivity as observed as no growth; Weak, sensitivity observed as weak growth, × growth comparable with that of the parental strain *E. coli* BW25113.

Interestingly, of the 11 isolates tested in the presence of SDS, eight demonstrate higher Q4 than Q2 percentages and of these five displayed higher Q4 than any other quadrant. Despite the few exceptions described above, the results obtained from this analysis are consistent with those described previously, indicating the method may be useful for large scale screening in the future.

3.3. DISCUSSION

This study has identified 54 nonessential genes which encode products critical for the formation and maintenance of the cell envelope barrier function. This is by no means a definitive list, in some instances the inability to identify genetically correct mutants from the library, may have limited the number of genes identified. For example, mutations in *lamB* and *lpp*, were predicted to have shown some degree of membrane defect under the conditions tested, based on their known functions and defects previously reported in the literature. However, we were unable to isolate genetically correct isolates for either of these mutants, in addition to numerous others, as shown by the significantly high error rates observed from this study (>30%). Additionally, by only selecting two markers of membrane permeability this may have further restricted the numbers of genes highlighted during this study. Studies by Tamae *et al* (199) and Liu *et al* (200), have shown that sensitivity to different antibiotic classes highlights different subsets of genes. Thus, by expanding our initial screens to include other non-bactericidal compounds of different functional classes we may have further identified other genes and pathways.

Closer examination of the published literature shows both the Tamae *et al* and Liu *et al* papers originated from the same research group, though only 22 genes are consistently between both articles, of which only 10 were found to be sensitive and PCR correct in this

study, of which one lost its phenotype after P1 transduction (*rimK*). In contrast to this study, Tamae *et al* failed to check the veracity of any of the mutants used during their study, while Liu *et al*, performed only minimal checks, selecting just one mutant from those shown to be sensitive to a particular class of antibiotics for PCR checking. Furthermore, in both articles only one copy of each mutant was examined for sensitivity, in contrast to this study where both copies of each mutant were screened, to account for potential genetic variations between the isolates. Additionally, by P1 transducing mutations of interest into the parental strain and testing the new mutant, we have further reduced the potential for other secondary site mutations, capable of obscuring the true phenotype.

In contrast to the above articles, Nichols *et al* performed stringent PCR analysis of the mutants used in their study, and although no error rates are quoted in the article, a number of mutants are excluded, due to the authors being unable to confirm the veracity of the mutation. However, Nichols *et al*, used a measure of colony size to determine fitness, and thus distinguish sensitive mutants, while this study focused on complete inhibition of growth. Despite using higher concentrations of both reagents in this study, the criteria of complete growth inhibition may account for the significant differences in the numbers of isolates perceived as sensitive. Interestingly, comparison of the vancomycin sensitivity results from all data sets (Tamae *et al*, Liu *et al*, Nichols *et al* and this study), indicates only six mutants are in total agreement, highlighting the variability associated with both the KEIO library and different screening methods. Though despite these inconsistencies the sensitivity observed during this study has been reconfirmed by multiple techniques, including PI and BOX viability analysis, confirming the results observed here were not an artefact of the screening method employed.

The significantly high error rates in the KEIO library, reported as part of this study, contrast with that previously reported by Baba *et al* (171) and Yamamoto *et al* (195), whom reported the collection contained <1% and 1.6% incorrect mutants, respectively. These inconsistencies are potentially an artefact of the methods employed in the other reports. Here we have performed three independent PCR reactions to validate each mutant, using a combination of gene specific flanking primers, together or in combination with internal kanamycin cassette primers, to check for both gene duplications, and confirm the junctions either side of the inserted cassette. We again applied the strict criteria that all three PCR reactions, must generate products of the correct size, to be deemed correct. In contrast, when the collection was constructed only the junctions either side of the inserted cassette were assessed, which failed to check for gene duplications. Further to this, when the collection was rechecked, internal primers to the disrupted genes were used to perform 'negative PCR' whereby the absence of a PCR fragment in the mutant but present in the parental strain, was deemed sufficient to annotate the mutation as correct; in other words they used absence of evidence as evidence of gene absence. Thus, although the error rate observed in our collection maybe an artefact of only having screened 457 isolates (potentially extending our investigations may reduce this value) we have shown that the identification of correct/incorrect mutants appears to in some instances be collection specific. Comparing our PCR data with that reported by Yamamoto *et al*, highlighted mutants which they have annotated as incorrect or a mixture, we have been able to isolate genetically correct colonies (i.e. *nohA* and *mdh*). However, in other instances the reverse is true; isolates found to be incorrect in our collection were annotated as correct in their study (i.e. *lamB*, *lpp* and *oxyR*). Nevertheless, while we may not have identified every gene in *E. coli* that is required for the

maintenance of outer membrane homeostasis we are confident that we genes identified in this study are absolutely required.

Of the genes identified, many of the phenotypes observed are easily explainable based upon previously investigation and known function. For example many of the mutants displaying reduced growth kinetics, increased durations of lag phase or early plateau into stationary phase are associated with cellular metabolism or the biogenesis of key cellular components. Such as *fabH* and *glnA* which encodes a β-ketoacyl-acyl carrier protein (ACP) synthase III, responsible for catalysing the first elongation reaction of type II fatty acid biosynthesis (207, 208) and glutamine synthase, catalysing the only enzymatic reaction in glutamine biosynthesis (209), respectively. The major peptidyl-prolyl isomerase (PPIase) periplasmic chaperone, SurA is involved in the transit of OMP precursors (including OmpA, OmpF and LamB) across the periplasmic space from the SEC transport machinery (IM) to BAM complex (OM) (14, 15). Additionally, *tatC* encodes a subunit of the IM Tat complex, required for the export of folded proteins to the periplasm (27, 29), including the amidases, AmiA and AmiC, required for peptidoglycan NAM cleavage during cell division (28, 32), respectively.

In contrast to other studies of a similar nature, we have characterised the membrane defect further by examining cell leakage and efflux activity in all mutants. Interestingly, there is a correlation between those mutants with 'leaky' phenotypes and those that produce significant vesicles as assessed by SEM analysis. This correlation has been reported previously for some of the mutants, such as for *tolA, tolQ, pal, rfaG, ompA, ompR* and *tatC* (8, 210-213) suggesting that the resultant proteins observed are most likely due to the production of vesicles and not as a direct result of periplasmic leakage. Interestingly, in a number of instances, mutants were identified where proteins were not identified in the culture

supernatant fractions but where vesicles were observed by SEM. This may be due to their size as it has been shown previously that these vesicles can range in size from 10 - 300nm in diameter (214) and larger vesicles may have been excluded during the supernatant filtering step prior to TCA precipitation (214). Alternatively, the vesicles may be present at lower frequencies and fail to contain sufficient protein concentrations to be detected using this technique. Thus, analysis via alternative techniques (including Bradford protein concentration assay) may be more suitable.

The SEM analysis performed to date, both confirm previous literature reports and advances our understanding of the similarities between different species, i.e. in the case of *tatC* and *msbB* mutants have elongated cells, with a clear division defect. This phenotype has been previously described for *E. coli tatC* (27) and similar replication defects have been described for *msbB* mutants in both *S.* Typhimurium and *Shigella flexneri* **(215, 216).**

Despite not offering a definitive list, this study has provided significant insight into the role of nonessential genes in maintaining the cell envelope barrier function, providing new insights as to the possible function of predicted gene products e.g. *yibP*, *yciM* and *plpA*. YibP is a predicted peptidoglycan hydrolase that maybe associated with cell division (217), however, it also has significant homology to the periplasmic RND component, AcrA (65% amino acid) (218). Given our results demonstrate that a *yibP* mutant has a significant defect in efflux it is tempting to speculate the protein encoded by this gene may be associated with efflux. YciM is a predicted heat shock protein, forming part of the σ^{32} regulon (219). It too had significantly reduced efflux however we do not have a plausible hypothesis to explain the defect. Notably, this gene is essential in *S*. Typhimurium (220). Our analysis failed to clarify the mechanism by *plpA* (*yraP)* contributes to membrane barrier function. Therefore, this gene is the focus of investigation in chapter 4.

A real benefit to this investigation was the development of the PI and Box staining assay. In most instances, the results were consistent with those obtained from growth on agar plates although puzzlingly when incubated with SDS a considerable proportion of the population localised to Q4. As described previously, Q4 staining should not be possible and thus poses an interesting question. SDS has been shown to solubilise cytoplasmic membranes (221). It is therefore tempting to speculate that the observed Q4 staining is as a result of 'dead cells' with a solubilised IM, thus removing the binding site for BOX. However, this requires further investigation to confirm this hypothesis, which is not within the scope of this study. Nevertheless, it seems this study could be expanded as a rapid high through-put assay for compounds which inhibit cell viability and membrane stability.

This study aimed to identify nonessential genes, which may form suitable (secondary) drug targets, for potential co-administration with previously ineffective antibiotics. The mutants require significant further investigation, regarding the impact of the gene deletion on pathogenic bacterial strains; do the mutations attenuate virulence, or increase susceptibility to the host immune system? This will be investigated further in chapter 5, by transferring a selection of these mutants to the broad host range pathogen *S.* Typhimurium for assessment in a murine model of infection.

CHAPTER 4

IDENTIFICATION OF A PATHWAY FOR THE TRANSPORT OF PHOSPHATIDYLGLYCEROL OF THE OUTER MEMBRANE

4.1. INTRODUCTION

The OM of Gram negative bacteria is composed of OMPs, LPS, lipoproteins and phospholipids, the first three components are trafficked and inserted into the membrane by means of the BAM complex, Lpt and Lol pathways, respectively (1). However, the mechanisms by which phospholipids are trafficked and inserted into the inner leaflet of the OM remains elusive. The inner leaflet, comprises 70-80% phosphatidylethanolamine (PE), 20-25% phosphatidylglycerol (PG) and 5% cardiolipin (CL) (222).

Biogenesis of phospholipids in *E. coli* occurs via a multi-step process, with the initial conversion of dihydroxyacetone phosphate to sn-glycerol-3-phosphate (G3P) via the G3P dehydrogenase, GpsA (223). This initial reaction creates the phospholipid backbone and is highly conserved in numerous bacterial species. The attachment of the first and second fatty acids generating monacyl-G3P and phosphatidic acid, is performed by PlsB and PlsC, respectively (223). While the CDP-diglyceride synthase (encoded from *cdsA*) converts phosphatidic acid to its activated intermediate CDP-diglyceride (CDP-diacylglycerol), which forms the basis for either PE or PG and CL (223). In the case of PE, phosphatidylserine synthase (CDP-diacylglycerol-L-serine phosphatidyltransferase) PssA and phosphatidylserine decarboxylase (Psd) catalyse the next two reactions. For PG and CL synthesis the *pgsA*-encoded CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase catalyses removal of the phosphate moiety from PG phosphate; CL synthase (*cls*) can then catalyze the condensation of PG to CL and glycerol (223).

The enzymes associated with phospholipid biogenesis are predominantly IM bound or associate with the IM giving rise to the hypothesis that phospholipids are flipped across the IM in a manner analogous to LPS. However, the mechanism by which they're trafficked across the periplasm and inserted into the inner leaflet of the OM is completely unknown. In

contrast, the pathways and proteins associated with maintaining phospholipid asymmetry at the OM are known. Although the biochemical mechanisms by which they function have not been completely resolved, their identification has enhanced our understanding of the maintenance of lipid asymmetry. Cell envelope stress is a common cause of migration of phospholipids into the outer leaflet of the OM. Indeed, exposure to chemicals such as EDTA strips 30-50% of LPS molecules from the surface and chelates magnesium ions creating destabilised regions, which are predicted to allow phospholipid localisation on the outer leaflet (71, 73, 224). In addition, antimicrobial cationic peptides produced by host cells during infection, have been shown to induce a flip-flop movement of phospholipids across the OM, enhancing permeability to these molecules (224). The Mla Pathway, PldA and PagP proteins counteract the accumulation of phospholipids in the outer leaflet but by different mechanisms.

The Mla pathway contains components localised to the OM, periplasm and IM, for the extraction and recycling of phospholipids from the OM to the IM. However, it is currently unknown whether this complex is specific for PE or whether it can recognise PG and CL too as the MlaC crystal structure was determined whilst bound to PE (71). The OM localised MlaA removes mislocalized phospholipids transferring them to the periplasmic MlaC, which in turn delivers the phospholipid to the MlaBDEF complex at the IM, where the fate of the phospholipid is unknown (71). PldA functions in an alternative manner, normally existing as an inactive monomer in the OM. The binding of calcium stimulates dimerisation, enabling PldA to sequester and degrade phospholipids, by catalysing the removal of sn-1 and sn-2 fatty acid chains from the backbone (72). In contrast PagP catalyses the transfer of palmitate from the sn-1 position to lipid A (73). The PagP active site is located on the outer leaflet of the OM for association with lipid A. Though the means by which PagP interacts with phospholipids is unknown, current hypotheses suggest localisation of phospholipids to the outer leaflet of the

OM allows PagP access to its' substrate (224). Interestingly, despite both these proteins functioning to maintain OM lipid asymmetry, only over expression of PldA (and not PagP) can compensate for the loss of the Mla pathway (71).

During this investigation sought to characterise *plpA*, a gene identified during our screen for mutations resulting in membrane defects (Chapter 3). Using a combination of bioinformatics analysis, *in vivo* and *in vitro* assays, we characterised the function of *plpA* and provide a context to its role in maintaining membrane barrier function. Here we provide evidence that PlpA functions as a PG transporter, moving PG across the periplasm to the OM.

4.2. RESULTS

4.2.1. *plpA* encodes a predicted OM lipoprotein with two BON domains

Having previously shown that *plpA* mutants result in membrane barrier defects, we initially set out to determine the type of product encoded by *plpA* and its cellular localisation. Bioinformatic analyses of the sequence indicated *plpA* was under the control of σ^E promoters (Fig. 4.1) and encoded a predicted OM lipoprotein as determined by the presence of a Lol-dependent targeting sequence, lipobox, and an N-terminal cysteine residue at position 19. This allows for attachment to the inner leaflet of the OM when acylated, consistent with other OM lipoproteins (30). The protein contains two separate Bacterial and OsmY Nodulation (BON) domains (Fig. 4.1), predicted to be associated with phospholipid binding, though this function has yet to be confirmed experimentally (225).

4.2.2. PlpA is OM localised, but not surface exposed

As *plpA* is predicted to encode an OM lipoprotein, we sought to confirm its cellular localisation. By immunoblotting cellular fractions using anti-PlpA ABs, PlpA was shown to

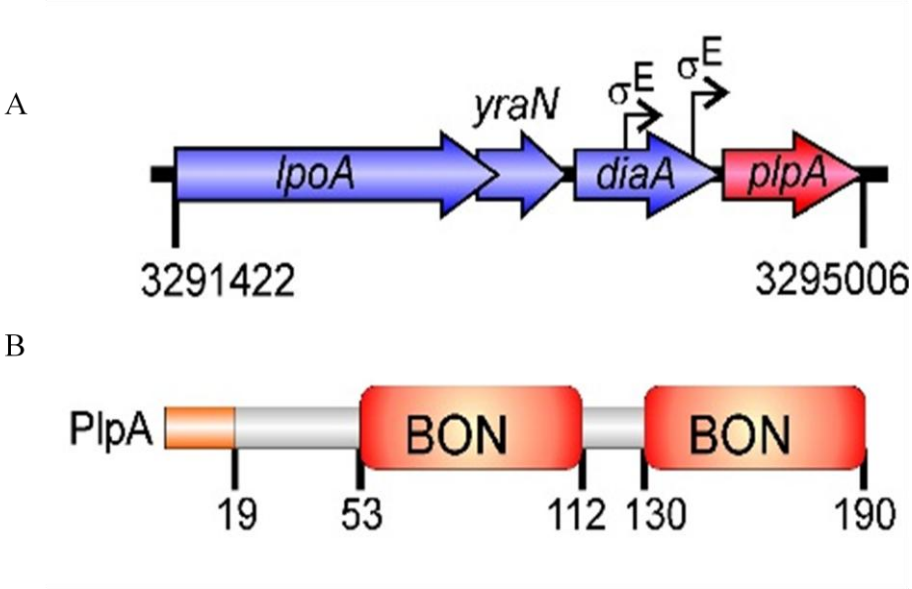


Figure 4.1. Gene location and protein structure of PlpA
A. The genetic organisation of *plpA*, with two upstream promoters under the control of σ^E. **B.** The domain architecture of PlpA, containing Lol dependant signal sequence, N-terminal cysteine residue at position 19 which is acetylated to allow anchoring into the inner leaflet of the OM and two BON domains.

be localised to the OM fraction of the parental *E. coli* K12 strain BW25113 and complemented mutant, but not the *plpA* mutant (Fig. 4.2). Recent investigations have revealed that certain OM localised lipoproteins have the capacity to be surface localised. Therefore, to determine whether PlpA was periplasmically located or surface localised we performed immunofluorescence using the same strain set. PlpA was only detected in the permeabilised parental strain *E. coli* BW25113 or the complemented mutant and not in the permeabilised *plpA* mutant or any of the non-permeabilised samples. These data indicate PlpA is localised in the periplasm. As expected, SurA is detectable in all the permeabilised samples due to its periplasmic localisation.

Furthermore, PlpA should be anchored to the inner leaflet of the OM by the N-terminal acylated cysteine. Therefore, mutation of the cysteine residue to alanine should prevent acylation and recognition by the Lol pathway. Immunoblotting the OM fraction from this strain in parallel to the parent, *plpA* mutant and complemented mutant, indicated the cysteine mutant failed to localise to the OM confirming the role of this N-terminal cysteine in targeting PlpA to the OM.

4.2.3. *plpA* is distributed throughout proteobacteria

Further bioinformatic investigations revealed three BON-domain proteins were encoded on the *E. coli* genome: PlpA, OsmY and YgaU (Fig. 4.3). PlpA and OsmY share a dual BON-domain architecture and sequence similarity (29.5% identity) but OsmY possesses a typical Sec-dependent signal sequence. YgaU is more distinct consisting of one BON and one LysM domain. Clustering analyses of sequences (CLANS) obtained by HMMER searches were used to determine their taxonomic distribution: PlpA and YgaU are distributed throughout the α-, β- and γ–*proteobacteria* whereas OsmY is more widely distributed (Fig.

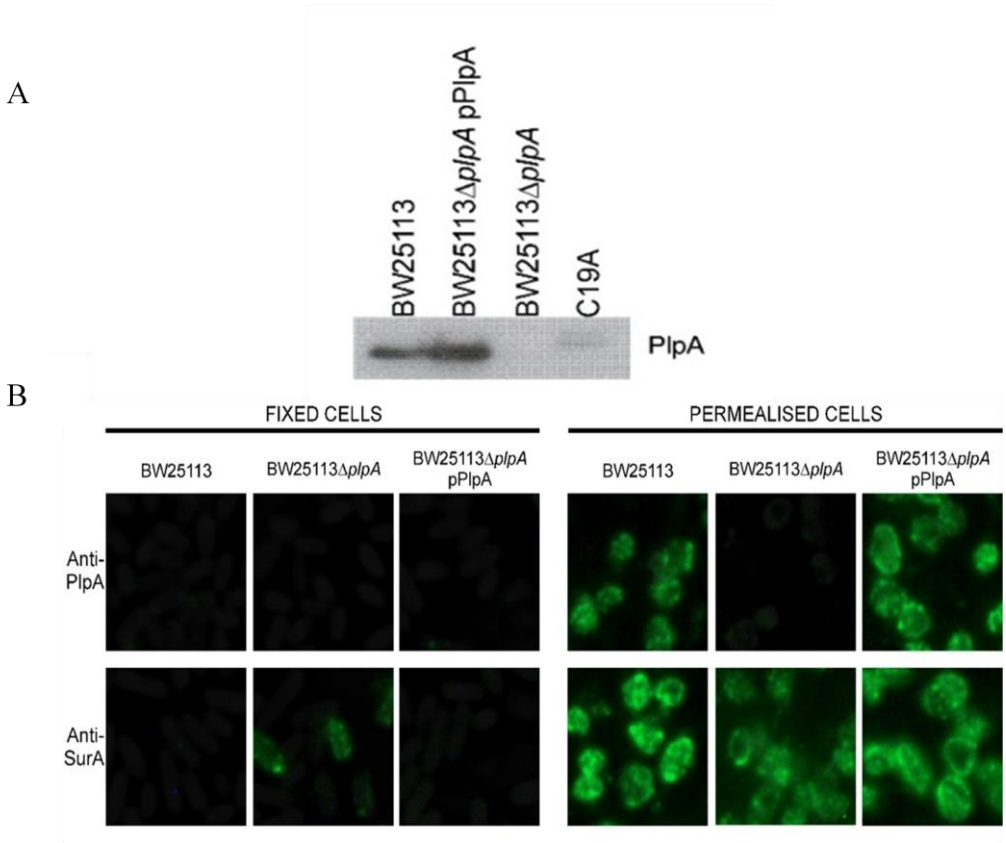


Figure 4.2. PlpA is localised to the inner leaflet of the OM
A. Western blot of OM fractions for parental strain *E. coli* BW25113, BW25113Δ*plpA* complemented with pET20b*plpA,* BW25113Δ*plpA* and BW25113*plpA*$_{C19A}$, probed with α PlpA primary AB and secondary anti-rabbit AB. Indicating PlpA can only be detected in either the parental strain or complement, though is absent in the mutant and not detectable in mutated cysteine version, due to the inability of the protein to anchor to the inner leaflet of the OM. **B.** Immunofluorescence of whole cells and permeabilised cells, stained with either α PlpA (top panel) or α SurA (bottom panel) primary AB, and secondary GAR alexa fluor488 AB. Indicating PlpA is only detectable in permeabilised parental strain, *E. coli* BW25113 and complemented mutant, but not *plpA* mutant. SurA is detectable in all permeabilised samples, but also non permeabilised *plpA* mutant, indicating the membrane defect allows immunoglobulin's access to the periplasmic space.

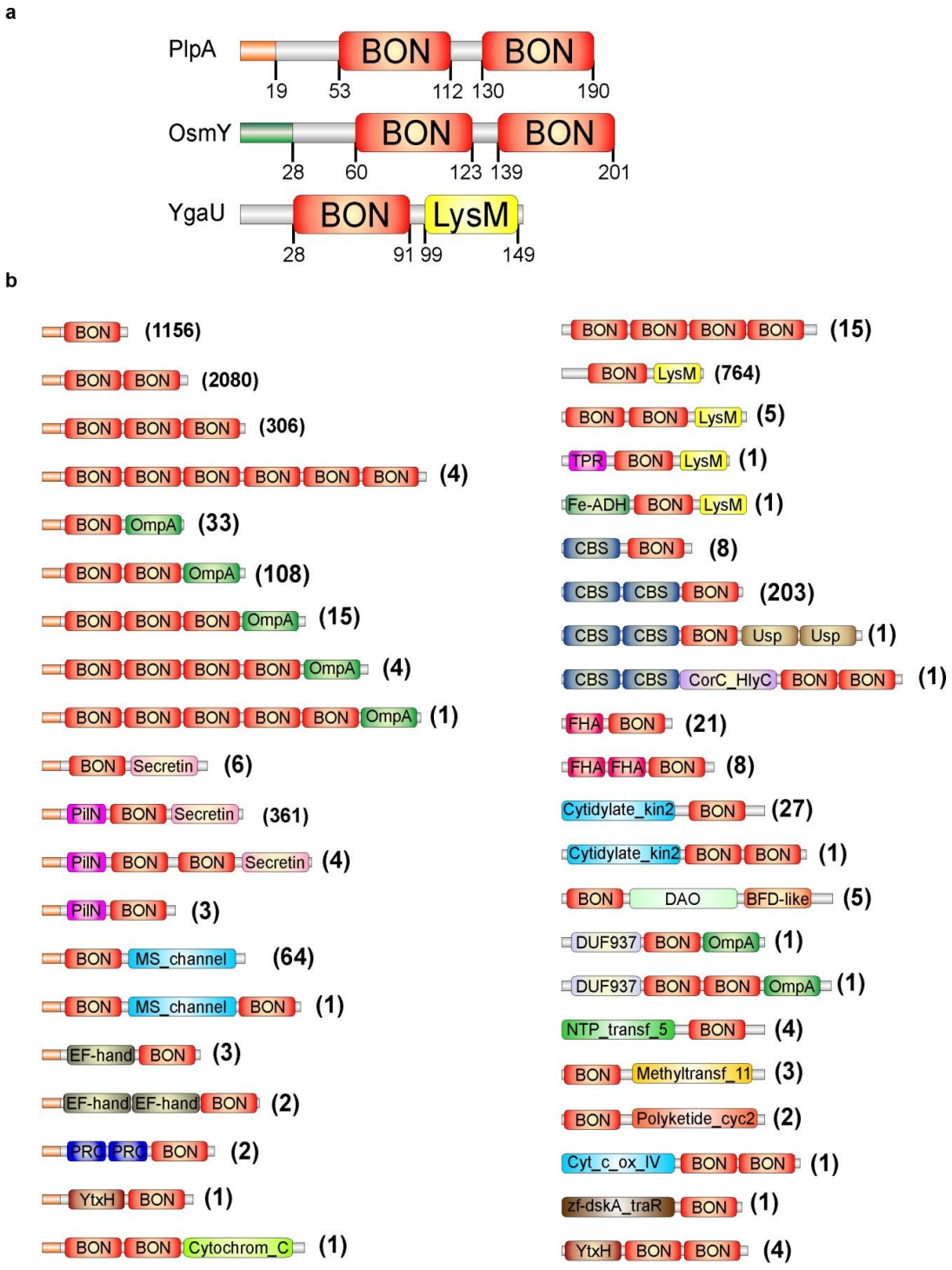


Figure 4.3. BON Domain containing proteins
A. Domain architecture of PlpA, OsmY and YgaU proteins. Highlighting PlpA and OsmY share the most similar protein domain structure, with each protein containing two BON domains, while YgaU is more distinct with one BON domain and C-terminal LysM domain. **B.** Architecture and distribution of BON domain containing proteins, across several bacterial phyla. Indicates BON domains are most commonly observed in seven formats, as depicted by the number of species for which they are observed in, shown in brackets next to each domain type.

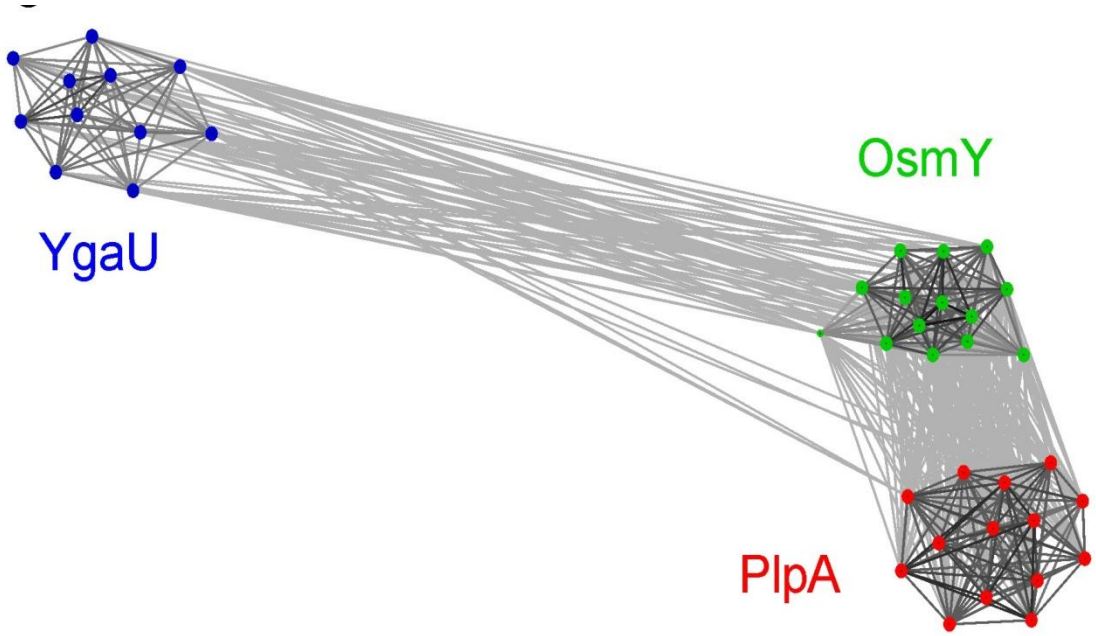


Figure 4.4. Clustering analyses of sequences (CLANS)
CLANS analysis for PlpA, OsmY and YgaU. Indicating all three proteins are evolutionary connected. PlpA and YgaU are distributed throughout the *α*-, *β*- and *γ–proteobacteria* whereas OsmY is more widely distributed.

4.4). The BON domain can be found in several different domain architectures, and our comprehensive analysis recovered seven predominant domains co-occurring with BON in major groups across different bacterial phyla (Fig. 4.3).

4.2.4. PlpA is not required for resistance to changes in osmolarity

Although the precise functions of YgaU and OsmY are unknown, both the respective genes have shown to be induced during hyperosmolarity (226, 227). As OsmY has been predicted to be required for resistance to changes in osmolarity and turgor pressure, and due to the structurally similarity of these proteins to PlpA, we next set to examine whether *plpA*, *osmY* or *ygaU* mutants were susceptible to elevated salt concentrations (Fig. 4.5). In all instances the survival of the mutants was comparable with that of the parental strain, when plated on LBA with increasing concentrations of sodium chloride. Indicating that if these proteins are required for survival during conditions of increased osmolarity, they maybe functionally redundant.

4.2.5. Some PlpA homologues can complement a *plpA* mutant

As *plpA* was shown to be widely distributed across a range of proteobacterial species, we next sought to determine whether these homologues performed similar functions, to *plpA* from *E. coli*. A selection of homologues were either cloned or *de novo* synthesised into the constitutive expression vector, pET17b. These constructs were transformed into the *E. coli* BW25113Δ*plpA*, and resistance to vancomycin (100 or 200 μg/mL) or SDS (4.8%) used as a measure for functional complementation. By initially testing the *E. coli* BW25113 pET17b (empty vector) or BW25113Δ*plpA* pET17b (empty vector) strains we were able to confirm that the presence of the plasmid did not affect the resistance of the parental strain BW25113

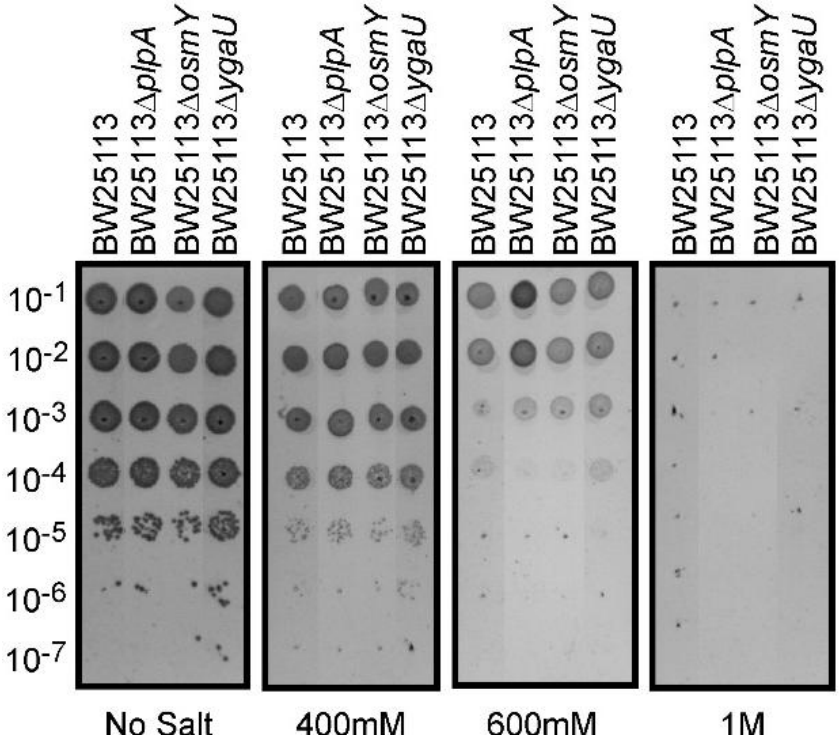


Figure 4.5. Single *plpA*, *osmY* and *ygaU* mutants are not susceptible to elevated sodium chloride concentrations

Comparison of growth on LBA supplemented with varying concentrations of sodium chloride, comparing the parental strain, *E. coli* BW25113, *plpA* mutant, *osmY* mutant and *ygaU* mutant, indicating no single mutant shows elevated sensitivity to NaCl compared to the parental strain, though growth of all strains is inhibited in the presence of 1 M NaCl.

or the susceptibility of the *plpA* mutant to either compound. By comparing the growth of the various strains (when sampled from a consistent inoculum) on LBA supplemented with vancomycin or SDS at the concentrations described above, we were able to show the homologues from *H. influenza, N. meningitidis, P. multocida and S.* Typhimurium complemented the phenotype of a *plpA* mutant completely, restoring resistance to both concentrations of vancomycin and SDS (Fig. 4.6). The *V. cholera* homologue was only capable of partial complementation, restoring resistance to 100 μg/mL vancomycin, but not 200 μg/mL or SDS. While OsmY was unable to complement, as judged by sensitivity to both compounds at all the concentrations tested. Indicating that despite the similar domain architecture of OsmY and PlpA, they're not paralogous.

4.2.6. *S.* Typhimurium *plpA* mutants have elevated sensitivity to vancomycin and SDS

As *E. coli* K12 is an evolved laboratory strain devoid of OM features including O-antigen, we next sought to examine whether a *plpA* mutant demonstrated similar phenotypes in a pathogenically relevant strain. We therefore constructed a *plpA* mutant in the broad host range pathogen *S.* Typhimurium, in both the virulent and attenuated *S.* Typhimurium strains SL1344 and SL3261, respectively. Screening the *plpA* mutants against their respective parental strains, indicated that *S.* Typhimurium *plpA* mutants were also susceptible to vancomycin and SDS as observed previously in *E. coli* (Fig. 4.7)

4.2.7. *S.* Typhimurium *plpA* mutants are mildly attenuated *in vivo*

As OM associated lipoproteins have been shown to be important for bacterial virulence (i.e. deletion of Lpp in *S.* Typhimurium results in attenuated virulence (228)), we choose to investigate whether PlpA contributes to virulence of *S.* Typhimurium. As infection

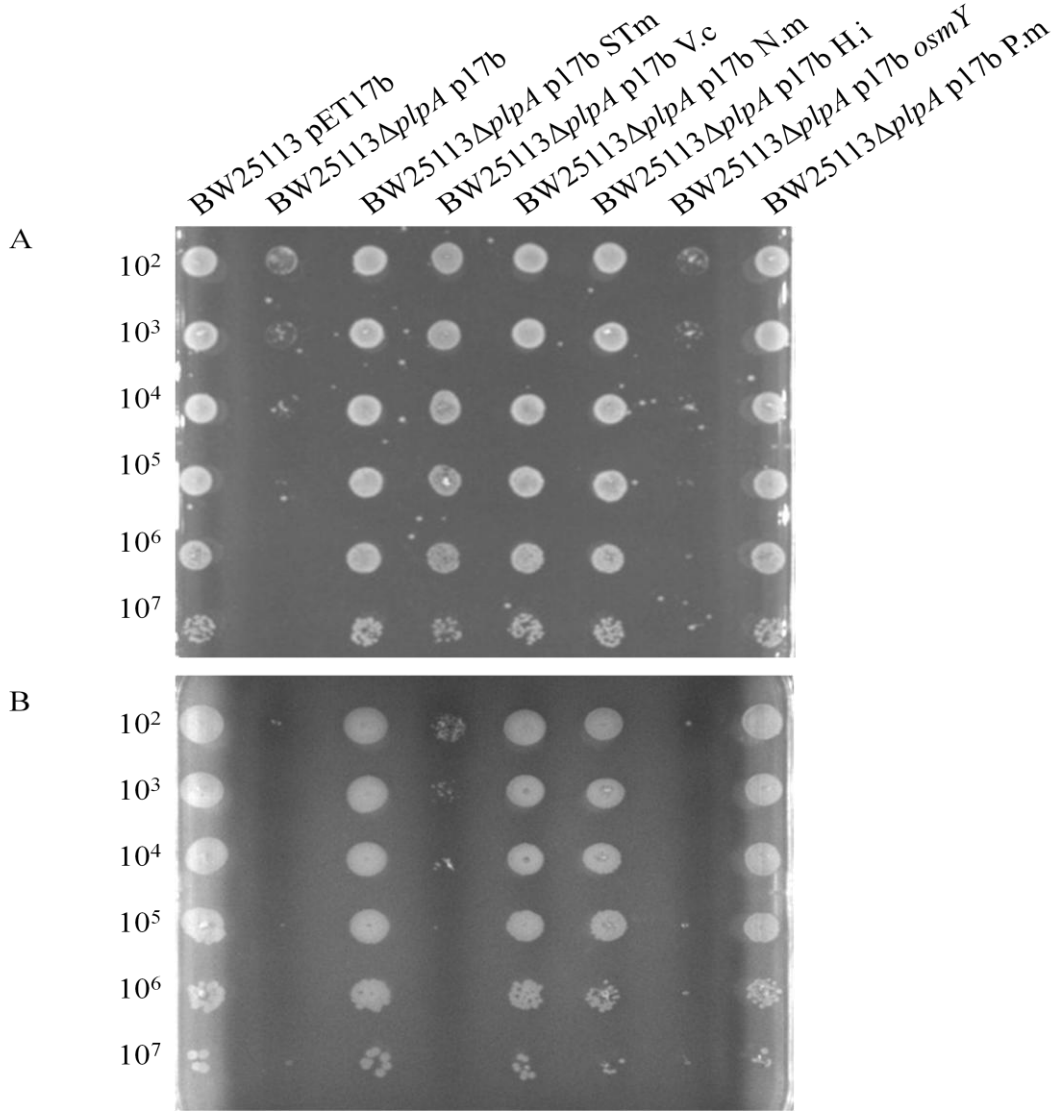


Figure 4.6. Complementation *plpA* mutant with various homologues
Examining sensitivity of *plpA* mutant complemented with *plpA* homologues from various species, STm; *S.* Typhimurium, V.c; *Vibro cholera*, N.m; *Neisseria meningitidis* H.i; *Haemophilus influenzae* P.m; *Pasteurella multocida* in the presence of A. vancomycin 100 μg/mL or B. SDS 4.8%, indicating the *plpA* mutant with empty vector (pET17b) is unable to grow in the presence of either compound, while other homologues complement, with the exception of *Vibro cholera* on SDS. While *osmY* is unable to complement in the presence of either compound.

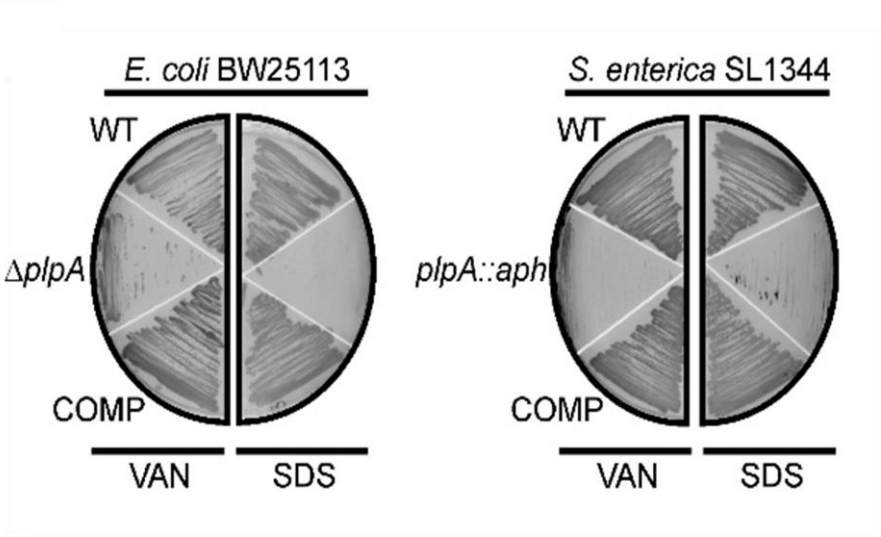


Figure 4.7. Sensitivity of *plpA* mutants in *E. coli* K12 and *S.* Typhimurium
Sensitivity of *plpA* mutants in *E. coli* K12, and *S.* Typhimurium, comparing the parental strains; BW25113 and SL1344, respectively, *plpA* mutants, and complemented mutants, expressing the respective *plpA* gene from pET20b or pQE60, respectively. Grown in the presence of either vancomycin 100 μg/mL or SDS 4.8%. In all instances the *plpA* mutants were shown to be susceptible, but resistance was restored by complementation.

most commonly occurs due to the consumption of contaminated food and water, we therefore choose to examine the effects of *plpA* mutation on the ability to colonise, cause systemic infection when administered via the most physiologically relevant route, oral gavage. Using virulent *S.* Typhimurium strain SL1344 and SL1344 *plpA::aph*, we assessed the ability of the mutant to colonise and infect C57BL6 mice, as determined by bacterial burdens four days post infection, in the spleen, liver, mesenteric lymph nodes (MEL) and Peyers patches (Fig. 4.8). The *plpA* mutant was shown to be mildly attenuated in its ability to colonise sites including the Peyers patches and MEL, though bacterial burdens were more comparable with the parental strain at systemic sites, including the liver and spleen.

4.2.8. *plpA* mutants are susceptible to innate immunity

As the oral infection suggested *plpA* mutants were mildly attenuated at gastrointestinal sites, but not at systemic sites, we questioned whether the observed attenuation was due to the environmental conditions of the gastrointestinal regions (i.e. presence of bile salts) and *plpA* mutants being more susceptible due to the membrane defect. We therefore choose to examine the ability of the *plpA* mutants to cause systemic infection, when administered via the IP route. Using the attenuated *S.* Typhimurium strain SL3261 we evaluated bacteria burdens over time, to investigate whether the slight reductions observed previously (in the spleen and liver) were an artefact of the reduced numbers of bacteria surviving the gastric transit during oral infection. The bacterial burdens were determined at days seven, 18 and 28 (Fig. 4.9). The bacterial counts at day seven indicated a significant attenuation in the recovered numbers of *plpA* mutants in the spleen ($p= 0.004$). However the bacterial burdens obtained at day 18, although still slightly reduced were more comparable with that observed for parental strain, and directly comparable by day 28.

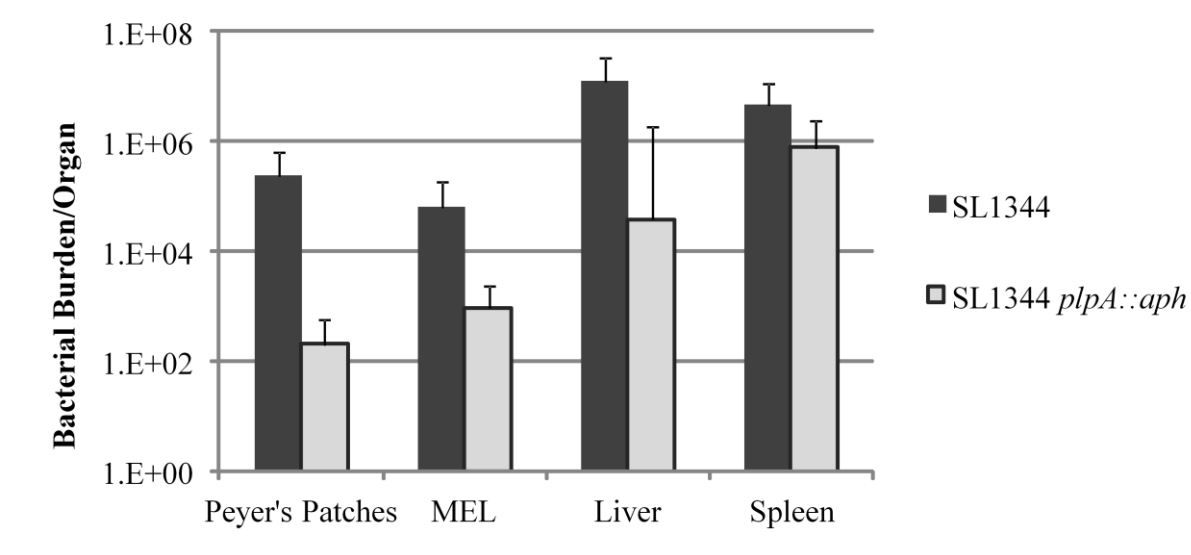


Figure 4.8. Oral Infection with *plpA* mutants
Bacterial burdens four days post oral infection, with virulent *S.* Typhimurium, SL1344 and SL1344 *plpA::aph*. MEL refers to mesenteric lymph nodes. Despite reduced bacterial burdens of *plpA* mutants in the Peyers patches and MEL, no statically significant differences were observed, between the mutant and the parental strain.

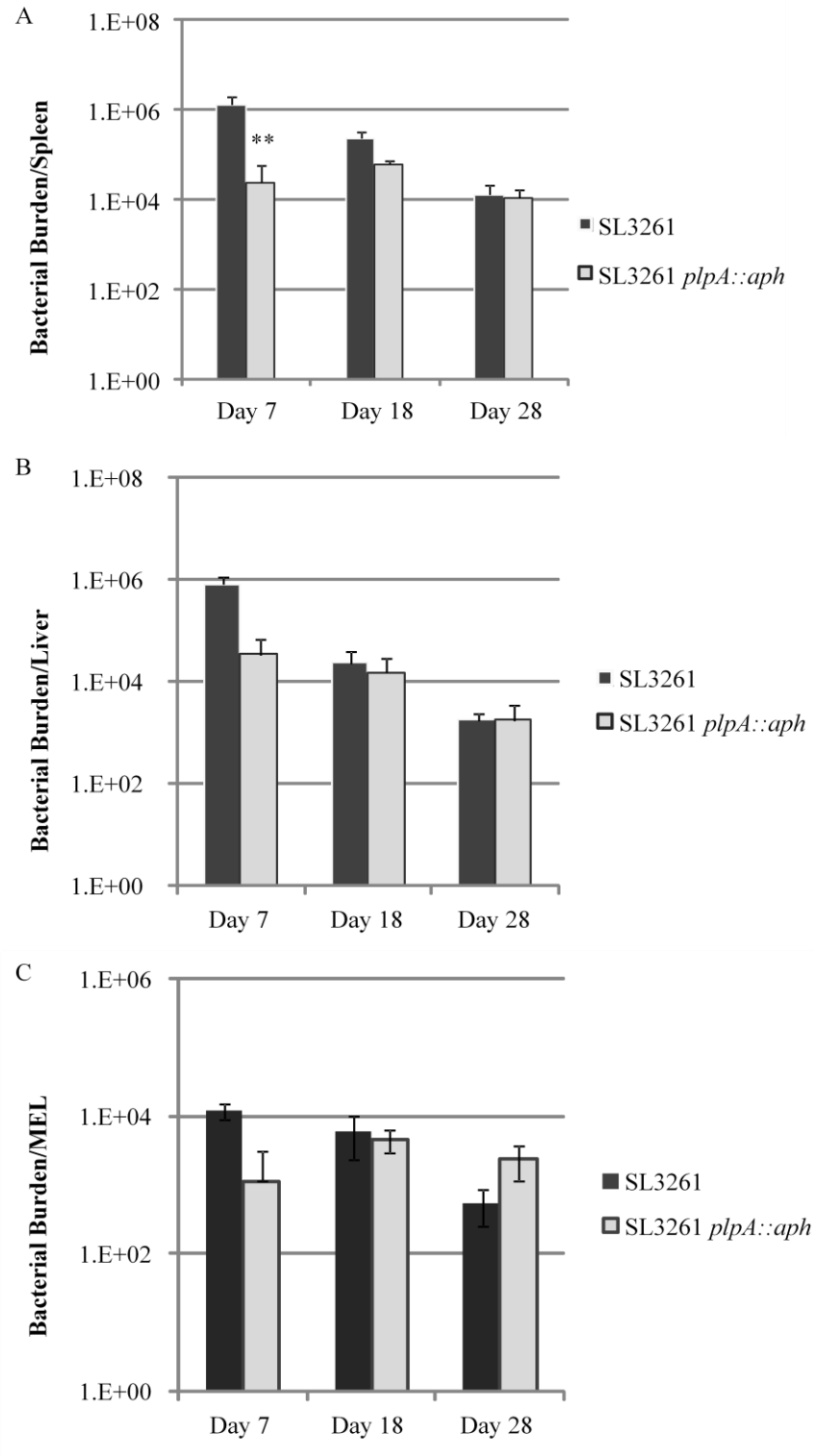


Figure 4.9. IP infection over a time course

IP infection of C57BL6 mice with attenuated *S.* Typhimurium strain SL3261 comparing the parent against the *plpA* mutant, over a time course, examining bacterial burdens in **A.** Spleen, **B.** Liver and **C.** Mesenteric Lymph nodes (MEL) at days seven, 18 and 28. The *plpA* mutants were significantly attenuated in the spleen at day seven, as determined using Mann-Whitney test, where * <0.05, **<0.009

As all groups were administered the same inoculum, we wanted to investigate the cause of this early attenuation. We therefore set to investigate whether this initial attenuation was due to susceptibility to innate immune responses, and not necessarily a defect in the ability of *plpA* mutants to cause infection. To investigate this possibility we performed a 24 h IP infection study using two separate mouse strains lacking the ability to produce specific adaptive immune responses (Fig. 4.10). RagB6 mice do not produce mature T or B cells, while CD30 OX40 mice fail to produce memory mediated AB responses due to the deficiency of CD^{4+} T cells (229, 230). The recovered bacterial burdens from the spleen of RagB6 mice infected with *plpA* mutants indicated a significant reduction ($p= 0.05$). While only a mild reduction is observed in CD30 OX40 mice, suggestive that *plpA* mutants maybe more susceptible to innate immune responses.

4.2.9. *plpA* mutants are slightly more susceptible to complement-mediated killing

To enhance the data obtained from the *in-vivo* studies, we next sought to investigate whether the *plpA* mutation affected recognition and activation of complement-mediated killing. Using healthy human serum we examined resistance of both *S.* Typhimurium SL1344 and SL3261 strain sets (Fig. 4.11). In both instances the *plpA* mutants were slightly more susceptible to complement-mediated killing, compared to their applicable parental strains.

4.2.10. PlpA is not involved in LPS biogenesis

As *plpA* mutants had a heightened sensitivity to innate immune responses *in vivo* and complement-mediated killing in healthy human serum, we next sought to investigate whether PlpA may be involved in LPS biogenesis and thus *plpA* mutants had an altered profile which would have affected resistance in both of these instances. To investigate this hypothesis we

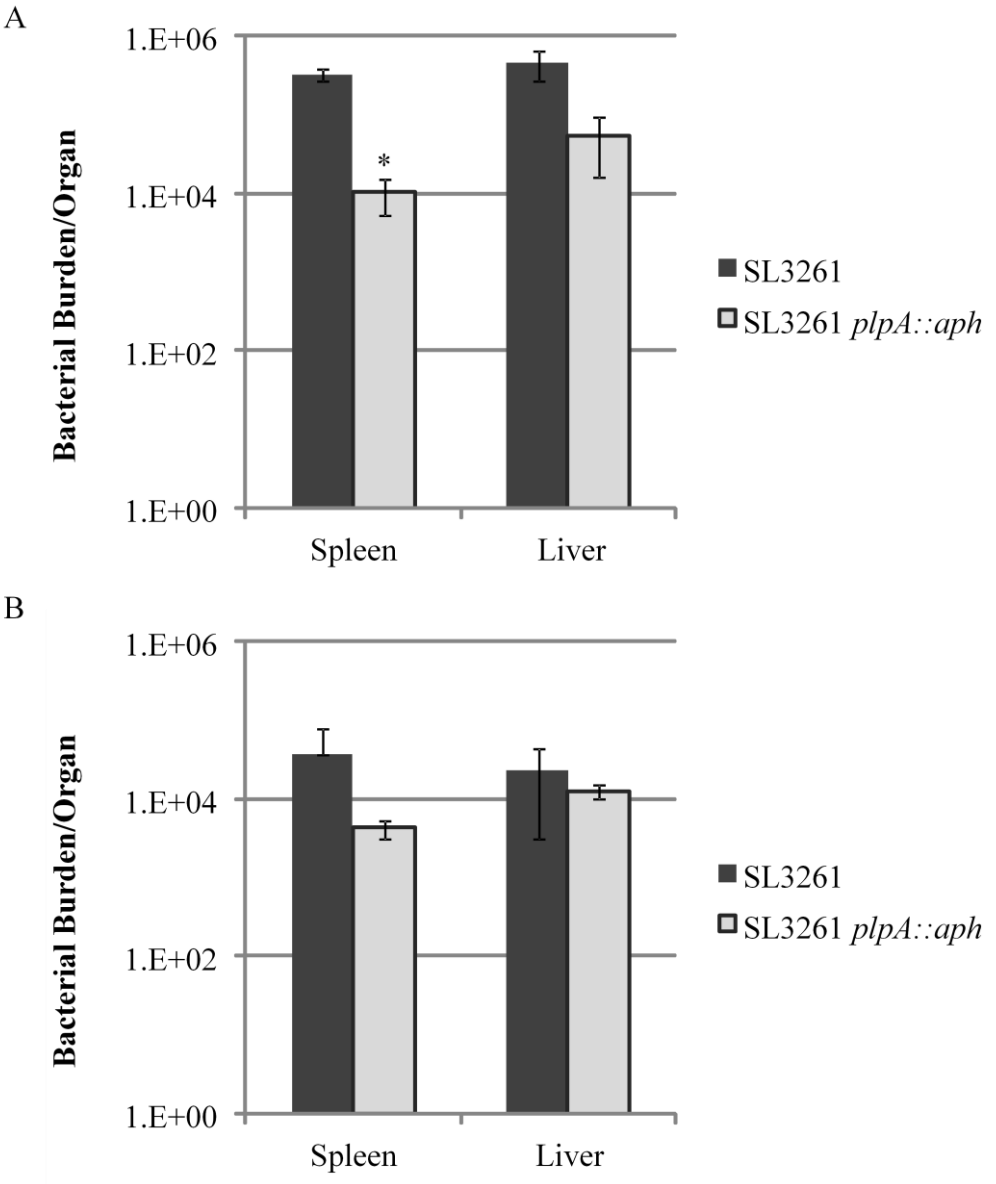


Figure 4.10. IP infection over 24h
IP infection of **A.** RagB6 or **B.** CD30 OX40 mice with attenuated *S.* Typhimurium strain SL3261 comparing the parent against the *plpA* mutant, over a 24 h period, examining bacterial burdens in the spleen and liver. The *plpA* mutants were significantly attenuated in the spleen of RagB6 mice, as determined using Mann-Whitney test, where * <0.05

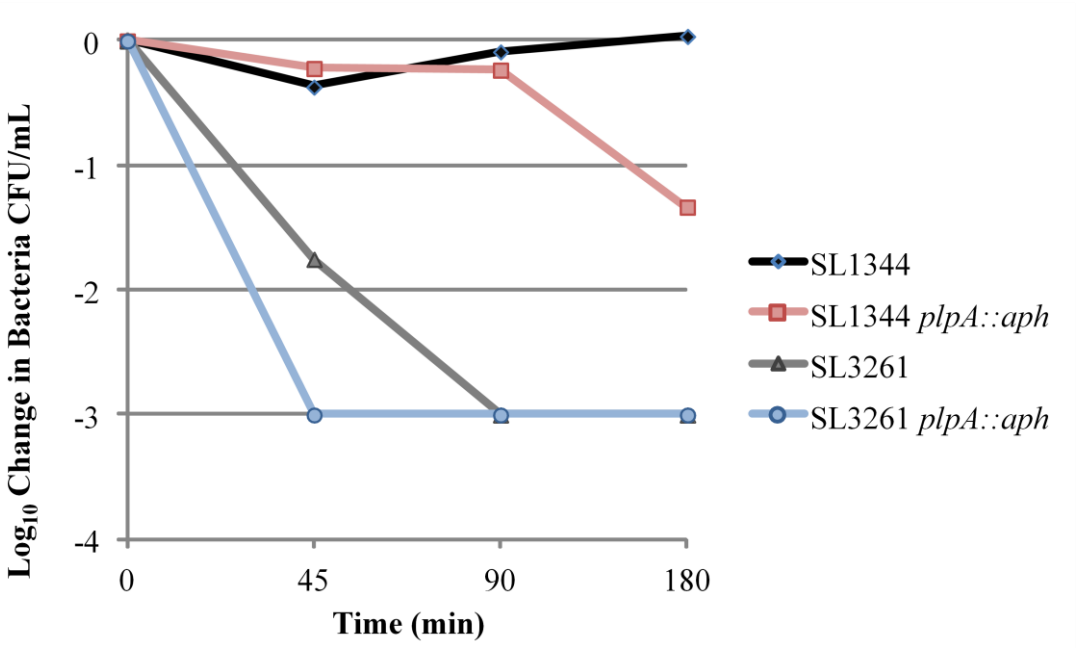


Figure 4.11. Serum resistance of *S.* Typhimurium strains in healthy human serum
Serum resistance of *S.* Typhimurium, virulent SL1344 and attenuated SL3261 strains, comparing survival of the respective parental strain against that of the *plpA* mutant. As expected *S.* Typhimurium, SL3261 is significantly more susceptible to complement-mediated killing, compared to the virulent SL1344. Though both strains demonstrate *plpA* mutants are slightly more susceptible than their respective parental strain

utilised two strain sets, the *S.* Typhimurium parent, *plpA* mutant and complement as described above and *E. coli* K12 strain BW25113 containing *wbbL* on the vector pET20b (as this gene is disrupted by an insertion element, preventing the production of long chain LPS). Examining the LPS profiles for both strain sets showed no differences between the parental strains and *plpA* mutants (Fig. 4.12). Confirming that PlpA is not required for the biogenesis of long chain LPS.

4.2.11. PlpA does not affect the biogenesis of OMP or BAM complex lipoproteins

As PlpA is an OM lipoprotein, we next sought to investigate whether it was required for OMP or lipoprotein biogenesis. As several components of the BAM complex and Lol pathway are OM localised lipoproteins, we initially examined whether disruption of *plpA* resulted in a defect in OMP biosynthesis. We prepared OM fractions and examined these by SDS-PAGE electrophoresis, showing there were no obvious differences in the OM profiles and the presence of key OMPs such as OmpA and OmpF/C (Fig. 4.13). To determine whether *plpA* maybe required for the function of the Lol pathway, we immunoblotted OM fractions for the BAM complex lipoproteins, BamC and BamE. Although a crude method, by comparing the levels of BamC and BamE between the parental strain, *plpA* mutant or complemented mutant, no differences were observed in the levels of any of these proteins suggesting PlpA does not play a role in trafficking of OM lipoproteins. Although an obviously visible defect would be expected if PlpA was involved in OMP or OM lipoprotein biogenesis, as a means to confirm this result further, we performed proteomics assessment of the purified OM fractions, indicating there were no differences in the ratios of various integral OMPs and lipoproteins between the parental strain and *plpA* mutant (Figure 4.2.11.B).

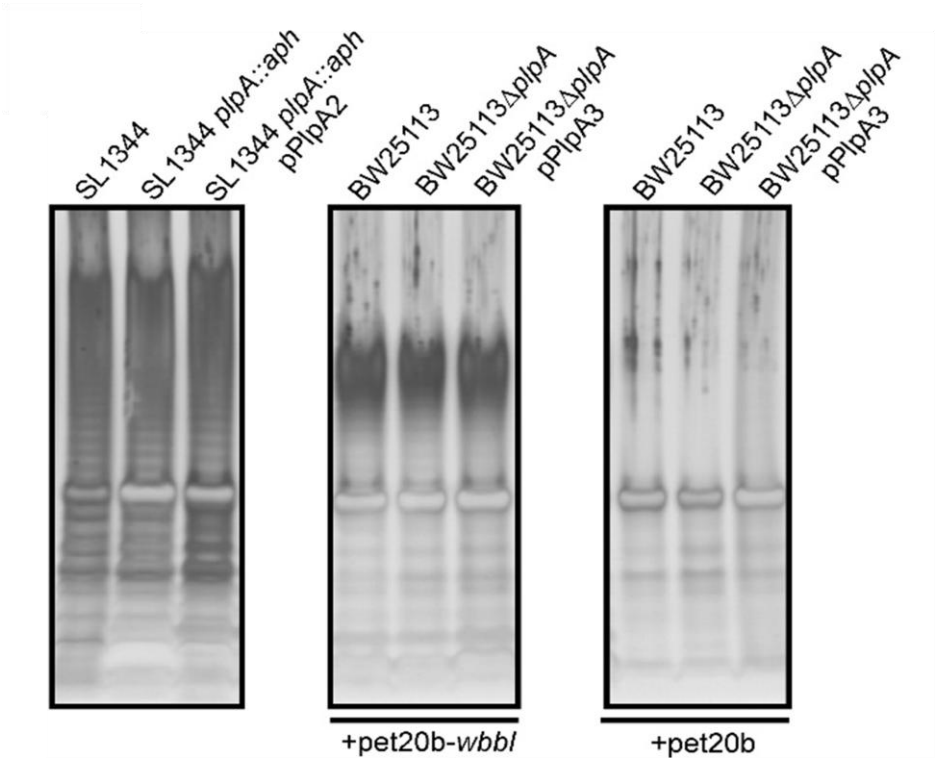
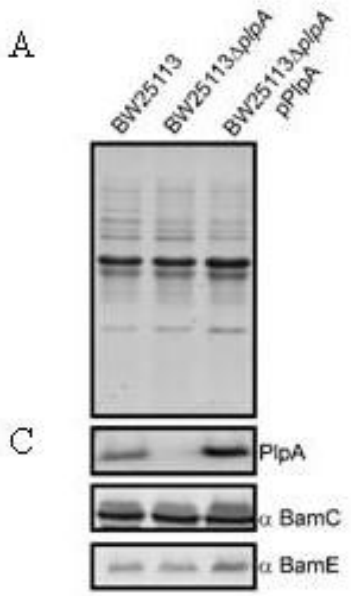


Figure 4.12. LPS profiles *S.* Typhimurium and *E. coli* K12 strains
LPS profiles for *S.* Typhimurium strain SL1344, *E. coli* K12 strain BW25113 expressing *wbbL* from pET20b and *E. coli* K12 BW25113 with no plasmid, for the respective parental strains, *plpA* mutant and complemented mutant (pQE60 for *S.* Typhimurium and pET17b *E. coli*). Indicating *plpA* is not required for long chain LPS and O-antigen production in either *S.* Typhimurium or *E. coli.*



B

Protein	BW25113	Δ*plpA*	Ratio
BamA	128664	130251	1.012
BamB	123736	125213	1.012
BamC	256912	263692	1.026
BamD	65740	67423	1.025
BamE	83748	87284	1.042
LolB	129252	138620	1.072
LptD	68316	74556	1.091
LptE	47932	52732	1.100
OmpA	643124	653032	1.015
OmpC	28692	29392	1.024
OmpW	24088	25846	1.073
TolQ	27084	28500	1.052

Figure 4.13. PlpA does not affect BAM complex lipoproteins or OMP biogenesis
A. SDS-PAGE gel of *E. coli* K12 BW25113, *plpA* mutant and complemented mutant. **B**. Proteomic analysis of OMPs from BW25113 and plpA mutant, indicating deletion of *plpA* does not affect OMP biogenesis. **C.** Western blots using α-PlpA, α-BamC and α-BamE primary ABs, samples ordered as described for **A** indicating no differences are observed in the biogenesis of BAM complex lipoproteins in a *plpA* mutant.

4.2.12. PlpA suppressor mutants against vancomycin have null mutations in *mlaA*

As we had performed several assays attempting to define the role of PlpA with no avail, we decided to utilise a different approach. As *plpA* mutants were susceptible to both vancomycin and SDS, we selected six independent *E. coli* BW25113 *plpA::aph* colonies, and plated these on LBA containing either 4.8% SDS or 200 μg/mL vancomycin. The plates were incubated at 37^{o}C until the appearance of suppressor mutants, which were passaged through liquid media (containing the supplement at the above concentration) and restreaked onto LBA containing the above, to confirm the resultant colonies were truly resistant, prior to stocking. The vancomycin suppressor mutants were resequenced and of the six independent suppressors the results highlighted an array of mutations. However two independent mutants, contained separate frame shifts mutants resulting in the introduction of stop codons in *mlaA*. To confirm this result, we therefore created the double mutant *E. coli* BW25113Δ*plpA, mlaA::aph* and re-examined the sensitivity to vancomycin. The double mutant was shown to restore resistance to vancomycin 200 μg/mL (Fig. 4.14).

As MlaA is known to work in conjunction with MlaB-F, functioning to recycle phospholipids from the OM, we predicted that if the *mlaA* null mutation was disrupting this pathway in order to counteract the *plpA* mutation, thus disruption of any gene associated with Mla pathway should provide the same result. We therefore constructed double mutants of *E. coli* BW25113Δ*plpA with mlaC/mlaD/mlaE/mlaF* and examined the sensitivity of these strains to vancomycin as described above. In all instances the combination of *mla* mutations with a *plpA* deletion was able to restore resistance to vancomycin, but interestingly not SDS. As PldA and PagP have also been shown function as an OM phospholipase and palmitoyl transferase, respectively (71), we also investigated whether disruption of these genes could compensate for the deletion of *plpA*, neither double mutant could restore resistance to SDS,

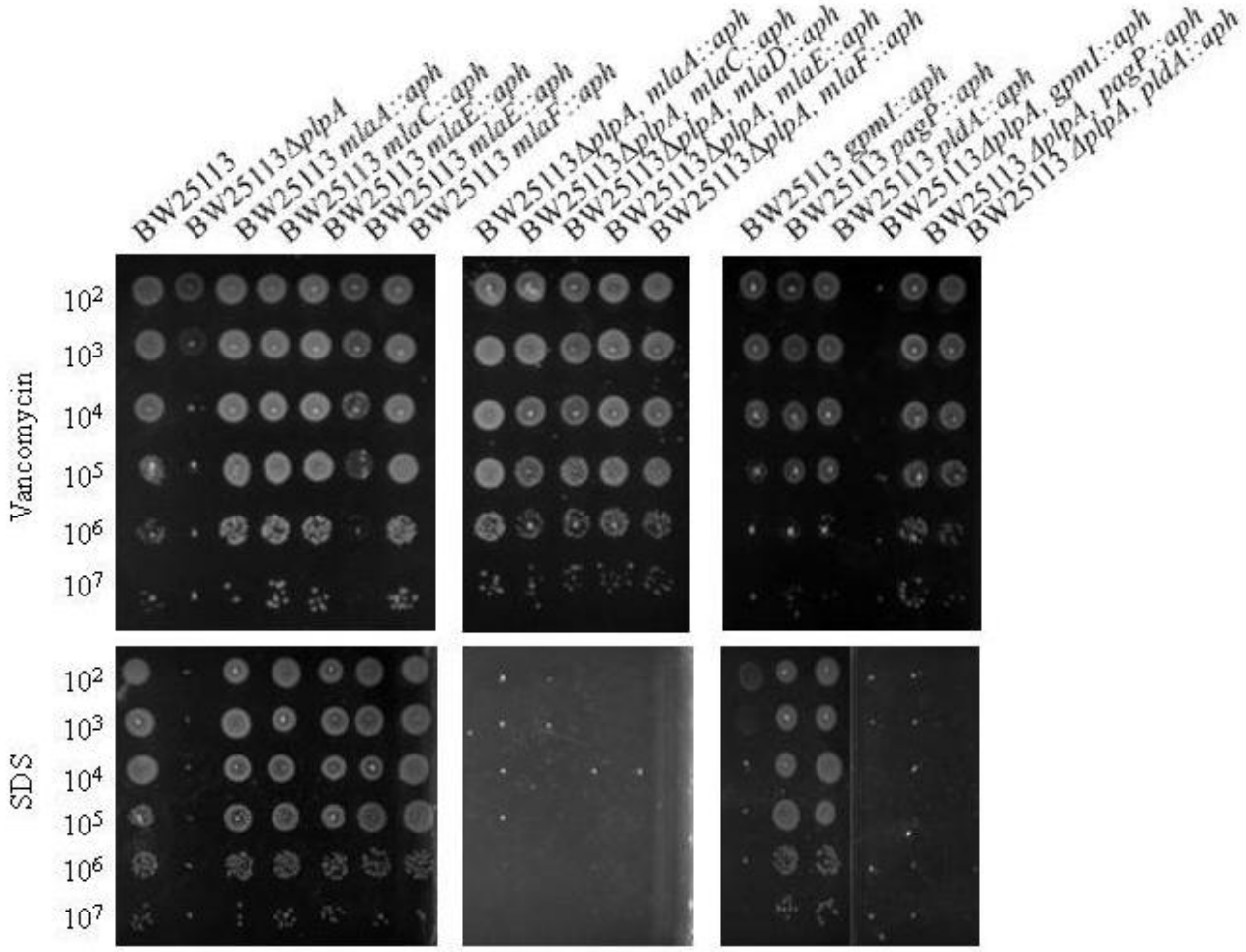


Figure 4.14. Double mutants of *plpA* and *mla, pagP* or *pldA* suppress sensitivity to vancomycin but not SDS

Sensitivity to vancomycin 200 μg/mL (top panel) or SDS 4.8% (bottom panel) of single or double mutants of *plpA* with, *mlaA/C/D/E/F*, *gpmI*, *pagP* or *pldA*. Indicating double mutants of *mlaA/C/D/E/F*, *pagP* or *pldA* can restore resistance to vancomycin, but not SDS, while double mutants with *gpmI* cannot restore resistance to either compound.

however both mutants could restore resistance to vancomycin. We additionally investigated the phosphoglycerate mutase, *gpmI* (231), though in contrast to the other double mutantstested, *gpmI* was unable to complement the phenotype in the presence of either compound. Unfortunately the data for the SDS suppressor mutants was not available at this time.

4.2.13. PlpA is associated with Phosphatidylglycerol insertion into the OM

The suppressor mutants highlighted that by creating null mutations in pathways and genes responsible for recycling or degrading OM phospholipids resistance to vancomycin could be restored. This raised the hypothesis that PlpA was responsible for inserting phospholipids into the membrane to balance the rates of recycling and removal. Thus in the absence of PlpA, the Mla pathway may not be counter balanced, resulting in the removal of excessive phospholipids. Therefore to investigate this hypothesis we performed lipidomics assessments of the OM of the parental strain, *E. coli* BW25113 and the *plpA* mutant. In collaboration with Jennifer Kirwan, we were able to determine the relative composition of various phospholipids in the OM of both strains (Fig. 4.15), indicating the *plpA* mutants lack PG and CL from the OM, but PE is still present, suggestive that PlpA is associated with OM trafficking of the phospholipids PG and CL.

4.2.14. Residues critical for PlpA function

In parallel to our investigations characterising the function of PlpA, our collaborators Prof. M. Overduin, Dr. T.J. Knowles and Dr. R. Maderbocus determined the NMR structure for PlpA (Fig. 4.16). The structural assessments were complemented by HSQC analysis using PG, indicating phospholipid binding was localised to residues on one face of the third α-helix.

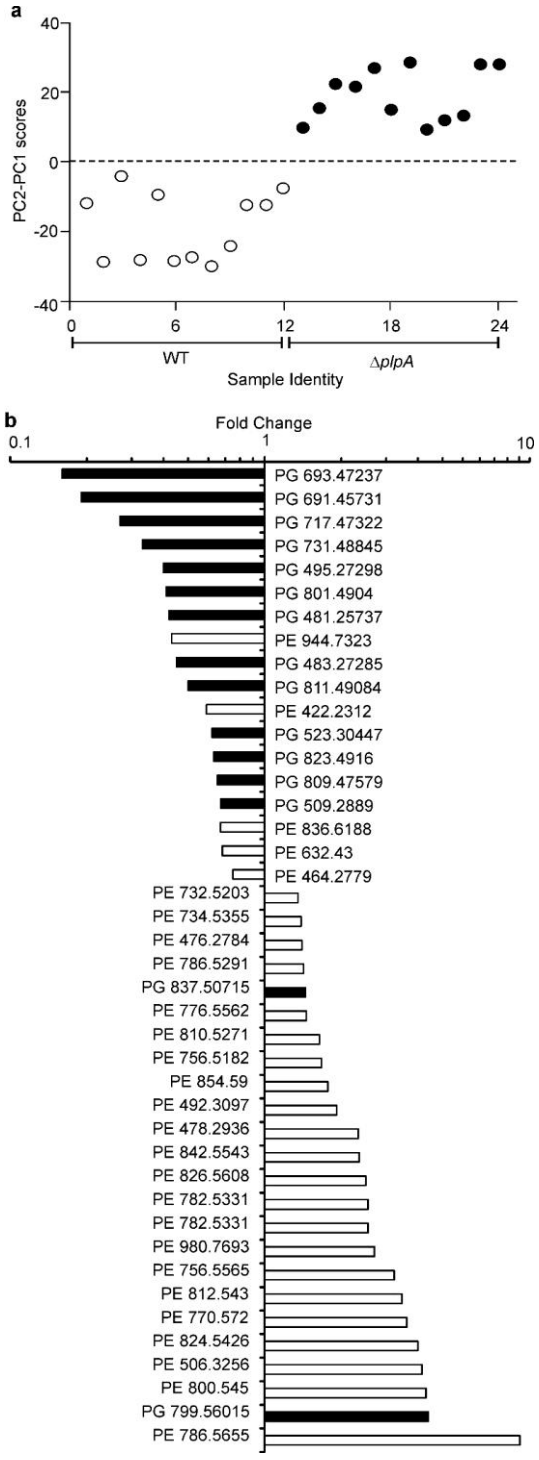


Figure 4.15. *PlpA* mutants lack PG in the OM, but have increased levels of PE
A. Principle component analysis of PG and PE as determined by lipidomics analysis of the OM of *E. coli* K12 parental strain BW25113 and *plpA* mutant, from two separate experiments. Indicating *plpA* mutants contain an OM with significantly altered lipid content, compared to that of the parental strain. **B**. Fold change of various lipid species in the OM, of the parental strain and *plpA* mutant, calculated from the principle component analysis shown in A. Indicating *plpA* mutants have significantly reduced quantities of PG, and elevated levels of PE, compared to the parental strain BW25113.

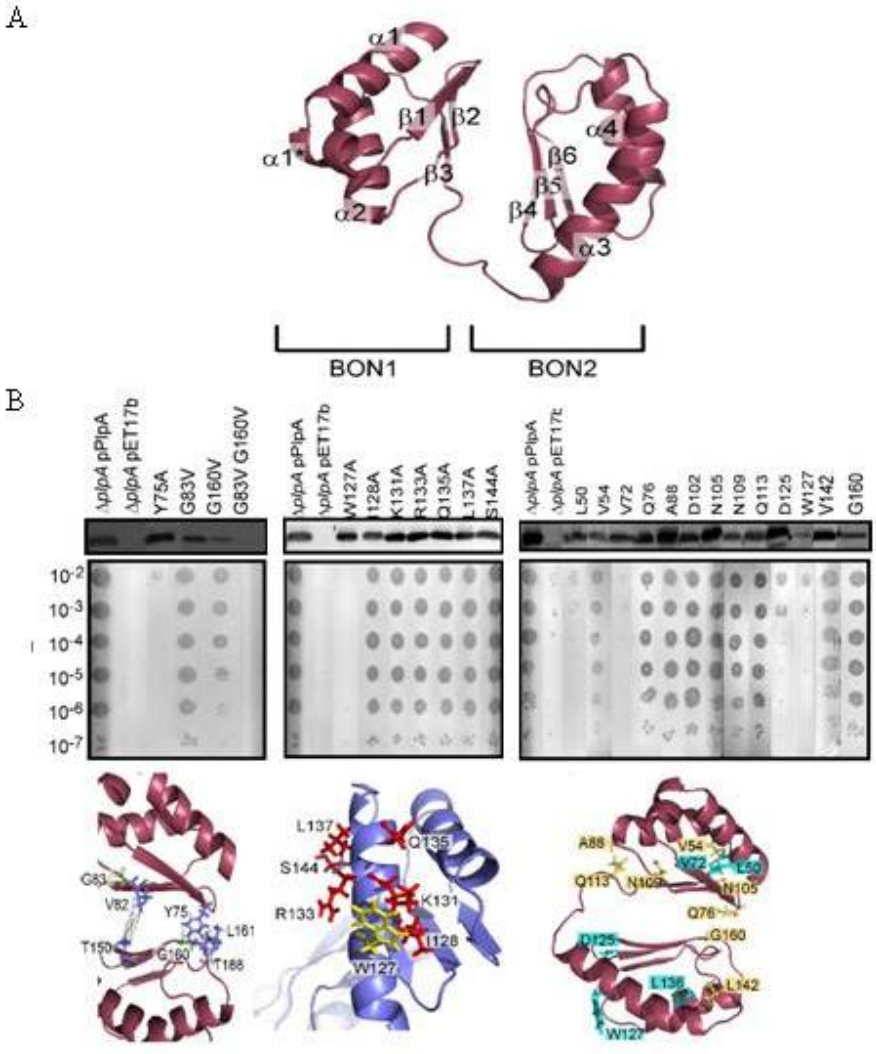


Figure 4.16. NMR structure of PlpA and residues critical to protein stability and function

A. NMR structure of PlpA, as determined by Dr. T.J. Knowles and Dr. R. Maderbocus, indicating PlpA is composed of two BON domains, each containing two α helices and three β sheets. **B**. Site directed mutagenesis and random linker insertion screen (affected residues listed above the relevant lane), examining the requirement for various residues to maintain protein stability, as determined by western blot of whole cell using α-PlpA ABs, and the ability of mutated proteins to complement the Δ*plpA* phenotype, in by examining growth in the presence of vancomycin 100 μg/mL. Below each plate structural representations of the positions of affected residues. Panel 1 refers to site directed mutants of the linker region connecting BON1 and BON2, panel 2 refers to site direct mutagenesis of the residues located within α3 (binding site for PG) and panel 3 refers to random linker insertion mutants distributed across both BON1 and BON2 domains.

To further confirm this analysis and determine which of the residues highlighted were critical for protein stability and function, we utilised two separate techniques, generating a series of site direct mutations and a random linker insertion screen, generating mutations described in table 2.4, in an effort to both confirm the earlier HSQC analysis, but also investigate whether any additional residues were of critical for stability and function. The presence of stable protein was assessed by immunoblotting whole cell lysate of each mutated versions, expressed in a *plpA* deletion background. Secondly we examined the ability of each mutant to grow in the presence of vancomycin or SDS, as a means of determining functionality. The NMR structure indicated that residues located at Y75 and V82 in BON1 and T150, G160, L161 and T188 in BON2 were required for the interaction between the two BON domains. Site directed mutagenesis, of Y75 highlighted the importance of this residue, whereby stable protein can be detected, though this mutation is unable to complement (Fig. 4.16). Additionally mutation of either conserved glycine residue (G83V or G160V, which are positioned at the terminus of the central β-strand in each BON domain) did not abolish function; however combination of these mutations, resulted in a loss of protein, suggesting these alterations resulted in a structurally unstable protein, that's rapidly degraded.

A series of random linker insertions were isolated with eight insertions in BON1 and six in BON2. Of the two BON1 insertions, L50 and V72 failed to complement the Δ*plpA* defect, though the remainder were well tolerated. Of the five insertions in BON2, three were well tolerated (positions L142, G160 and A170). While the remaining insertions (positions D125 and W127), failed to complement despite producing a stable protein. With both of these insertions localised to α3, reaffirming the importance of α3 in PlpA function.

4.3. DISCUSSION

During this study we have characterised the function of PlpA from *E. coli* K12 and *S.* Typhimurium. Based on the evidence presented as part of this study we hypothesis PlpA is a required for the trafficking and insertion of PG and CL molecules into the inner leaflet of the OM, as shown by the model in Fig. 4.17. In brief, PlpA binds PG and CL molecules by α3 of BON2. Consistent with other OM lipoproteins; PlpA (with bound PG or CL) is collected by the IM LolCDE complex – 1, which becomes rearranged for the transfer of PlpA to LolA – 2, which delivers PlpA and its cargo to the OM by LolB - 3, PlpA is inserted into the leaflet of the OM by LolB – 4, whereby PlpA delivers its phospholipid cargo by an unknown mechanism. This pathway works in synergy with the Mla pathway whereby when MlaA detects the presence of phospholipids in the outer leaflet - 5, these are removed and transferred to MlaC – 6, for traffic back through the periplasm, before being returned to the IM MlaBDEF complex – 7, to maintain the asymmetric structure.

Consistent with this hypothesis, PlpA is not surface exposed and instead anchored to the inner leaflet of the OM by N-terminal acetylated cysteine residue, as observed for several other OM lipoproteins, but in contrast to that of OM BamC and Lpp lipoproteins (7, 30, 65). Thus PlpA is optimally positioned for insertion of phospholipids into the inner leaflet.

Additionally the indication that both *mla* pathway mutants, *pagP* and *pldA* mutants can counteract the phenotype of a *plpA* mutant, further support the theory that PlpA adds phospholipids to the OM, to balance the rate of phospholipid recycling by Mla, PagP and PldA. We therefore suggest in the absence of *plpA*, unregulated recycling of phospholipids leads to excessive quantities being removed from the OM, creating ‘gaps’ or highly unstable regions, resulting in the serve membrane defect observed.

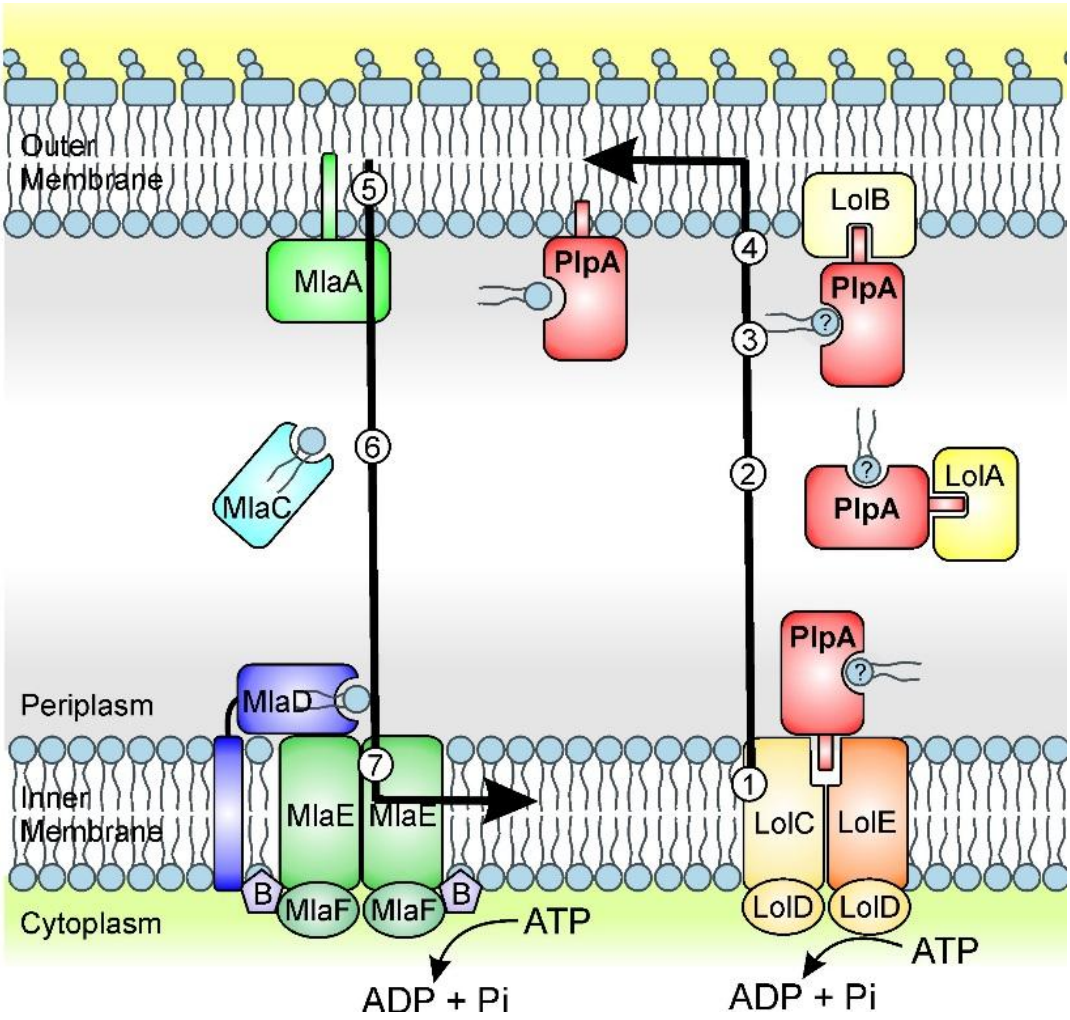


Figure 4.17. Predicted model of PlpA function
Predicted model of PlpA transport of PG and CL to the OM in a multistep process. PlpA is trafficked to the OM via the Lol pathway, with potentially bound phospholipid. 1. PlpA is collected by the IM LolCDE complex, which becomes rearranged, 2. PlpA is transferred to LolA, transporting PlpA and it cargo to the OM, 3. PlpA is transferred from LolA to LolB, 4. PlpA is inserted into the leaflet of the OM by LolB, whereby PlpA delivers its phospholipid cargo by an unknown mechanism. This pathway works in synergy with the Mla pathway whereby, 5. MlaA detects the presence of phospholipids in the outer leaflet, which are removed and transferred to MlaC, 6. MlaC traffics phospholipids back through the periplasm, 7. To the IM MlaBDEF complex, where the phospholipid is recycled, to an unknown fate, ensuring membrane asymmetry is maintained at all times.

PagP and PldA are known to be regulated by PhoP/Q and the presence of calcium, respectively (71). Though the means by which the Mla pathway is regulated has yet to be determined. Answers to these questions may further elucidate the synergy the between PlpA and Mla functions. While the crystal structure of MlaC was solved bound to PE, the substrate specificity of Mla pathway (to our knowledge) has not been investigated and determined exclusively. Raising the question of the phospholipid composition of the membrane of a *plpA* double mutant with *mlaA/C/D/E/F, pagP* or *pldA*, which is capable of restoring resistance to vancomycin. For example if the PlpA and Mla pathways are shown to share similar substrate specificity, is the emphasis on the maintaining specific quantities of phospholipids in the membrane or maintaining the balance of phospholipid insertion/recycling from the membrane. In order to investigate hypothesis further we are planning lipidomics analysis examining the lipid content of *plpA, mlaA* double mutants to compare with the parental strain, *E. coli* BW25113, in order to determine how the double mutation affects the relative concentrations of various phospholipid types.

We have conclusively shown PlpA only binds PG and CL via α3 on BON2, and although we failed to show a role for BON1 in phospholipid binding, we have confirmed the importance of residues within this domain and the connecting β strands required for the interaction of BON1 and BON2. Mutation of residues outside of BON2 (Y75 or both conserved glycine residues simultaneously), significantly abrogates the function and stability of the proteins, respectively. Furthermore using a random linker insertion screen, we highlighted other residues also critical for function (i.e. positions L50 and V72 in BON1) in addition to confirming the role residues D125 and W127 (located in the helix of BON2 associated with phospholipid binding) in *plpA* function.

Despite significantly enhancing our understating of phospholipid transport *plpA* has highlighted the continuing dilemma that an alternative pathway for the transport of PE to the OM must also exist, as PlpA is only capable of interactions with PG and CL, as shown by HSQC analysis. Previously BON domains have been hypothesised to bind phospholipids (225), however this is the first reported experimental evidence confirming this role. This may suggest potential roles for other BON domain containing proteins, such as OsmY and YgaU, which require extensive further investigation. As although both OsmY and YgaU have been previously associated with hyperosmolarity, consistent with data reported by others (201), we were unable to identify a defect for either single mutant, in the presence of increasing concentrations of sodium chloride. While we observed no difference in their growth compared to that of the parental strain, *E. coli* BW25113, Nichols *et al* reported that *ygaU* mutants were fitter than the parental strain when grown in the presence of increasing concentrations of sodium chloride (201).

Although we have confirmed that PlpA is not a paralogue of OsmY, this does not eliminate the possibility that OsmY may be associated with other forms of phospholipid transport, such as that for PE. As the data presented as part of this study indicates that cells lacking *plpA* contain a membrane composed of PE only, therefore it's possible that OsmY may at least have a minor role in PE transport.

However, we have shown *plpA* is conserved across a number of species and clearly performs a similar function, due to the ability of homologues to complement a *plpA* deletion. Though further investigation into the substrate specificity of other *plpA* homologues will provide a more in-depth view of phospholipid transport, i.e. are these homologues restricted to PG and CL binding or are they also capable of PE transport, particularly in the case of the *V. cholera* homologue, which was only able to partially compensate a *plpA* deletion.

Consistent with the idea that *plpA* homologues present in other species performs similar roles, we were able to demonstrate that *S.* Typhimurium *plpA* mutants demonstrated a similar phenotype to that observed in *E. coli* K12. Further to this we have shown that loss of *plpA* results in mild attenuation of *S.* Typhimurium due to elevated sensitivity to innate immune mechanisms. Although our investigations to date have not confirmed the exact mechanism responsible, we have shown that this sensitivity is independent of T cell and B cells, through the use of RagB6 and CD30 OX40 mice, which are deficient for the production of mature T and B cells, and memory AB responses due to the deficiency of CD^{4+} T cell responses, respectively (229, 230), while *plpA* mutants are increasingly attenuated in colonisation of gastrointestinal sites after oral infection and significantly more susceptible to innate immunity when administered systemically. It is therefore tempting to speculate this sensitivity maybe due to antimicrobial peptides released during the onset of infection. Further to this we have shown these innate immune responses would likely be enhanced by complement-mediated killing in serum. As mice do not have an effective complement system (161), it's likely that the effect observed during the animal studies was as a result of other means. Though if PlpA were to be pursued as a potential drug target, additional investigations regarding the impact of the deletion on adaptive immune responses would be required to confirm, that the mutation does not alter AB responses against the bacteria and that later stage bacterial burdens, beyond day 28 were not more persistent than that of the parent.

Our investigations have confirmed that *plpA* is not required for the biogenesis of other OM components, such as LPS, OMP or lipoprotein, all of which are associated with aspects of bacterial virulence. Mutations resulting in rough LPS structure have been previously associated with increased bacterial susceptibility during murine infection, while over production of long chain LPS and O-antigen has been linked to increased resistance to

complement-mediated killing (168, 216). Incontrast, mutant strains defective for the production of particular OMPs and lipoproteins, such as OmpA and Lpp, have been shown to be attenuated in pathogenic *E. coli* and *S.* Typhimurium, respectively (228, 232, 233) further confirming that the attenuation observed is not as a result of defects in the biosynthesis of other cellular components.

This study is the first reported pathway for the transport and insertion of PG and CL phospholipids into the inner leaflet of the bacterial OM. We have additionally presented the first experimental evidence for the phospholipid binding by BON domains, in addition to the first NMR structure for a duel BON domain protein. Although this study highlights the requirement for further investigation to determine the means by which PE is inserted into the membrane. The results reported as part of this study have significantly advanced our understanding of bacterial membrane biogenesis.

Having extensively characterised *plpA* mutation and determined its role in membrane homeostasis in both *E. coli* K12 and *S.* Typhimurium, the following chapter will focus on characterising a subset of other mutations also identified in chapter 3.

CHAPTER 5

ROLE OF NONESSENTIAL GENES IN MAINTENANCE OF S. TYPHIMURIUM OUTER MEMBRANE BARRIER FUNCTION AND PATHOGENESIS

5.1. INTRODUCTION

As described previously, the OM excludes potential toxic and bactericidal compounds, whilst still allowing for the secretion and assembly of surface structures and uptake of nutrients that enable bacteria to colonise diverse and difficult environments (1, 2). In aiding bacterial survival, the selective nature of the OM also hinders antibacterial treatment by limiting the entry of antimicrobials (191, 234).

Our previous investigations on non-essential genes required for the maintenance of membrane homeostasis were performed in the evolved laboratory strain *E. coli* K12 BW25113. *E. coli* K12 has been significantly manipulated over time, subjected to mutagenizing agents including; ionizing radiation and UV light, which has resulted in the numerous genetic alterations, including the inability to synthesis OM structures, such as O-antigen (178). Therefore, we chose to further investigate the roles of some non-essential genes in a more physiologically relevant viz. *S.* Typhimurium. As discussed previously, *S.* Typhimurium, causes infection in a variety of hosts, including humans, mice, cattle, pigs and birds (148), most commonly as a result ingestion of contaminated food and water. During the course of infection *S.* Typhimurium faces an array of challenging environments during transit through the gastrointestinal tract, before transversing specialised epithelial to reside intracellularly in macrophages and DCs (155).

In a number of instances the nonessential genes described in chapter 3 have been previously characterised in pathogenic organisms, including *S.* Typhimurium. For example, genes associated with LPS modifications (*rfa*, *msbB* and *htrB*) have been shown upon deletion to significantly attenuate the ability of bacteria to cause infection in mice (216, 234-236). Transcriptional regulators, Hfq and OmpR are both required for virulence, through the regulation of small non-coding RNAs and a number of virulence genes, respectively (237,

238). Other OM features such as OmpA have been shown to be critical for virulence in pathogenic *E. coli* K1 and UPEC strains, where it promotes persistence in the bladder and has been thoroughly investigated as potential vaccine candidates (232, 233, 239). Efflux pumps and their individual components, including AcrA, AcrB and TolC have been extensively studied by others (206, 234, 240, 241).

The nonessential components of the BAM complex have been extensively studied in *E. coli* and shown not to be required for AT biogenesis, but are responsible for maintaining the stability of the complex (BamCE stabilise the interaction between BamAD) and the insertion of a number of other OMPs, whereby *bamB* single mutants and *bamC bamE* double mutants have reduced OMP profiles in the OM, including reductions in OmpA levels (67, 100). Investigations in *Salmonella* have characterised *bamB* and *bamE* mutants, from *S.* Enteritidis and *S.* Typhimurium, respectively (242, 243). In both cases the mutants were shown to have elevated sensitivity to a range of compounds, including the antibiotics such as rifampicin and vancomycin (*bamB* only) and SDS-EDTA (*bamE* only) (242, 243). Both mutations cause attenuation in virulence when administered orally, though *bamB* mutants are also less capable of causing lethal infection, whereby more than 50% of the cohort survive beyond 20 days, in comparison to the parental strain, which causes lethality in 10 days (242). Similarly, *bamE* mutants are incapable of competing with wild type strains *in vivo,* with a competitive index of between 0.08-0.12 when administered at equal inoculums, via the IP route (243).

The periplasmic chaperones SurA, Skp, DegP and FkpA have all been associated with transit of OMP and AT precursors across the periplasm to the BAM complex, preventing their aggregation during transit and promoting their degradation, upon either accumulation or misfolding (19, 22, 244, 245). Deletion of these chaperones has been investigated in a number

of organisms, resulting in varying degrees of attenuated virulence (18, 37, 246-248). For example the disruption of *fkpA* in *S.* Typhimurium, results in only minor defects in survival in the murine macrophage cell line J774 and epithelial cell line Caco-2, with no difference in LD_{50} or rate of lethality when compared to the parental strain (248). *In vivo* competition against the parental strain shows no significant difference, though when combined with a *surA* mutation, the mutant is significantly attenuated with a competitive index of 0.0121 (248).

The recently discovered TAM complex has been shown to be required for AT passenger domain secretion in *C. rodentium* and *E. coli*, whereby deletion of *tamA* results in the loss of AT protein p1121 from *C. rodentium* OM (103). Furthermore, a double *tamAB* mutant in *E. coli* MG1655 over-expressing the ATs, Ag43 and EhaB resulted in significant reductions in their ability to autoaggregate, a phenotype previously shown to be induced by both of these ATs (103, 125, 249). Additionally, *ytfP* is also transcribed within the same genomic operon, as *tamA* and *tamB*, though its contribution to the function of this complex is currently unknown.

During this study we aimed to investigate the role of a selection of nonessential genes, required for maintaining membrane barrier function and examine how these contributed to virulence in *S.* Typhimurium, using both *in vitro* assays and the murine model of *in vivo* infection.

5.2. RESULTS

5.2.1. Creation of single gene disruption mutants in *S.* Typhimurium

To further elucidate the roles of non-essential genes in membrane homeostasis, a selection of the mutations highlighted in chapter 3 (including those which were initially shown to be sensitive prior to P1 transduction), were transferred to the broad host range

pathogen *S.* Typhimurium, to assess the impact of these deletions in a more clinically relevant strain. Several other genes were also included in this study, due to their proposed roles in the biogenesis of OM complexes and virulence factors. The mutants were constructed using either, Datsenko and Warner method for single gene inactivation or gene doctoring techniques, using primers described in appendix Table 1. All resultant colonies were screened by PCR (Fig. 5.1), to confirm recombination of the resistance cassette and loss of the original gene. The mutations were subsequently P22 transduced into the *S.* Typhimurium strains; SL1344 (virulent) and SL3261 (attenuated) and reconfirmed by PCR prior to further use. In all instances, the mutations were recreated in both the virulent SL1344 and attenuated SL3261 strains; to account for potential differences in sensitivity caused by the *aroA* mutation in SL3261.

In all instances the growth kinetics were assessed under standard laboratory growth conditions (LB broth at 37^{o}C). In contrast to that observations for *E. coli* BW25113, none of the mutants demonstrated reduced growth kinetics when compared to the parental strains (Fig. 5.1).

5.2.2. Screening for membrane defects in *S.* Typhimurium mutants

Having determined the growth kinetics for all of the mutants, and shown no significant differences, we used the streak plating techniques highlighted in chapter 3 to assess the ability of the *S.* Typhimurium parental and mutant strains to grow on LB agar and LB agar supplemented with vancomycin (100-200 μg/mL) or SDS (4.8%). Several of the mutants selected for examination were initially shown to be sensitive in *E. coli* K12, however lost their phenotype upon P1 transduction. Though having already constructed these mutations we

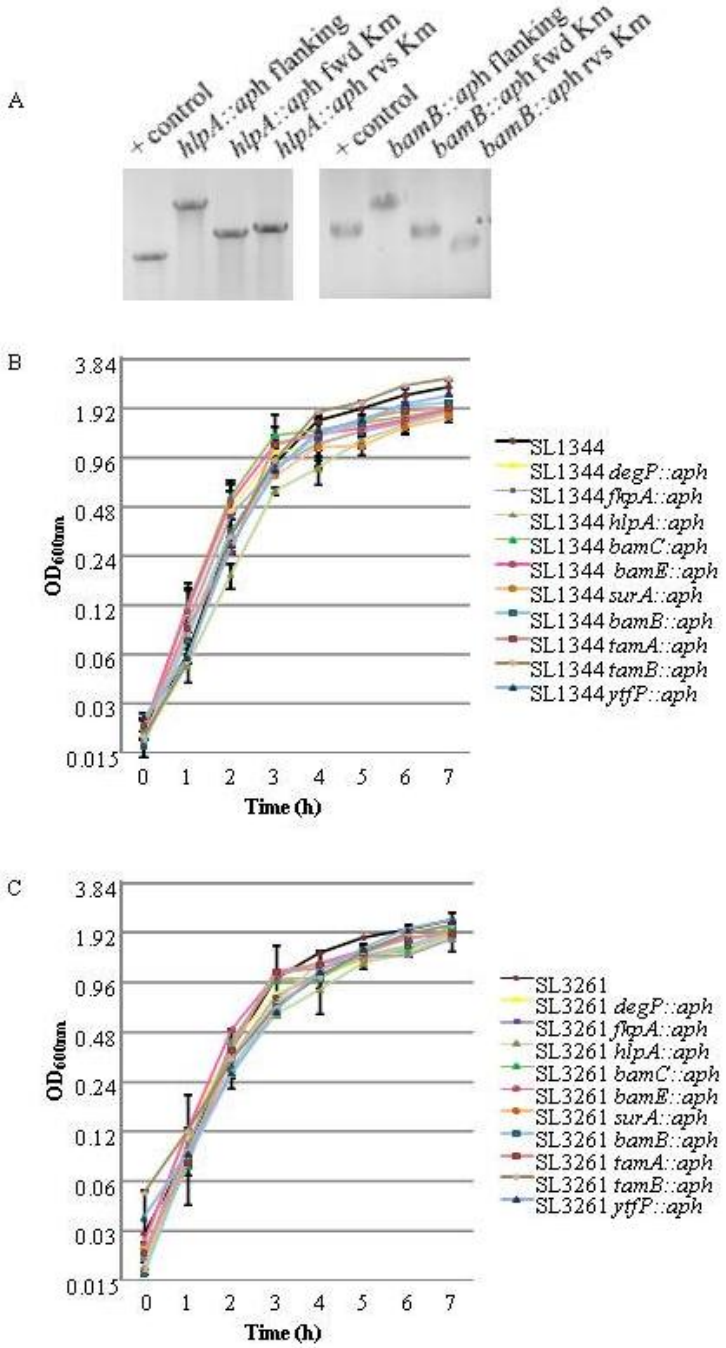


Figure 5.1. – Creation of *S.* Typhimurium mutants and Growth kinetics
A. PCR gels confirming the mutations for *S.* Typhimurium, SL1344*hlpA::aph* and SL1344*bamB::aph*, + control refers to SL1344 with gene specific flanking primers, flanking refers to gene specific flanking primers in combination, fwd Km refers to internal kanamycin forward primer in conjunction with reverse gene specific primer, while rvs Km is the opposite, forward gene specific primer with reverse internal kanamycin. **B.** Growth kinetics for SL1344 strains and **C.** SL3261 strains, under standard laboratory conditions, in LBB at 37^{o}C. Results represent the average optical density (OD) from three independent assays, error bars represent the calculated standard deviation from the three data sets.

felt that screening them in *S.* Typhimurium would still provide useful information. Other mutants (including the *tamAB* and *ytfP*) had also been shown to have heightened sensitivity vancomycin by other groups. Interestingly, a number of the mutants failed to display the same phenotype as that observed for the corresponding *E. coli* mutants (Table 5.1 and Fig. 5.2). The presence of long chain LPS and branch modifications, which are present in *S.* Typhimurium, are known to enhance resistance to antimicrobial compounds (234). Therefore, the phenotypes of the mutants was re-examined using higher concentrations of vancomycin (150 and 200 μg/mL). In the presence of increased concentrations of vancomycin, several mutants showed elevated sensitivity; in *S.* Typhimurium SL1344 both *hlpA* and *surA* mutants were reduced in growth and completely inhibited at 150 and 200 μg/mL of vancomycin, respectively. *S.* Typhimurium SL3261 mutants behaved similarly. Furthermore, *S.* Typhimurium SL3261 *bamC* and *bamE* mutants also showed weak and complete inhibition of growth at 200 μg/mL vancomycin, respectively. Due to the majority of the *S.* Typhimurium mutants demonstrating a phenotype similar to that of the parental strain, only a selection of were pursed for further investigation; *bamB, surA, tamA, tamB, ytfP*.

5.2.3. Membrane barrier defects are not a marker for serum sensitivity

Complement glycoproteins responsible for complete-mediated killing of bacteria, affect the stability of the membrane, resulting in susceptibility to changes in osmotic pressure and increased potential for cell lysis **(250)**. However, several investigations have shown Gram-negative pathogens resist complement-mediated killing through binding of TAA stalk domains to complement inhibitor factors H, C4BP and C3, as shown for YadA and UspA2 from *Yersinia enterocolitica* and *Moraxella catarrhalis,* respectively (144). As the some of the genes being investigated were shown to induce membrane defects and are associated with

Table 5.1. – Sensitivity of various *S.* Typhimurium mutants to vancomycin and SDS

	Compound and Concentration [a]				*E. coli* K12 [b]	
Strain	SDS 4.8%	vancomycin 100 μg/mL	vancomycin 150 μg/mL	vancomycin 200 μg/mL	SDS 4.8%	vancomycin 100 μg/mL
SL1344	×	×	×	×	NA	NA
SL1344 *bamB::aph*	Weak	✓	✓	✓	✓	✓
SL1344 *bamC::aph*	×	×	×	×	×	×
SL1344 *bamE::aph*	×	×	×	×	×	✓
SL1344 *degP::aph*	×	×	×	×	×	✓*
SL1344 *fkpA::aph*	×	×	×	×	×	✓*
SL1344 *skp::aph*	×	×	Weak	✓	×	✓*
SL1344 *surA::aph*	Weak	×	Weak	✓	✓	✓
SL1344 *tamA::aph*	×	×	×	×	×$^{+}$	×$^{+}$
SL1344 *tamB::aph*	×	×	×	×	×$^{+}$	×$^{+}$
SL1344 *ytfP::aph*	×	×	×	×	×	×
SL3261	×	×	×	×	NA	NA
SL3261 *bamB::aph*	✓	✓	✓	✓	✓	✓
SL3261 *bamC::aph*	Weak	×	×	Weak	×	×
SL3261 *bamE::aph*	×	×	Weak	✓	×	✓
SL3261 *degP::aph*	Weak	×	×	×	×	✓*
SL3261 *fkpA::aph*	×	×	×	×	×	✓*
SL3261 *skp::aph*	×	×	Weak	✓	×	✓*
SL3261 *surA::aph*	✓	×	Weak	✓	✓	✓
SL3261 *tamA::aph*	×	×	×	×	×$^{+}$	×$^{+}$
SL3261 *tamB::aph*	×	×	×	×	×$^{+}$	×$^{+}$
SL3261 *ytfP::aph*	×	×	×	×	×	×

[a] – Defects in membrane barrier permeability as determined by inhibition of growth; ✓ sensitivity as observed as no growth, Weak; sensitivity as reduced growth, × growth comparable with the parental strain

[b] - * Where sensitivity was not maintained after transduction, $^{+}$ highlighted as sensitive by others

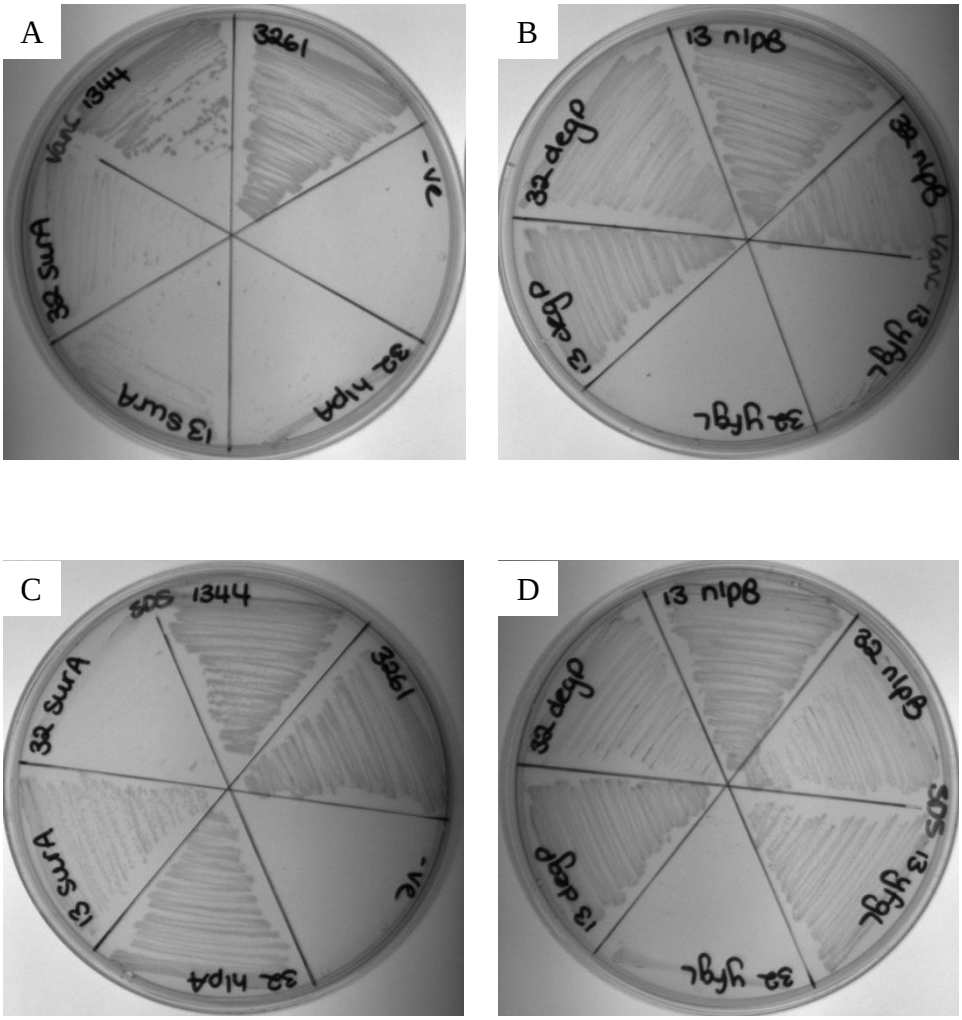


Figure 5.2. Representative plates from *S.* Typhimurium screening.
Images A and B represent growth on LBA supplemented with 200 µg/mL vancomycin, while C and D show growth in the presence of 4.8% SDS, for various *S.* Typhimurium strains. Annotations marked on the plates denote the location of various mutants, while 13 denotes SL1344, and 32 represents an SL3261 background.

the biogenesis of ATs, we choose to investigate how their deletion may affect their resistance to complement-mediated killing. The *surA, tamA, tamB* and *ytfP* mutants all demonstrate elevated sensitivity to human serum, compared to the parental strain, *S.* Typhimurium SL1344 Fig. 5.3). In contrast, the *bamB* mutant failed to show any alteration in serum resistance, with comparable survival to that of the parent strain. This is surprising given this mutation induced a severe membrane defect, more so than that observed for *S.* Typhimurium *surA* mutants, whereby *bamB* mutants were susceptible to vancomycin 100 μg/mL (presented as part of this study), in contrast to *surA* which only demonstrated complete inhibition of growth at 200 μg/mL vancomycin under the same test conditions. Interestingly, the OM protein TamA and its IM counterpart TamB, shows the most severe susceptibility, while the predicted cytoplasmic component of this complex (YtfP), although significantly more serum sensitive than the parental strain, can survive for longer than the *tamA* and *tamB* mutants.

5.2.4. Some mutants display reduced virulence during systemic infection

Having shown that the mutants are in most instances susceptible to complement mediated killing, we wanted to further investigate how this may impact upon *in vivo* infection. We examined the ability of the mutants to colonise and cause persistent infection using the murine model for systemic *S.* Typhimurium infection, using the SL3261 mutants administered via the IP route. The results show both *surA* and *bamB* mutants are highly attenuated in both spleen and liver colonisation at all time points ($p = 0.004$ for both mutants in spleen) (Fig. 5.4). The *ytfP* mutant showed severe reductions in bacterial colonisation at day 7 ($p= 0.004$), but recover by day 18 ($p= 0.7758$), with comparable burdens by day 28 ($p= 0.1091$), in both the spleen and liver, indicating the defect was not site specific.

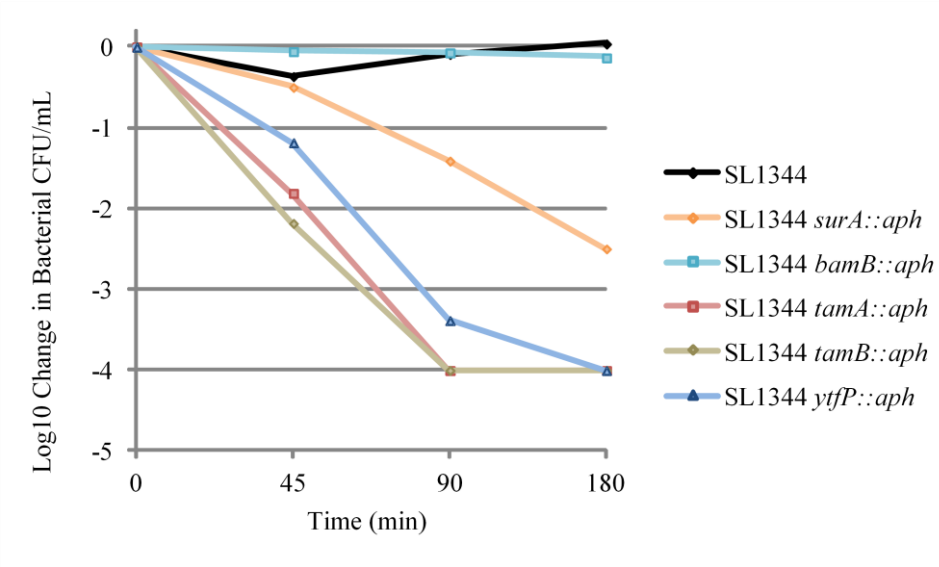


Figure 5.3. Serum resistance of various *S.* Typhimurium mutants
Serum resistance of various SL1344 mutants when incubated with healthy human serum, for various durations. Bacterial resistance determined as no change in Log_{10} of bacterial CFU/mL, as calculated from three biological replicates.

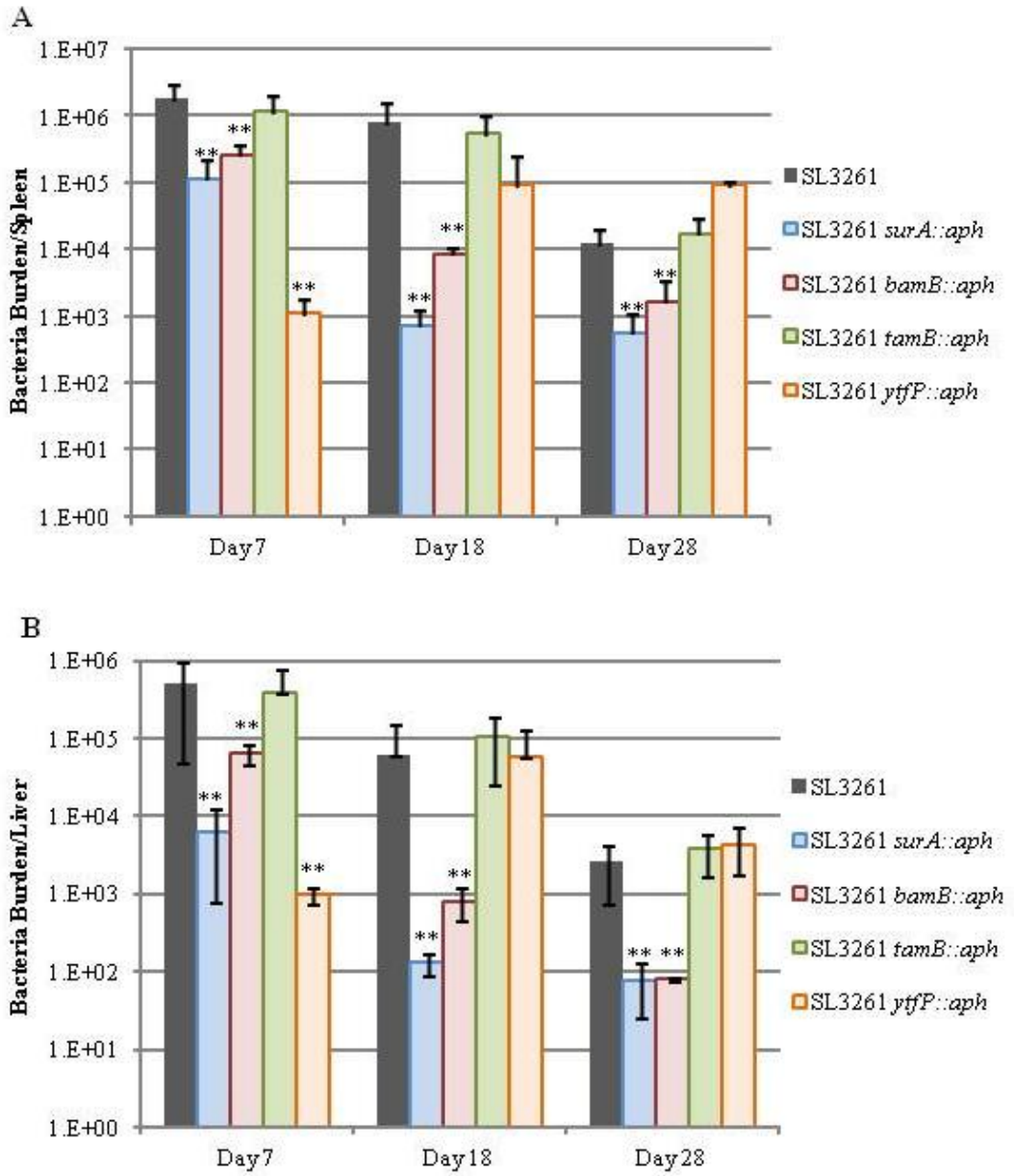


Figure 5.4. Bacterial burdens of various *S*. Typhimurium SL3261 mutants
Bacteria burdens per organ, (A) Spleen, (B) Liver, for C57BL6 mice infected with SL3261 mutants via IP route over a time course. Significance determined using Mann-Whitney t-test, whereby * <0.05, **<0.009

Unfortunately, at the time of completion a *tamA* mutant was not available. However, a *tamB* mutant failed to show any defect in colonisation or persistence. In contrast, when *tamA* or *tamB* mutants are competed against wild type *C. rodentium,* both fail to colonise efficiency (103). Therefore to determine whether a similar phenotype would be observed with *S.* Typhimurium *tamB* and *ytfP* mutants, we performed *in vivo* competition assays, evaluating bacterial colonisation when either mutant was co-administered with the *S.* Typhimurium parental strain, SL3261 (Fig. 5.5). The results show the competitive index for each mutant against the parental strain, over time. The *tamB* mutants demonstrated a mild attenuation at day seven, though at time points day 18 and 28, the *tamB* mutants were shown to colonise and persist to a degree comparable with or better than the parental strain (spleen day 28, competitive index 22.7). While the *ytfP* mutant demonstrated a significant decrease in its competitive ability at day seven, with competitive index of 0.002 and 0.04, for spleen and liver respectively, with recovering competitive indices at days 18 and 28, though this mutant failed to colonise quite as effectively as the parental strain, with competitive indices of 0.57 and 0.43 for the spleen and liver respectively, at day 28.

5.2.5. Oral administration marginally affects *S*. Typhimurium *bamB* colonisation

The previous assessments of *in vivo* infection were performed using IP administration, modelling systemic infection. As this is not a true representation of the normal route of *Salmonella* infection, which is commonly oral-gastric, we examined ability of the *bamB* mutant colonise and cause infection four days after oral gavage. The results do not show significant attenuation, though a slight reduction is observed in bacterial numbers at all sites (spleen, liver, mesenteric lymph nodes or payers patches) (Fig. 5.6).

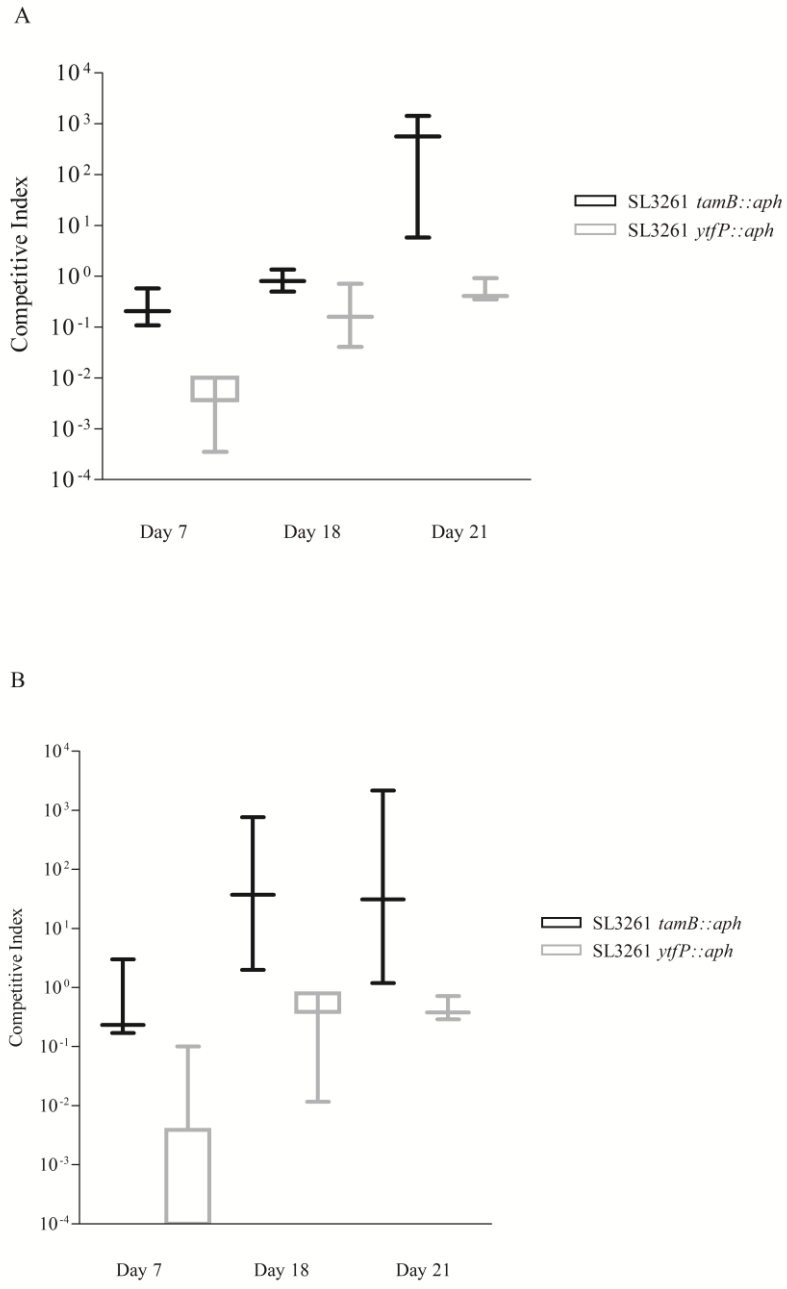


Figure 5.5. Bacterial burdens of *S.* Typhimurium Competition Strains
Bacteria burdens per organ, (A) Spleen, (B) Liver, for C57BL6 mice infected with SL3261 in competition with either SL3261 *tamB::aph* or SL3261 *ytfP::aph*, via IP route, over a time course. Mutants bacterial burdens determined by selecting for kanamycin resistance and comparing with LBA only.

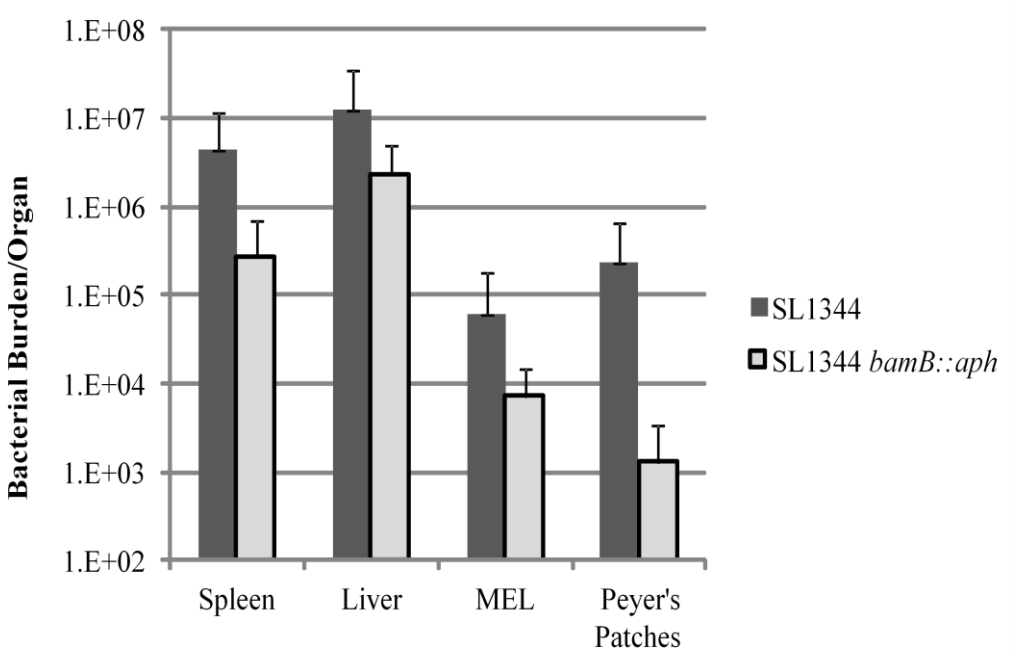


Figure 5.6. Bacterial burdens of *S.* Typhimurium *bamB* mutant Oral Challenge
Bacteria burdens per organ, for C57BL6 mice infected with SL1344 or SL1344 *bamB::aph* via oral gavage. Bacterial burdens determined after four days. MEL refers to mesenteric lymph nodes.

5.3. DISCUSSION

A selection of single gene disruption mutants, associated with the BAM complex and periplasmic chaperones which were highlighted as inducing severe membrane defects in *E.coli* K12 were transferred to the broad host range pathogen *S.* Typhimurium, to further investigate the phenotypes affected. In addition, other genes associated with the TAM complex required for AT passenger domain secretion, were also disrupted. In all instances the mutations were introduced into the virulent and attenuated *S.* Typhimurium strains, SL1344 and SL3261, respectively. To account for potentially heightened sensitivity of *S.* Typhimurium SL3261, caused by the *aroA* deletion, which is known to induce membrane defects resulting in elevated sensitivity to EDTA, complement mediated killing and albumen (251). In addition to altering innate immune responses, as has been shown by the lack of pro-inflammatory cytokine stimulation in gnotobiotic pigs (252). During our investigations we have shown that the barrier defects previously observed were not an artefact of using a laboratory adapted *E. coli* K12 strain, and represent true phenotypes, reproducible in a pathogenic strain. The enhanced susceptibility to both a previously ineffective antibiotic and detergent, highlighting the potential for these proteins to formulate secondary drug targets for future drug development.

To complement these investigations we have investigated both innate and adaptive immune responses to a selection of these mutants. The phenotypes observed for the *surA* mutant are unsurprising, in addition to inducing a severe membrane defect, the protein encoded serves as a major periplasmic chaperone **(18, 248)**. Transporting a number of OMP and AT precursors from the Sec Machinery at the IM to the BAM complex (18). The decrease in serum resistance in this strain maybe in part attributed to (possible) reduced efficiency of AT biogenesis in this mutant. Although the levels of *S.* Typhimurium AT proteins were not

examined during this study, it has been previously shown that deletion of *surA* does affect the biogenesis of the type Va protein EspP (16). Although no studies have yet to confirm the role of SurA in the biogenesis of TAAs, DegP has been shown to degrade misfolded YadA precursors **(253)**, confirming a role for periplasmic chaperones in TAA biogenesis. Although investigations performed to date implicate only TAAs in mediating serum resistance (138, 144), this phenotype has not been explored for the type Va proteins from *S.* Typhimurium, thus their contributions cannot be excluded at this time.

Consistent with previous observations from *S.* Typhimurium, *Shigella flexneri* and UPEC strain UT189 (18), during this study we have also confirmed *surA* is critical for virulence. Our investigations with *bamB* mutants from *S.* Typhimurium, complement previous reports from *E. coli* and *S.* Enteritidis. BamB is not essential for secretion of the EAEC or UPEC ATs Pet and Sat, respectively (100). Consistent with the hypothesis that the elevated sensitivity to complement-mediated killing observed with other mutants is as a result of reduced ATs in the OM.

In contrast to the well documented association of BamB with the OM BAM complex, investigations with *S.* Enteritidis have shown BamB also affects the transcription of a several SPI1 and SPI2 associated genes, including T3SS structure and effector proteins, resulting in a attenuated virulence in this mutant **(242)**. Consistent with the role of SPI1 and SPI2 T3SSs with invasion and survival of *Salmonella* in macrophages, *bamB* mutants were shown to be significantly attenuated at all time points when assessing systemic infection during this study, though bacterial burdens were only mildly reduced after oral challenge. Though closer inspection of the results presented here with that from *S.* Enteritidis investigations **(242)**, indicates the differences in bacterial burdens between the mutants and their respective parental strains are marginal. Thus, potentially having extended our study over a longer

duration (six days instead of four) with a larger cohort of animals, may have yielded statistically significant differences.

The TAM complex has been shown to be associated with secretion of AT passenger domains, working in conjunction with the BAM complex. TamA forms the OM protein with similar homology to the Omp85 family proteins, including BamA (103). While TamB is an IM bound protein, which protrudes into the periplasm, interacting with the POTRA domains of TamA (103). The heightened sensitivity of *tamA* and *tamB* mutants to complemented-mediated killing is consistent with the role of this complex. Although the degree of mature AT proteins present in the OM of these single mutants has not been investigated during this study, it has been shown that *tamAB* double mutants have reduced levels of at least one AT in *S.* Typhimurium, ApeE (Personal communication Dr. Matthew Belousoff, Monash University).

In *C. rodentium*, *tamA* and *tamB* mutants, show significant attenuation in virulence, when completed against the relevant parental strain (103). Unfortunately, the *tamA* mutant was not available at the time of performing the *in vivo* studies, though in contrast to *C. rodentium* we failed to observe any attenuation in virulence for a *tamB* mutant when administered singly and only a mild reduction in competitive index when competed against the parental strain at day seven, which was not replicated at the later time points. These may in part be due to the route of administration used in this study, whereby two of three characterised *S.* Typhimurium AT proteins (MisL and ShdA) are associated with intestinal colonisation (123, 124, 135). Repeating these studies using oral gavage, may therefore prove to be more insightful.

In the case of *ytfP*, although transcribed within the same operon as *tamA* and *tamB*, its associated function with this complex has yet to be determined. However, disruption of *ytfP*

in *S.* Typhimurium resulted in significantly attenuated virulence (day 7 IP), similar to that observed previously for *plpA* mutants (chapter 4). This may be indicative of heightened sensitivity to innate immune responses, as observed with *plpA* mutants, though this hypothesis remains to be tested.

Based on the results presented as part of this study and in consideration with that presented by others previously, SurA and BamB make attractive drug targets. Their cellular localisation within the periplasmic space and inner leaflet of the OM, respectively, would provide ease of access for small inhibitory molecules (capable of penetrating the OM). Both of these mutations result in significant attenuation, though we hypothesised this attenuation stems from different means. The TAM complex provides an alternative target for antibacterial drug development, due to its association with the secretion of AT protein passenger domains.

The results presented during this study highlighting the critical need for further investigation into the immune responses stimulated by these mutants. To ensure they do not adversely affect the stimulation of pro-inflammatory cytokines, required for clearance and control of bacterial infections. Or the development of suitable memory mediated AB responses. Further studies are planned to examine how AB responses to various surface structures (including LPS, purified ATs and OMPs) are affected in these mutants, to ensure that a balanced AB response is maintained, and not predominately enhanced in for potentially anti-bactericidal ABs, such as to LPS.

Despite requiring further investigation, this study has provided key insight to the virulence associated phenotypes of a number of genes, and investigated the impact of their deletion on bacterial virulence and the host immune response; critical investigations if the proteins encoded are to be pursued as potential drug targets for future development. As several of the genes investigated during this study are known to be associated with AT protein

biogenesis and secretion, the phenotypic roles of these proteins from *S.* Typhimurium will be investigated further in the next chapter.

CHAPTER 6

CHARACTERISATION OF THE AUTOTRANSPORTERS OF *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM

6.1. INTRODUCTION

The T5SS or AT pathway is the simplest and most widespread protein secretion pathway in Gram negative bacteria, with the majority of the characterised proteins shown to have a role in virulence. *S.* Typhimurium contains genes encoding five AT proteins, four of which belong to the Va subclass; ApeE, MisL, SapP (STM0373) and ShdA, and a single trimeric Vc protein; SadA (STM3691). Three of the Va proteins (MisL, SapP and ShdA) are predicted to form traditional passenger domain structures, which fold to form a right handed β-helical structure, conserved across 97% of the Va proteins (94). The remaining Va protein (ApeE) is predicted to form a globular passenger domain, rich in α-helices, observed in <3% of classical AT proteins. The Vc protein encoded by *S.* Typhimurium is SadA, similar in domain organisation to Va proteins, with the key difference being Vc proteins have a smaller translocation domain and must form homotrimers in the OM to produce a functional 12 stranded β barrel (116-118). The passenger domains of the three individual polypeptides combine to form the functional region of the protein, which is comprised of the head, neck and stalk domains **(254-256)**.

Of the five AT proteins in *S.* Typhimurium only three have been previously characterised; ApeE, MisL and ShdA. ApeE is regulated by the transcriptional repressor *apeR* and *phoBR* regulon, with expression induced under phosphate limiting conditions **(257)**. The protein encodes an esterase, responsible for the hydrolysis of fatty acid naphthyl esters, though it does not hydrolyze peptide bonds or act as a protease **(258)**. Despite ApeE having first been described over a decade ago, the physiological relevance of these findings with regards to pathogenesis has yet to be determined.

MisL (membrane insertion and secretion) is produced from the gene of the same name located on SPI3 and transcriptionally activated by MarT, also located on SPI3, which is

predicted to relieve HNS mediated gene silencing **(259, 260)**. Despite previous attempts, expression of *misL* from its native promoter has not been detected *in vitro.* However, expression has been shown during *in vivo* oral infection of chickens, where MisL is required for virulence (261). Furthermore during murine infection, MisL is required for persistent colonisation of the caecum and faecal shedding (123). When expressed under the control of an inducible promoter *in vitro*, MisL increases binding to the ECM molecule fibronectin and promotes bacterial invasion of T84 human epithelial cells derived from colonic carcinoma (123).

The final characterised AT protein, ShdA, is also an intestinal colonisation factor. Like MisL, ShdA is associated with colonisation of the caecum (lumen and mucosal layer), and is required for continued faecal shedding during the course of infection (124). Prolonged shedding is associated with the ability of ShdA to bind fibronectin and collagen I by means of an ionic interaction that mimics the binding of the natural host molecule, heparin (124, 135, 137, 262).

Deletion of either *misL* or *shdA* individually does not significantly attenuate *S.* Typhimurium virulence in the mouse model (135, 260). However, when either mutant is competed against its respective parental strain (or relevant other mutant), reductions in mutant strain colonisation of the Peyers patches and caecum are observed (123, 136). Further to this, the combined deletion of *misL* and *shdA*, results in significant reductions in the numbers of recovered double mutants from the spleen, the caecum, Peyer's patches and small intestine when competed against a *phoN* mutant (which does not display a colonisation defect) (123, 136). Despite investigations into the role of these proteins in gut colonisation, no further investigations have been performed into their role in systemic infection and it remains unclear if a double mutant is avirulent.

As some of the genes examined in the previous chapter are associated with AT biogenesis and demonstrated varying degrees of attenuation *in vivo* we chose to investigate the role of *S.* Typhimurium ATs in pathogenesis, characterising the roles of individual proteins, in addition to examining the potential for functional redundancy.

6.2. RESULTS

6.2.1. Construction of *S.* Typhimurium AT deletion strains

In order to examine the role of various AT proteins from *S.* Typhimurium, we constructed a series of both single and multiple deletion strains, in both the *S.* Typhimurium pathogenic (SL1344) and attenuated (SL3261) strains. Single gene disruption mutants for all *S.* Typhimurium ATs were constructed using the Datsenko and Warner method for single gene inactivation (as previously described) with the exception of the *S.* Typhimurium SL1344*shdA::aph* mutant which was kindly provided by Dr. R. Kingsley. These mutations were subsequently P22 transduced back into the both the attenuated and pathogenic strains prior to use (Fig. 6.1), with the exception of *S.* Typhimurium SL1344 *shdA::aph*, which despite several attempts we were unable to transduce the single mutation into *S.* Typhimurium SL3261.

In order to further examine the potential for functional redundancy amongst these proteins, we constructed a series of multiple deletion mutants in both *S.* Typhimurium SL1344 and SL3261. These multiple deletions were produced by combining the single mutations created above, using P22 transduction. Upon confirmation of the mutation, the cassette was then removed as described previously (263), and the next mutation added via P22 transduction. In each instance, both the gene being disrupted and previously deleted genes were PCR checked, to confirm these had not reverted to WT during the transduction or had

undergone abnormal recombination events between the remaining FRT sites located at various positions around the chromosome (Fig. 6.1). We constructed multiple deletions based on the type V classification and predicted AT structure, thus producing a 3 in 1 mutant where the classical ATs were deleted (Δ*sapP*, Δ*misL* and *shdA::aph*), combined with either the esterase or trimeric AT (3 in 1 containing either Δ*apeE* or Δ*sadA*) termed 4 in 1 *apeE* or *sadA* (referring to the gene deleted) or all five ATs simultaneously (Δ*sapP*, Δ*misL*, Δ*apeE*, Δ*sadA* and *shdA::aph*), termed 5 in 1.

6.2.2. Virulence of single and multiple AT deletion strains

6.2.2.1. Single mutants are mildly attenuated after oral administration

As *S.* Typhimurium infection most commonly occurs as a result of consuming contaminated food and water, we initially examined the ability of the mutants to cause infection when administered via a physiologically relevant route, oral gavage. We examined the bacterial burdens in the spleen and liver at four and six days post infection with virulent *S.* Typhimurium SL1344 (Fig. 6.2). At both time points the *S.* Typhimurium SL1344 *sapP::aph* mutant was attenuated in both the spleen and liver, with bacterial burdens reduced by approximately 1.5 and 3 logs compared to that of the parental strain. In contrast the *S.* Typhimurium SL1344 *sadA::aph*, showed enhanced survival at day four, with slightly reduced and equal bacterial burdens by day six. While both *S.* Typhimurium SL1344 *apeE::aph* and SL1344 *misL::aph* mutants demonstrated only slight decreases in bacterial burdens at day four (approximately 0.5 log decrease in spleen bacterial burdens), which were enhanced by day six (to approximately 1.5 log decrease in bacterial burdens).

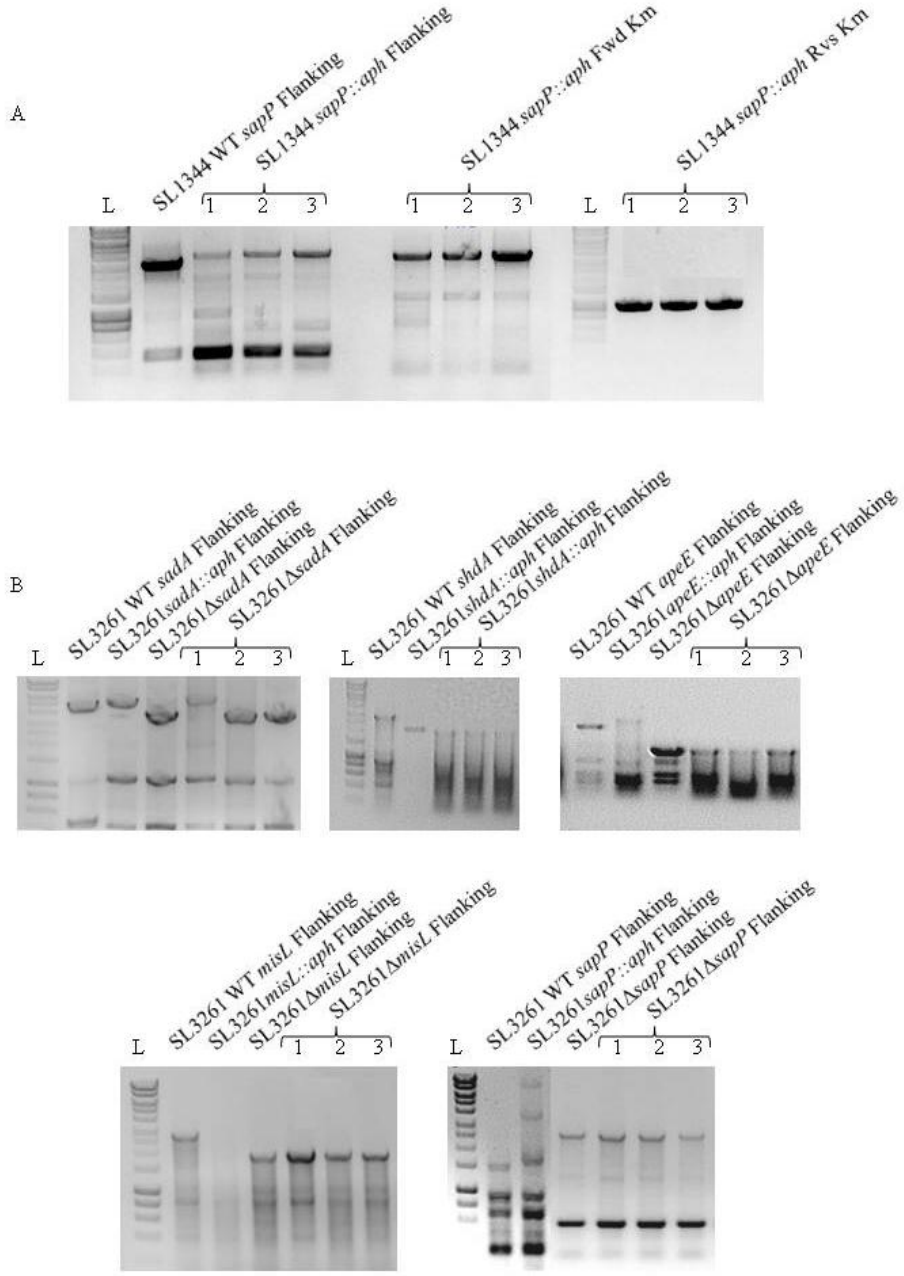


Figure 6.1. Construction of *S.* Typhimurium single and multple deletion strains
PCR confirmation of the mutants constructed in *S.* Typhimurium strains **A.** Single *S.* Typhimurium SL1344*sapP::aph*, using gene specific flanking primers to compare the fragement size between the parental strain, and test colonies. Fwd Km refers to the use of the internal kanamycin forward primer in conjunction with the gene specific reverse primer, while Rvs Km refers to the opposite, an internal reverse kanamycin primer in conjunction with the gene specific forward flanking primer. **B**. Confirmation of the pentuplet mutant in *S.* Typhimurium SL3261, using gene specific flanking primers in each instance, comparing the parental strain, with that of a known disruption and deletion mutant (where appropriate).

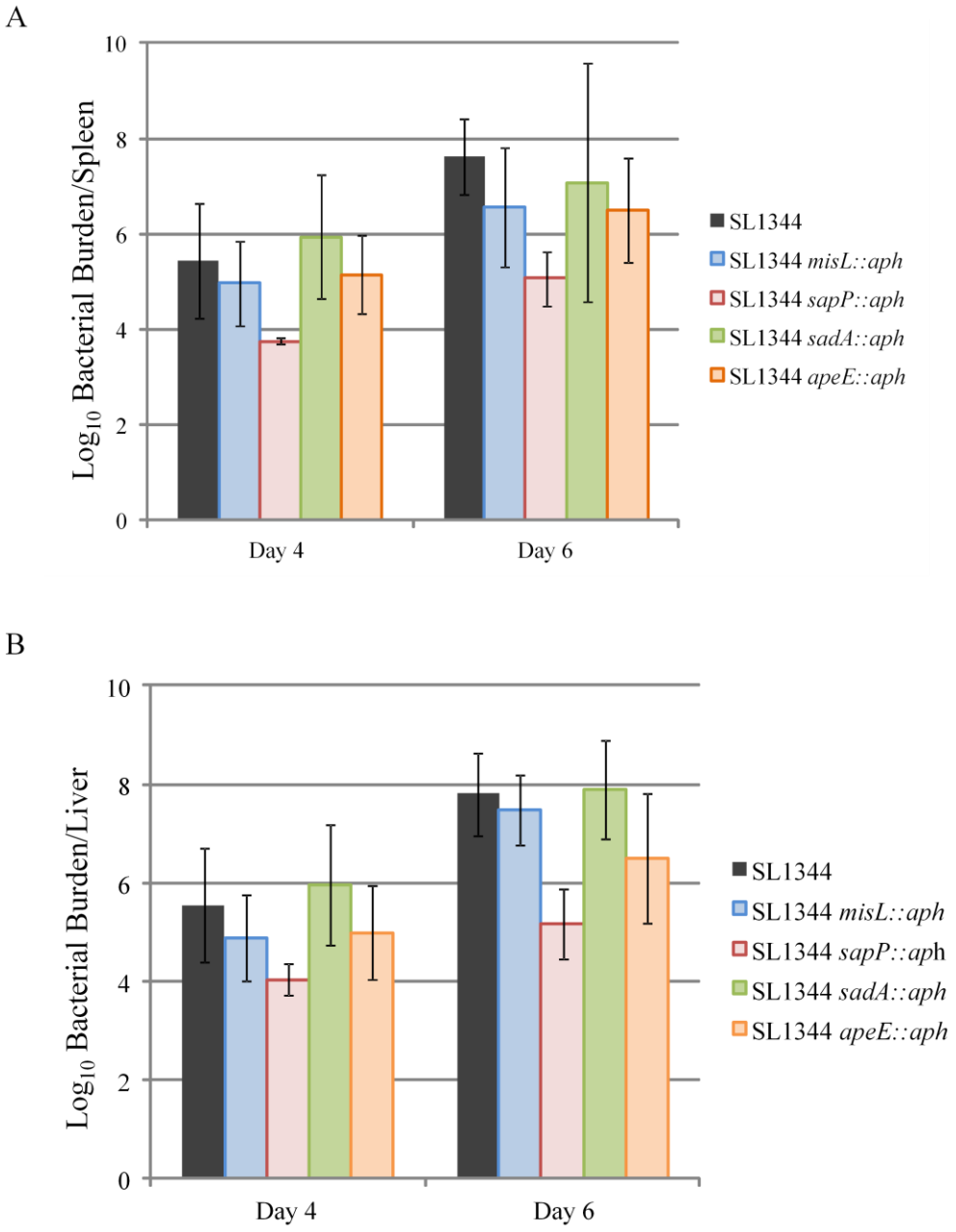


Figure 6.2. Bacterial burdens post four and six days oral infection
Bacterial burdens in the **A**. spleen, **B.** liver, post four and six days infection with single mutants in *S.* Typhimurium SL1344 background.

6.2.2.2. Single mutants are also attenuated during later stages of systemic infection

Despite oral infection being the most physiologically relevant route, we questioned whether the ATs being investigated may also be required for systemic infection. For example, as *sapP* mutants were attenuated after oral administration, we questioned whether this AT maybe required for murine infection generally or whether its roles were limited gastrointestinal colonisation, in a manner analogous to MisL and ShdA which are required for intestinal colonisation and persistent infection (123). We also choose to investigate the role of MisL in systemic infection, as this mutant had not previously been investigated in this manner (all prior studies administering *misL* mutants via IP, were done so in competition). Finally, *sadA* mutants were not attenuated after oral challenge despite the fact several other TAAs, including YadA and UpaG, are required for serum resistance, biofilm formation and adherence and invasion of specific cell types (119, 143), phenotypes that are associated with causing and maintaining persistent systemic infection. Therefore, we chose to examine the ability of the *S.* Typhimurium SL3261 *sapP*, *misL* and *sadA* single mutants to cause systemic infection over a time course of seven, 21 and 35 days after IP administration (Fig. 6.3).

At days seven and 21 the *sadA* mutant demonstrated no attenuation in virulence, in either the spleen or liver and was therefore excluded from the day 35 study. The *sapP* and *misL* bacterial burdens were slightly reduced at day seven, though not significant in either the spleen or liver. In contrast, at days 21 and 35 the *sapP* mutant was significantly attenuated at both sites ($p= 0.0286$), while the *misL* mutants were significantly reduced in spleen at day 21 and the liver at day 35 ($p= 0.0286$) suggesting that both SapP and MisL are required for systemic virulence in the murine model.

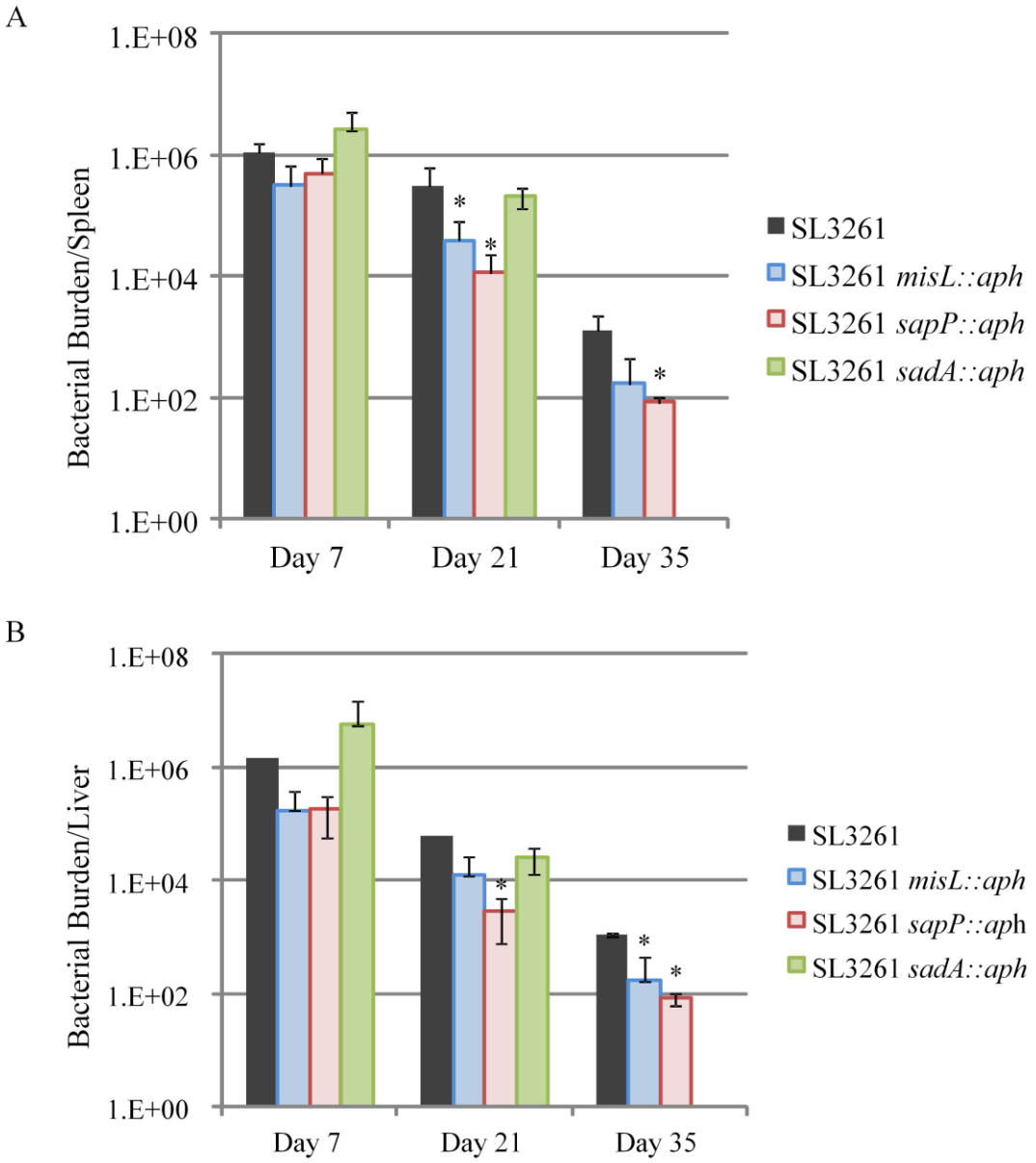


Figure 6.3. Bacterial burdens after systemic infection with *S.* Typhimurium
Bacterial burdens from **A.** spleen and **B.** liver of C57BL6 mice dosed with various *S.* Typhimurium single mutants, in SL3261 strain. Examining bacterial colonisation and persistance over a period of seven, 21 and 35 days.

6.2.2.3. Multiple deletion strains show graded attenuation in systemic infection

As the single *sapP* and *misL* deletion mutants showed significant attenuation in virulence when administered IP, we questioned whether these reductions in bacterial burdens could be enhanced in the absence of the other Type Va AT ShdA. Additionally despite *sadA* mutants showing no attenuation in virulence, we questioned whether the deletion of this protein was compensated for by the Type Va ATs. Therefore, we chose to examine the virulence profiles of the *S.* Typhimurium SL3261 multiple deletion mutants after systemic infection over a time course of seven, 21 and 35 days. Based on the previously reported roles of MisL and ShdA in intestinal colonisation (123, 136) combined with our earlier results indicating *sapP* mutants are attenuated when administered orally, we felt commencing our investigations with the multiple deletion strains using systemic (IP) infection would be more insightful. We feared if oral administration was used and the mutants were unable to colonise the gut and achieve systemic infection, we may gain limited information from these experiments, thus IP administration was selected (using the SL3261 variants) for our investigations despite this not being a physiologically relevant route of infection.

Interestingly, despite our previous investigations showing that both *misL* and *sapP* single mutants were attenuated at days 21 and 35, no significant attenuation is observed when these mutation are combined with disruption of *shdA*, at any time point examined in either the spleen or liver (Fig. 6.4). Our previous investigations had suggested SadA was not required for virulence in the murine model, though the combination of this mutation, with that of *misL*, *shdA* and *sapP*, does significantly reduce the spleen bacterial burdens at day 21 (P=0.0286), However, the reductions in the liver are not significant. By day 35 this mutant appears to colonise and persist to the same degree and better than that of the parent in the spleen and liver, respectively. As ApeE is the only remaining AT in this strain, this result may be

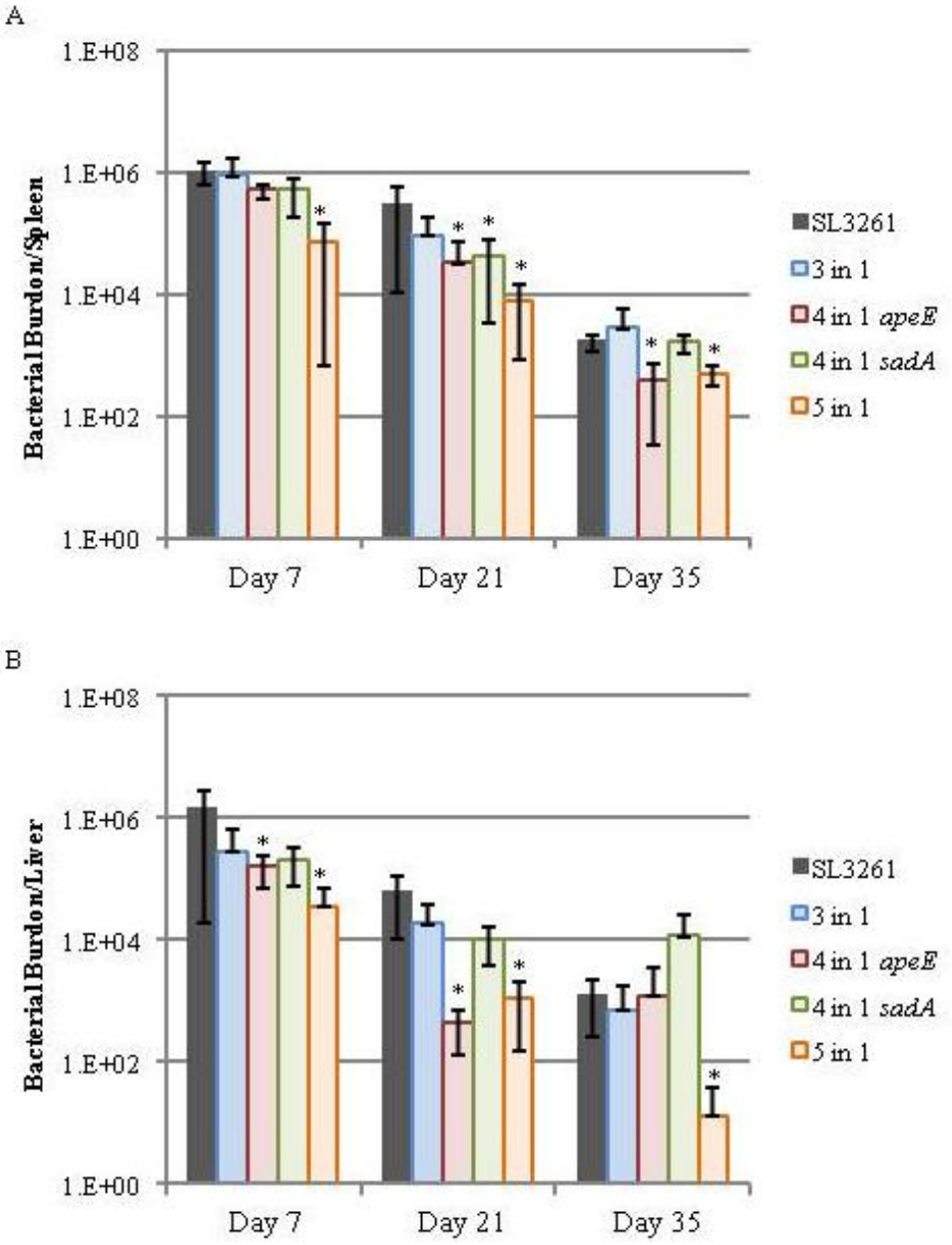


Figure 6.4. Virulence of *S.* Typhimurium multiple AT deletion mutants
Systemic infection of C57BL6 mice with attenuated SL3261 and various multiple AT deletion strains. Bacterial burdens determined per organ for Spleen **(A)** and Liver **(B)** at seven, 21 and 35 days post infection, via the IP route. Deletion of multiple AT proteins results in a gradual attenuation in virulence, at day 7. However by day 21 ApeE appears to provide a more critical role in persistent infection in the liver and day 35 in the spleen, where by the 4 in 1 *apeE* mutant is comparable with a 5 in 1 mutant. Similarly mutants with only functional ApeE appear to persist better than parental strain in the liver at day 35 and to equal degree as wild type in the spleen. By day 35, deletion of all 5 ATs results in significant attenuation predominantly observed in the liver.

indicative of a role for ApeE in persistent infection, particularly that of the liver. Consistent with this hypothesis, a four in one mutant of *misL, shdA, sapP and apeE* was shown to be significantly attenuated in the liver at both days 7 and 21 (P=0.0286), though comparable with the parental strain at day 35, while in the spleen this quadruple mutant was attenuated at both days 21 and 35 (p=0.0286). This suggests that ApeE may be required for persistent murine infection. However, this result does not differentiate between whether this effect is specific to the loss of ApeE alone, or is a result of combination with the deletion of other type Va ATs. Interestingly, when all five AT proteins are deleted simultaneously, the pentuple mutant is significantly attenuated at all timepoints, in the colonisation of both the spleen and liver (p=0.0286 spleen and liver).

6.2.3. Bioinformatics Analyses of *S.* Typhimurium AT proteins

As MisL and ShdA were largely already characterised, we chose to focus our investigations on ApeE, SadA and SapP. Therefore, we examined each of these genes and their encoded amino acid sequences using bioinformatics, to examine the protein domain structure and confirm their predicted type V subclassification. In addition, we examined their distribution across other Gram-negative organisms, in an attempt to identify characterised homologues present in other species.

6.2.3.1. Structure and distribution of ApeE

ApeE has been previously described and shown to be encoded by a 1971 bp gene of the same name, producing a mature protein of approximately 67 kDa (post signal sequence cleavage) with esterase activity (257, 258, 264). Analysis of ApeE amino acid sequence indicates the protein contains three distinct domains; a Sec-dependant signal sequence, predicted to be cleaved after amino acid residue 25, Pfam: Lipase_GDSL domain (29 -342)

and C-terminal Pfam: Autotransporter domain, located between residues 386 - 645, encoding the catalytic passenger domain and membrane spanning, 12 stranded β-barrel, respectively (Fig. 6.5). Models of the ApeE tertiary structure show the GSDL lipase passenger domain folds to form a globular domain rich in α-helical (Fig. 6.5), bringing together a triad of catalytic residues, containing; serine, aspartate and histidine, located at positions 10, 301 and 304 (residues are numbered based on post signal sequence cleavage) (Dr V. Bavro, personal communication).

To determine the prevalence of *apeE* across other Gram negative bacterial species, the amino acid sequence was used to probe all Gram-negative genomes available in NCBI. The protein was shown to be highly conserved and present in most *Salmonella spp* in addition to significant distribution across both *Pseudomonadacae* and *Xanthomonadacae* species, where it's annotated as EstA and EstE, respectively (126, 265) (Fig. 6.5). Interestingly the protein is also shown to be present across a diverse number of proteobacteria and one cyanobacteria, however, as described previously by Carinato *et al* (258) no homologue is found in *E. coli*.

6.2.3.2. Structure and distribution of SadA

The 4,383 bp gene STM3691, encodes the only Type Vc TAA in *S.* Typhimurium termed SadA; Salmonella Adhesin protein A (266). Structural analysis shows the sequence contains all the features of a TAA; including a conserved N-terminal extended signal peptide region (ESPR) predicted to be cleaved after amino acid 50, a 1333 amino acid passenger domain. Which is composed of three structurally conserved regions; head, neck and stalk (118, 267), as depicted by Pfam:Hep_Hag (PF05658) motifs (head), a single Pfam:HIM (PF05662) motif (neck) and a series of small unconserved repeats comprising the stalk (118).

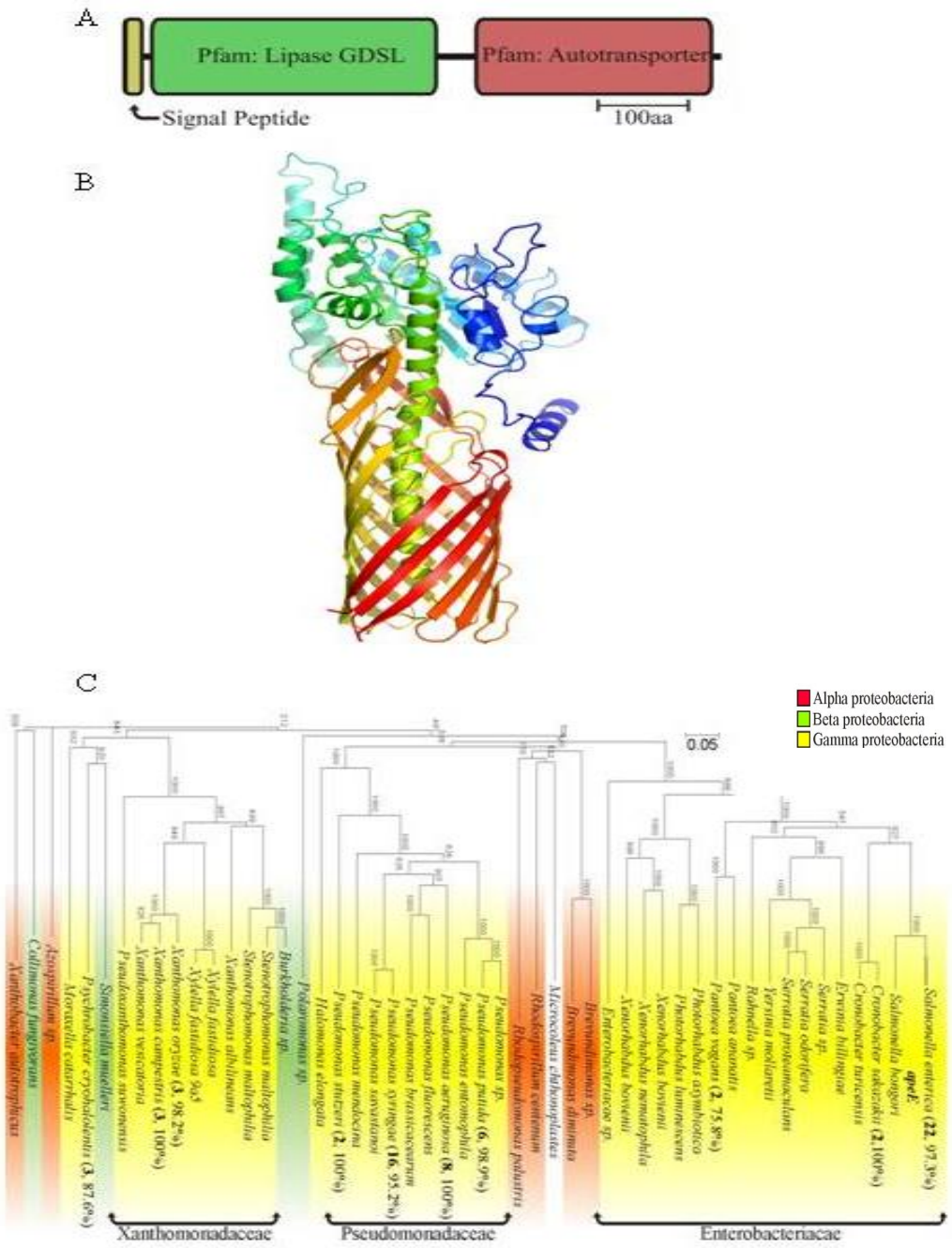


Figure 6.5. Structure and distribution of ApeE

A. Predicted domain structure of ApeE as determined by SMART analysis of the amino acid sequence. Protein contains a Sec-dependant signal sequence, predicted to be cleaved after residue 25. GSDL Lipase passenger domain and, Pfam; Autotransporter domain, encoding a 12 stranded β-barrel. **B.** Predicted tertiary structure of ApeE, as modelled by Dr. V. Bavro **C.** NCBI BLASTP search probing all Gram negative bacteria genomes indicates ApeE is present in all sequenced *Salmonella* strains. ApeE homologues are present in a number of proteobacteria, including human, animal, insect and plant pathogens.

While the 78 amino acids C-terminal translocator domain, identifiable as the Pfam:YadA (PF03895) forms a homo-trimer to create the 12 stranded β-barrel (Fig. 6.6).

To identify SadA homologues all Gram-negative genomes, NCBI was probed using the protein sequence and BLASTP search. For which only resultant sequences with the same domain architecture and >50% similarity were accepted. Homologues were identified in *E. coli, Shigella sonnei, Citrobacter youngae* and *Salmonella enterica* serovars, as shown in Fig. 6.6. In all instances *sadA* is present in the same genomic position, adjacent to *lld* locus, as shown in Fig. 6.6. Sequence alignments of *Salmonella sadA* homologues indicated over 98% similarity, with increasing variability between *E. coli* homologues. However, the homologues from intestinal *E. coli* strains were more conserved than those from extra-intestinal, uropathogenic and environmental strains (174). Through from this multiple sequence alignment the positional homologue, UpaG (uropathogenic *E. coli* strain CFT073) was identified (119).

6.2.3.3. Structure and distribution of SapP

The 978 amino acid protein SapP; *Salmonella* Adhesion and Pathogenicity Protein, is encoded by the gene STM0373. Structural analyses of the amino acid sequence indicated the protein contains a Sec dependant signal sequence, predicted to be cleaved after amino acid 27, the passenger domain; comprising of a Pertactin like region (forming a predicted single stranded right-handed β helix), an AC domain and an α helix, in addition to the 250 amino acid Pfam: Autotransporter domain, which encodes the 12 stranded β-barrel (Fig. 6.7). Modelling of the SapP tertiary structure indicates the AC domain is exposed on the surface, while the α helix resides in the lumen of the barrel, consistent with other Type Va proteins.

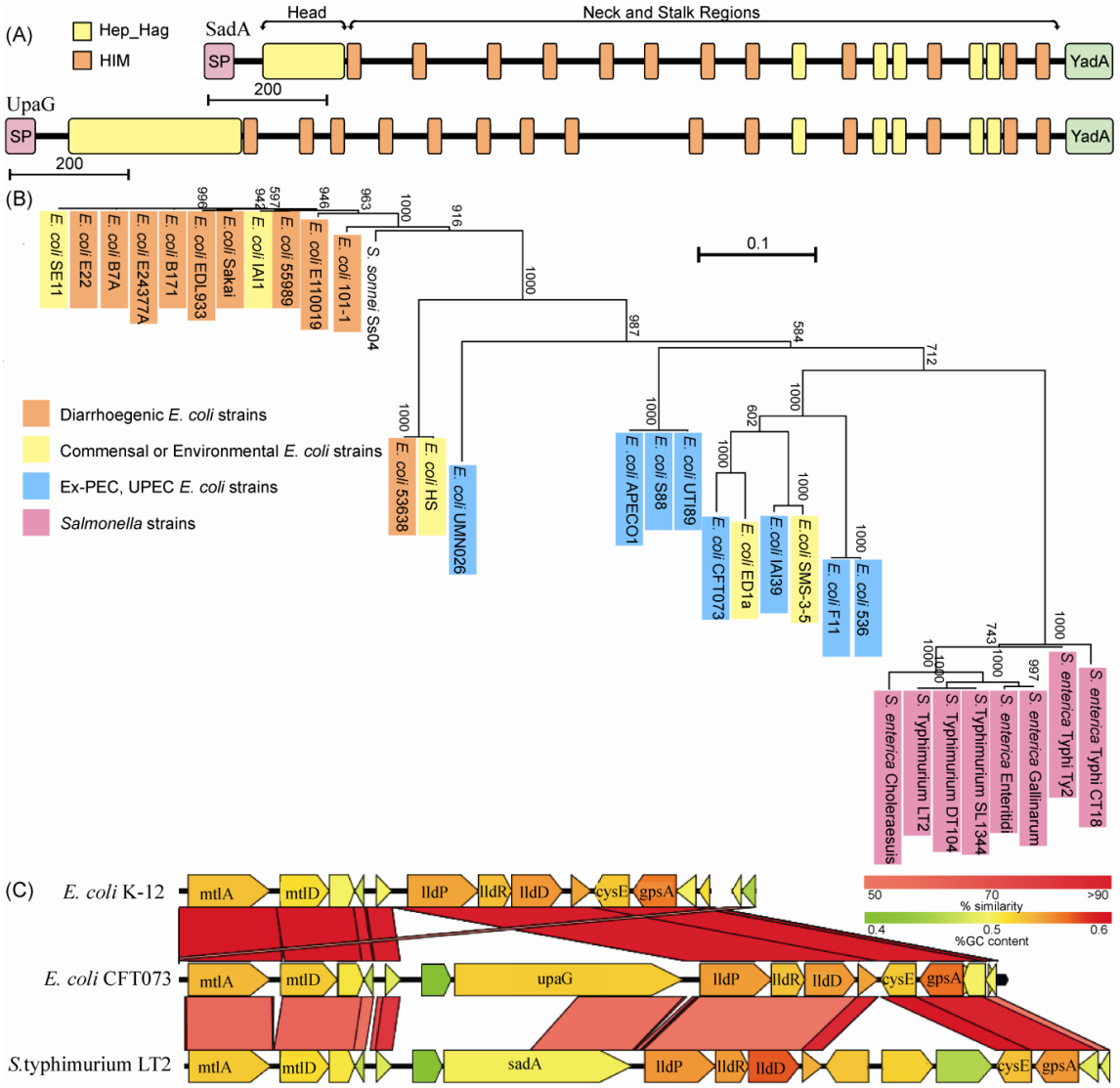


Figure 6.6. Domain organisation and distribution of SadA

In silico analysis of SadA **A).** Schematic diagram comparing SadA and its homology UpaG. Structural domains are shown as coloured boxes, with SP (pink) referring to the signal peptide, the passenger domain which is composed of a head (yellow), with alternating neck (orange) and stalk (black) domains, the translocation (green) domain is described as YadA like domain, whereby each single polypeptide contributes four β strands to the final 12 stranded β barrel. UpaG encodes a larger head domain, with increased neck and stalk domains. **B).** Unrooted phylogram of full length *sadA* orthologues from 32 genomes, where bootstrap values taken from 1000 replicates. Genomes are highlighted according to pathogenic groups. **C).** Genomic region alignments for *S.* Typhimurium with the orthologues regions in *E. coli* CFT073 and *E. coli* K12. Figure taken from Raghunathan *et al* (174).

To identify SapP homologues, all Gram-negative genomes in NCBI were probed using the protein sequence (Fig. 6.7). SapP is present in numerous *Enterobacteriaceae* including *Salmonella* species, *E. coli, C. rodentium, Klebsiella oxytoca* and *Yokenella regensburgei,* to name but a few. Closer inspection shows *sapP* positional homologues are identifiable in *E. coli, C. rodentium* and *E. cloacae* though not in *Klebsiella pneumonia* (Fig. 6.7).

6.2.4. ApeE and SadA are expressed *in vitro* and surface localised

6.2.4.1. ApeE is detectable at the OM

As with all AT proteins, ApeE and SadA are predicted to be localised to the OM, with the passenger domains exposed on the extracellular side. In the case of ApeE the passenger domain is predicted to remain attached to the translocator domain, as is observed for other GSDL ATs (127). To investigate this hypothesis we prepared OM fractions using Triton X 100 extraction. The membranes were treated with urea, to remove loosely associated material or aggregates prior to western blotting using anti-ApeE antibody (Fig. 6.8). ApeE is only detectable as full length (67 kDa) protein, localised to the OM fraction in the *S.* Typhimurium strain SL1344 and complemented mutant. In contrast, ApeE is not detectable in the *apeE* null mutant OM fraction. This result confirms that ApeE does localise to the OM and is not cleaved from the β-barrel.

6.2.4.2. SadA is expression is enhanced at higher temperatures

SadA is a predicted to form a TAA, forming a homotrimer of 426 kDa, localised to the OM with the passenger domain attached. However, expression of previously characterised *S.* Typhimurium ATs, MisL and ShdA is only detectable under *in vivo* conditions, despite varying media types and pH *in vitro* (123, 124). Therefore to confirm the expression of *sadA*

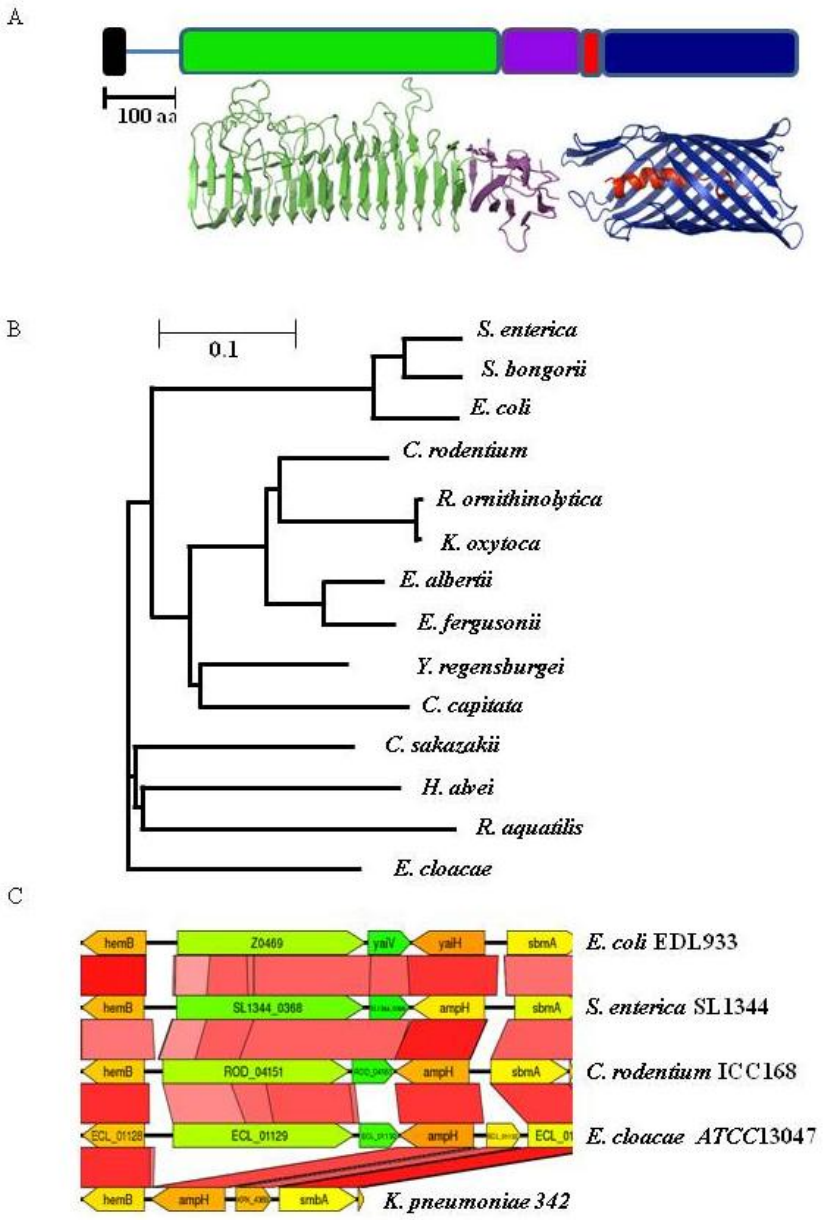


Figure 6.7. Structure and distribution of SapP

A. Predicted domain structure of SapP as determined using the amino acid sequence, with the predicted tertiary structure of each domain shown below. Protein contains a Sec-dependant signal sequence, predicted to be cleaved after residue 27. Pertactin like passenger domain, with a right handed β helical structure (green), AC domain (purple), α helix residing in the barrel lumen (red) and the 12 stranded C-terminal β-barrel. **B.** NCBI BLASTP search probing all Gram negative bacteria genomes indicates SapP is broadly distributed, with homologues present in *Salmonella* strains and closely related species such as *E. coli, C. rodentium,* in addition to more diverse species including *H. alvei* and *E. cloacae.* **C.** Genomic alignments for *S. enterica, E. coli, C. rodentium, E. cloacae and K. pneumoniae* indicating *sapP* is present in the same genomic location in each instance, with the exception of *K. pneumonia,* which does not encode a *sapP* homologue.

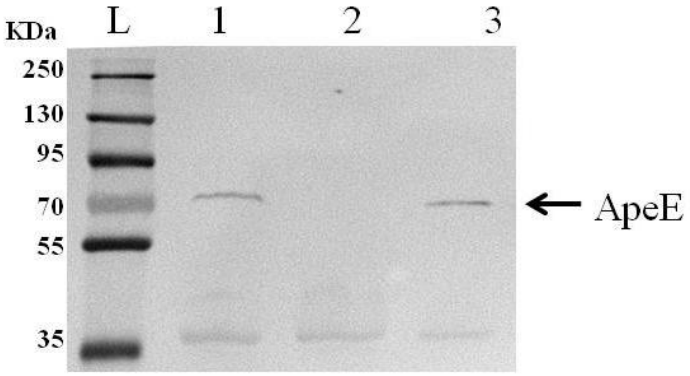


Figure 6.8. OM localisation of ApeE.
Western blot of Triton X 100 extracted *S.* Typhimurium OM fractions, post urea washing, probed with α-ApeE antibodies. L; Ladder, 1. SL1344, 2. SL1344 *apeE::aph*, 3. SL1344 *apeE::aph* pQE60*apeE* (note compliment sample diluted 1:10 compared to samples in lanes 1 and 2). Full length (67 kDa) ApeE protein is only detectable in the OM fractions of the parental strain and complemented mutant.

in vitro RT-PCR was performed after growth in LB broth at 37^{o}C using the primer combination, *sadA* internal forward and reverse. The *sadA* transcript was detectable in the parental strain, but absent from the *sadA* mutant confirming the gene is transcribed under *in vitro* conditions (Fig. 6.9).

Having confirmed the presence of *sadA* transcript, we next sought to confirm that the protein was detectable and localised to the OM. OM fractions were prepared from *E. coli* BL21 pET22b with and without SadA, the *S.* Typhimurium parental strain SL1344, *sadA* mutant or complemented mutant grown in different media types and at different temperatures. OM preparations were western blotted using anti-SadA primary antibodies. Over expression of SadA in *E. coli* produced two proteins corresponding to the monomeric form (approximately 142 kDa) and the trimeric form of SadA (over 400 kDa) indicating SadA adopts its native conformation, when over expressed in *E. coli*. SadA was weakly detected in OM of *S.* Typhimurium SL1344, when grown in LBB at 37^{o}C and absent in the mutant (Fig. 6.9).

Testing a range of growth temperatures (25^{o}C, 37^{o}C, 42^{o}C) and media's; PCN (268), shown to enhance expression of SPI2 (160), MM5.8 Bis/Tris media (269), known to mimic the intracellular conditions of the macrophage vacuole (270) and standard M9 minimal media, which more closely replicate the environment *in vivo*, SadA expression was observed when cultured at either 37^{o}C or 42^{o}C, though protein levels were enhanced when grown in either PCN or MM5.8 media, and significantly at 42^{o}C.

Despite confirming SadA expression and localisation to the OM, this technique failed to confirm that SadA was present on the outer leaflet with a surface exposed passenger domain. Therefore, immunofluorescence was performed using non-permeabilised *S.* Typhimurium SL1344 and *sadA* mutant (Fig. 6.9). Non permeabilised *S.* Typhimurium cells,

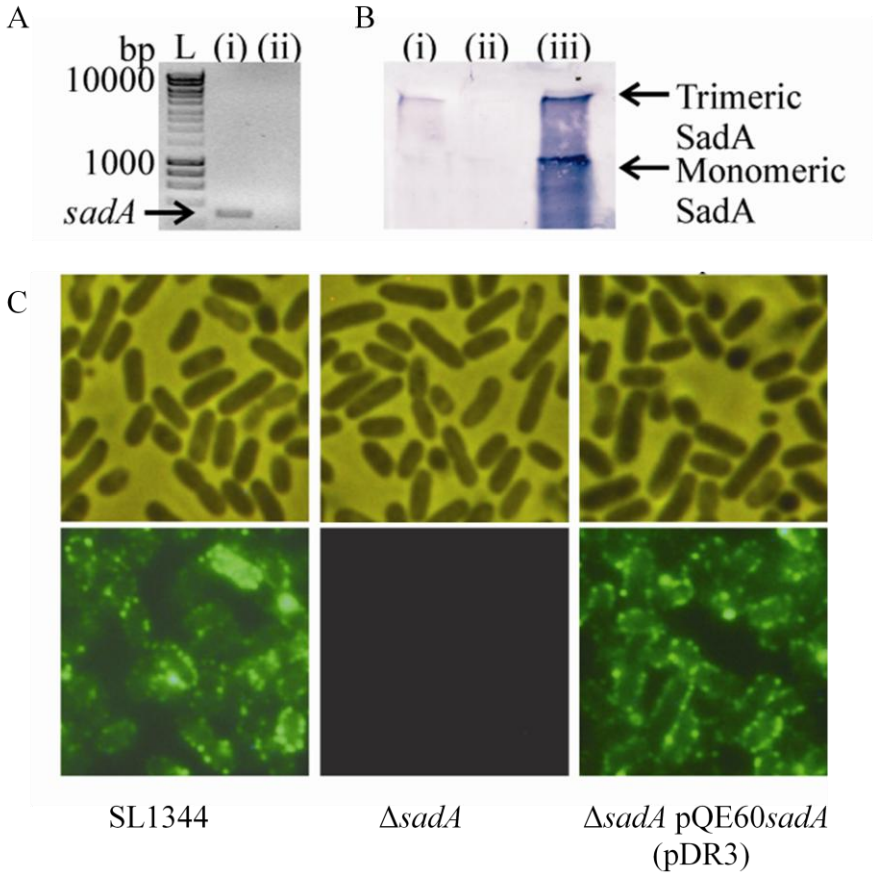


Figure 6.9. Expression and localisation of SadA
A). RT-PCR using primers internal to *sadA*, L refers to Hyperladder 1, i). parental strain SL1344, ii). SL1344 Δ*sadA.* The results confirming *sadA* transcript can only be detected in the parental strain and not the *sadA* mutant. Confirming *sadA* is expressed under standard growth conditions at 37^{o}C in LBB. **B).** Western blot of OM prep for i). SL1344, ii). SL1344 Δ*sadA* iii). SL1344 Δ*sadA* pQ60*sadA* (pDR3), blotted using α-SadA primary antibodies. **C).** Immunofluorescence of SL1344, SL1344 Δ*sadA* and SL1344 Δ*sadA* pQ60*sadA* (pDR3). Using whole non permeabilised cells, staining with α-SadA primary antibodies and GAR alexa fluor488 secondary antibody, indicate SadA displays punctuated labelling at the surface in the parental strain and mutant, but absences in the *sadA* mutant strain. Figure adapted from (174)

stained with anti-SadA primary antibody and Alexa-flour secondary antibody displaying punctuated labelling in both the parental strain and complemented mutant, that was absent in the *sadA* mutant (174). The distribution of AB labelling observed for SadA is consistent with previous observations for YadA (253) though contrasts with other reports suggesting ATs are polarly localised (271). As some TAAs are capable of non-specific binding of immunoglobulin's (143), the following controls were also tested; a). labelling with secondary antibody only and b). labelling with an alternative primary antibody for which there is no corresponding protein in *S.* Typhimurium, anti-Pet antibodies (AT from EAEC). In both instances no labelling was observed, confirming the labelling with anti-SadA.

6.2.5. Characterisation of ApeE catalytic activity

6.2.5.1. ApeE demonstrates esterase activity against TWEEN 80

As the bioinformatic analyses showed ApeE encodes a surface localised GSDL lipase passenger domain with esterase activity, we sought to investigate the substrate specificity of this protein using BioLog Phenotype Microarrays. These assays compare the growth kinetics of parental *S.* Typhimurium with an *apeE* mutant, under 2000 different conditions (data not shown). From these experiments we observed the growth of the *apeE* mutant was consistent with that of the parental strain for the majority of the conditions tested, though in keeping previous observations, the growth kinetics were significantly reduced when using TWEEN 80 as the sole carbon source. To confirm this result, we examined the growth kinetics of *S.* Typhimurium SL1344, *apeE* mutant and the complemented mutant, in M9 modified minimal media supplemented with 1% TWEEN 80 as the sole carbon source for growth (Fig. 6.10). The results clearly indicate deletion of *apeE* renders the bacteria unable to utilise TWEEN 80 as a sole carbon source for growth.

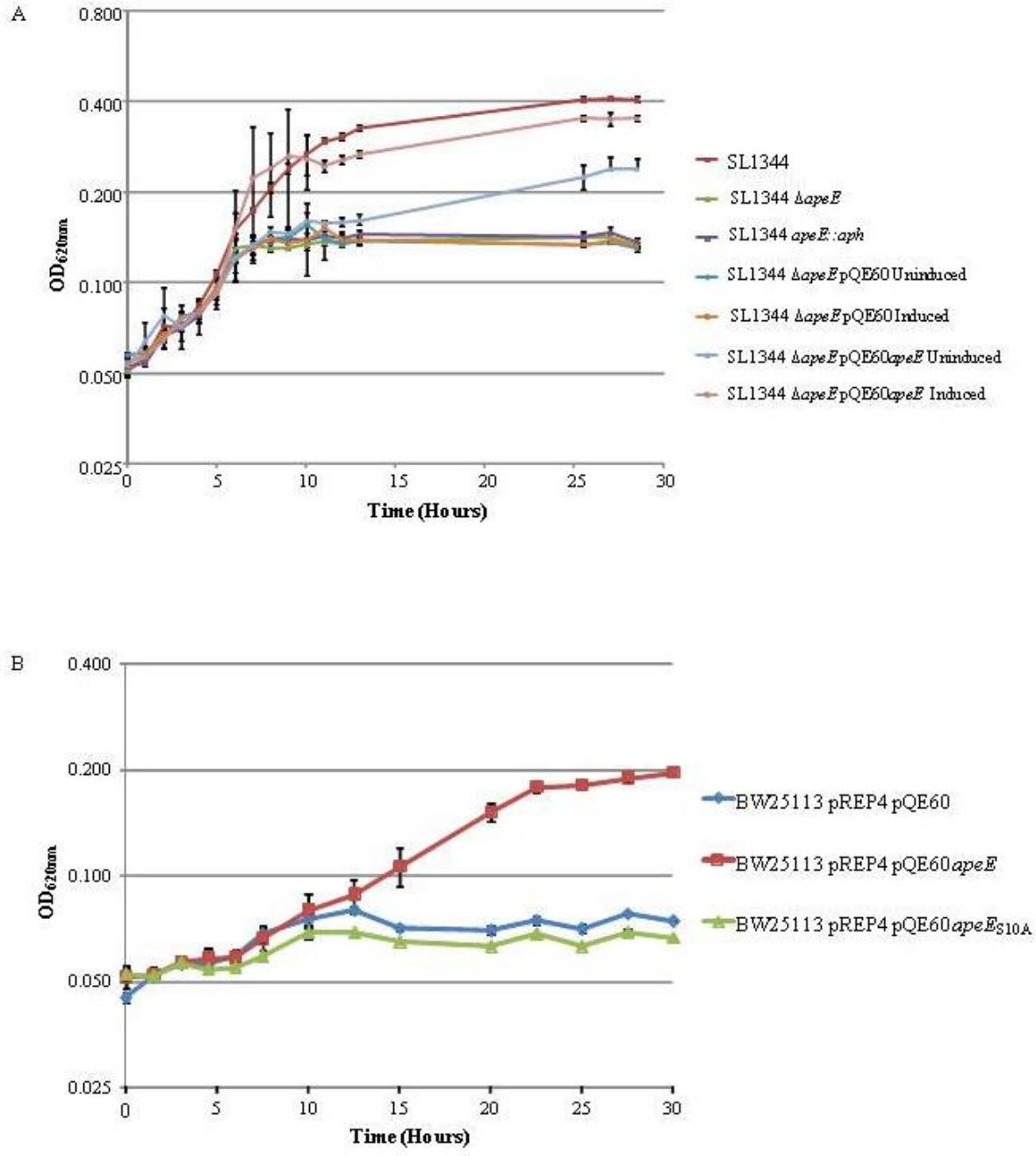


Figure 6.10. Growth kinetics when using TWEEN 80 as the sole carbon source
A). Growth kinetics for *S.* Typhimurium strains in modified M9 media containing 1% TWEEN 80 as the sole carbon source for growth. Indicating only the parental strain and complimented mutant can utilise TWEEN 80 for growth **B)**. Growth kinetics for BW25113 strains, expressing wild type ApeE protein or $ApeE_{S10A}$ mutant protein, whereby the catalytic serine has been mutated to alanine, in BW25113

To confirm ApeE was responsible for this phenotype, we choose to examine the growth of *E. coli* K12 strain BW25113 under the same conditions, as this strain does not contain a natural homologue of ApeE. By comparing *E. coli*, BW25113 containing either the empty vector or plasmid expressing WT ApeE we were able to show that ApeE enables *E. coli* to utilise TWEEN 80 as a carbon source (Fig. 6.10).

In the case of the ApeE homologue EstA catalytic activity is as a direct result of the catalytic serine at position 14 (126). To investigate whether the ApeE serine at position 10 performed a similar role, we mutated this residue to alanine using site directed mutagenesis, to create $ApeE_{S10A}$ (courtesy of Dr. A. E. Rossiter). By examining the growth of *E. coli* BW25113 expressing either the WT ApeE (as above) or $ApeE_{S10A}$ mutant, we were able to confirm that the ability of the bacteria to utilise TWEEN 80 as a carbon source is dependant upon functional catalytic activity (Fig. 6.10).

6.2.5.2 ApeE hydrolyses TWEEN 80 to utilise the fatty acid for growth

The requirement for a catalytic activity suggests that ApeE must cleave TWEEN 80, in order to be used as a carbon source. TWEEN 80 is a polyester of the glucose alcohol sorbitan and a fatty oleic acid, where ApeE is predicted to hydrolyze the molecule between these two components. To determine if the bacteria utilized the sorbitan or fatty acid component as the carbon source, we removed the ability of the bacteria to take up all long chain fatty acids. To do this we obtained the *fadD* and *fadL* mutants from the KEIO Library. These genes encode for components of the Fatty Acid Degradation pathway, with *fadD* encoding the IM component, *fadL* encoding the OM component and while *fadH* acts as the transcriptional regulator for the system. By expressing either functional ApeE or $ApeE_{S10A}$ in these mutant backgrounds we were able to show that utilization of TWEEN 80 as a sole

carbon source, is dependent on a functional FAD system to take up the fatty acid chain (Fig. 6.11), whereby deletion of either the IM or OM FAD components renders the bacteria unable to grow, even in the presence of catalytically active ApeE. Additionally, it should be noted, that all strains show comparable growth kinetics when supplemented with glucose as the carbon source.

6.2.5.3. ApeE degrades the tomato cuticle

Previously, our collaborators had noticed that numerous *Salmonella enterica* serovars (including Typhimurium, Senftenburg, Thompson, Agona, Arizona and Gallinarum) locally degraded the cuticle of tomato skins, during tomato binding assays. As long chain fatty acids are a component of the tomato cell wall biopolymer, cutin (272), we hypothesized that ApeE may be responsible for this phenotype. Having confirmed the role of ApeE in the utilization of TWEEN 80 and the similarity of the structures of TWEEN to that of oleic acid, we sought to investigate whether ApeE was potentially involved in the adhesion and/or survival of *S.* Typhimurium to tomato skins. With the assistance of Dr. R. K. Shaw and Prof. G. Frankel we investigated the ability of *S.* Typhimurium parental strain, *apeE* mutant, complemented mutant and *E. coli* BL21 strains with and without ApeE to adhere and survive on tomato skins for a period of 20 h (Fig. 6.12). The results show that although ApeE is not required for adhesion or short term survival of *S.* Typhimurium on the tomato skin, as determined by the recoverable bacterial counts 20 h post inoculation. The presence of ApeE, results in the appearance of a ‘zone of degradation’ surrounding the bacterial cell for both *S.* Typhimurium and *E. coli* strains, as observable in the SEM images for the parental strain, complemented mutant and *E. coli* BL21 expressing ApeE, but not the *S.* Typhimurium *apeE* mutant or BL21 empty vector, consistent with ApeE functioning as an esterase, locally degrading the tomato

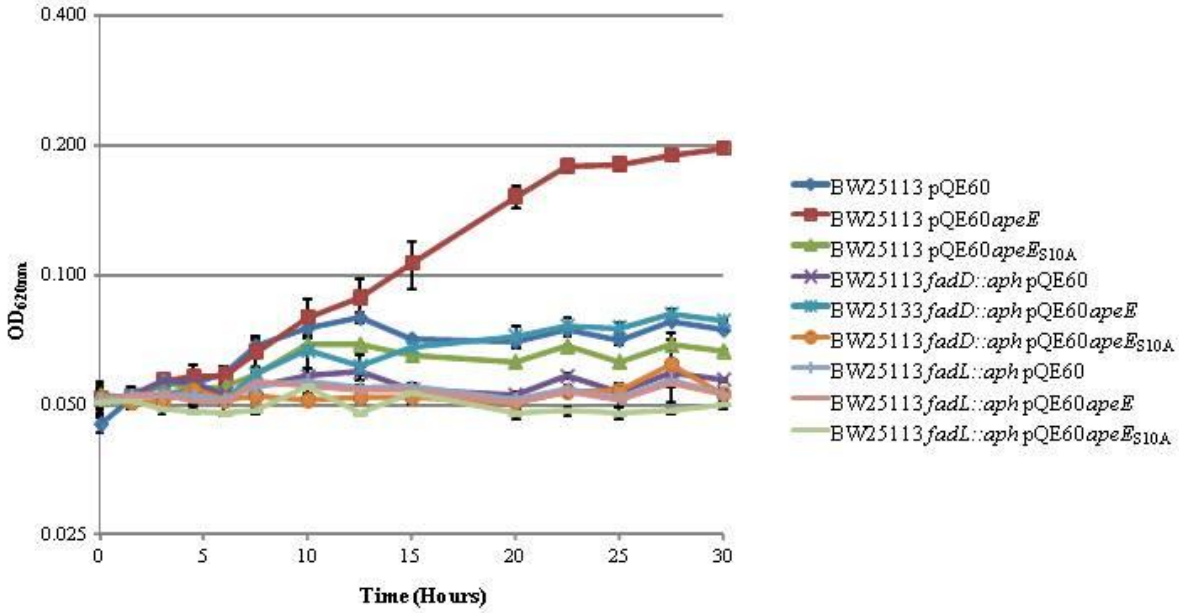


Figure 6.11. Growth kinetics for FAD mutants with TWEEN 80 as the carbon source
Growth kinetics for BW25113 parental strain, *fadD* or *fadL* mutant strains, expressing wild type ApeE protein or an ApeE serine mutant protein, whereby the catalytic serine has been mutated to alanine, with TWEEN 80 as the sole carbon source for growth. Indicating the requirement for both catalytically functional ApeE and FAD system to utilise TWEEN for growth.

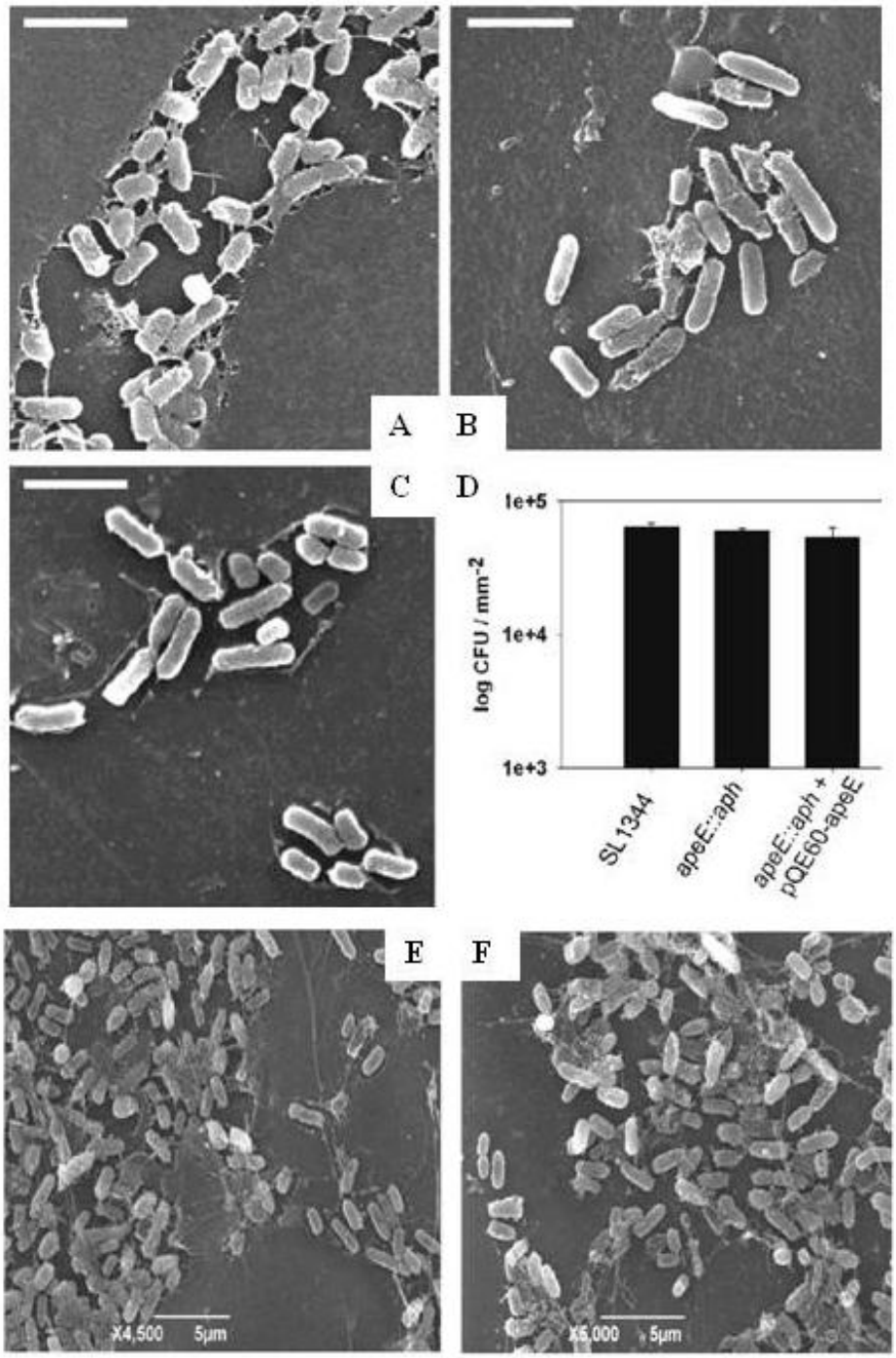


Figure 6.12. Adherence and survival of *S.* Typhimurium and *E. coli* expressing ApeE A). SL1344, **B).** SL1344*ΔapeE,* **C).** SL1344*ΔapeE* pQE60*apeE* **D)**. Adherence of various *S.* Typhimurium strains post 20 hours incubation, **E).** BL21 pET22b*apeE* **F).** BL21 pET22b empty vector. Samples were induced with 10 mM IPTG, where appropriate and incubated on tomato skins for 20 hours at room temperature. Zones of degradation are clearly visible in the strains expressing ApeE, but absent from the mutant and empty vector strain.

cuticle and possibly utilizing this as a carbon source for growth. However, the absence of any change in bacterial numbers post 20 h incubation contradicted the hypothesis that this phenotype was required to obtain a carbon source for growth.

To address this issue, we extended the incubation period to four days, to investigate whether the bacterial survival rates observed after 20 h incubation were an artifact of the relatively short incubation period selected previously. In order to provide a more physiologically relevant environment for this experiment we elected to use whole tomatoes, as opposed to discs of tomato skins utilized previously. Bacterial cultures (adjusted to the same inoculum) were spotted onto the surface of the tomato and incubated for four days in sealed containers at room temperature. After four days incubation bacterial counts were determined by removing the treated area (tomato skin and the underlying fleshy tissues) and homogenizing in PBS, prior to plating on LB agar. Samples of the tomato skin and underlying fleshy tissues were also collected in parallel for TEM analysis. Using this extended incubation period, we demonstrated that only strains expressing ApeE (i.e. the parental *S.* Typhimurium strain SL1344, complemented mutant or *E. coli* strain BL21 expressing *apeE* from pET22b), were recoverable post four days (Fig. 6.13). TEM analysis of the cuticle samples from tomato sections treated with strains expressing *apeE* showed the cuticles were significantly damaged, with necrosis into the underlying fleshy tissues (Fig. 6.13). In contrast, the tomato section treated with the *apeE* mutant showed no damaged to the cuticle.

The results presented as part of this study highlight ApeE mediates the survival of *Salmonella enterica* serovars on tomatoes, enabling the bacteria to not only degrade the cuticle providing a means of accessing the fleshy, sugar rich, underlying tissues, but also utilize the cuticle as a carbon source for survival during the nutrient limited environment of the outer tomato surface.

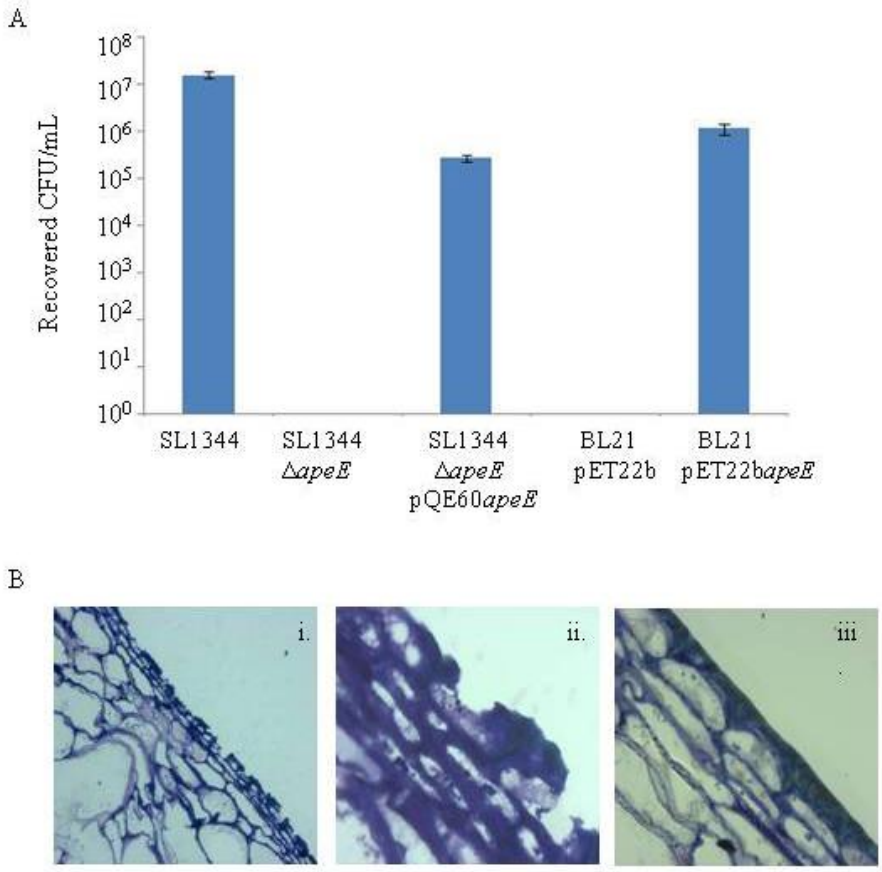


Figure 6.13. Survival of *S.* Typhimurium and *E. coli* strains post four days incubation on tomatoes

A). Bacterial survival, as determined by recoverable CFU/mL, post four days incubation on tomatoes, incubated at room temperature, under sealed conditions. **B).** TEM analysis of tomatoes, sampling from the cuticle down into the underlying fleshy tissue. i). SL1344, ii).Increased magnification of the cuticle, when incubated with SL1344, iii). SL1344 Δ*apeE*. Indicating only strains with ApeE are capable of survival on tomato skins under extended durations (four days) or causing necrosis of the tomato cuticle, providing access to the underlying fleshy tissues.

6.2.6. Deciphering the mechanisms by which Type V proteins contribute to virulence

6.2.6.1 S. Typhimurium ATs promote adhesion to ECM molecules

As ATs and TAAs are commonly associated with binding to ECM molecules, we sought to investigate whether SapP and SadA were also capable of this phenotype. Previous investigations with MisL and ShdA have shown both of these proteins are capable of binding to ECM molecules, including fibronectin and collagen IV (MisL only) (123, 124). Additionally the SadA homologues UpaG and EhaG have been shown to bind fibronectin, laminin, and collagen I, II, III, and V (128). To examine the possibility that the SapP and SadA may also be involved in binding other ECM molecules, we investigated this hypothesis by expressing these proteins in the *E. coli* K12 strain BL21, to exclude the possibility that the results maybe distorted by the presence of other OM features such as long chain LPS (Fig. 6.14). The results show that in contrast to other TAAs and characterised homologues, SadA does not promote binding to collagen III, IV or fibronectin, while SapP can promote binding to all of these molecules, to varying degrees.

6.2.6.2. SadA does promote adhesion and invasion of epithelial cells

As several characterised TAAs have been shown to be associated to binding to various different cell types, we next sought to examine whether SadA mediated adhesion to epithelial cells. To investigate this hypothesis we examined the ability of both the *S.* Typhimurium parental strain and *sadA* mutant, alongside the *E. coli* K12 strains over-expressing SadA to adhere too and invade human intestinal cell line; CaCo-2 (Fig. 6.15). No differences were observed between the *S.* Typhimurium strains, however expression of SadA from *E. coli* M15 significantly increased their ability to adhere to and invade CaCo-2's, $P=<0.05$ and $P=<0.01$, for adhesion and invasion, respectively.

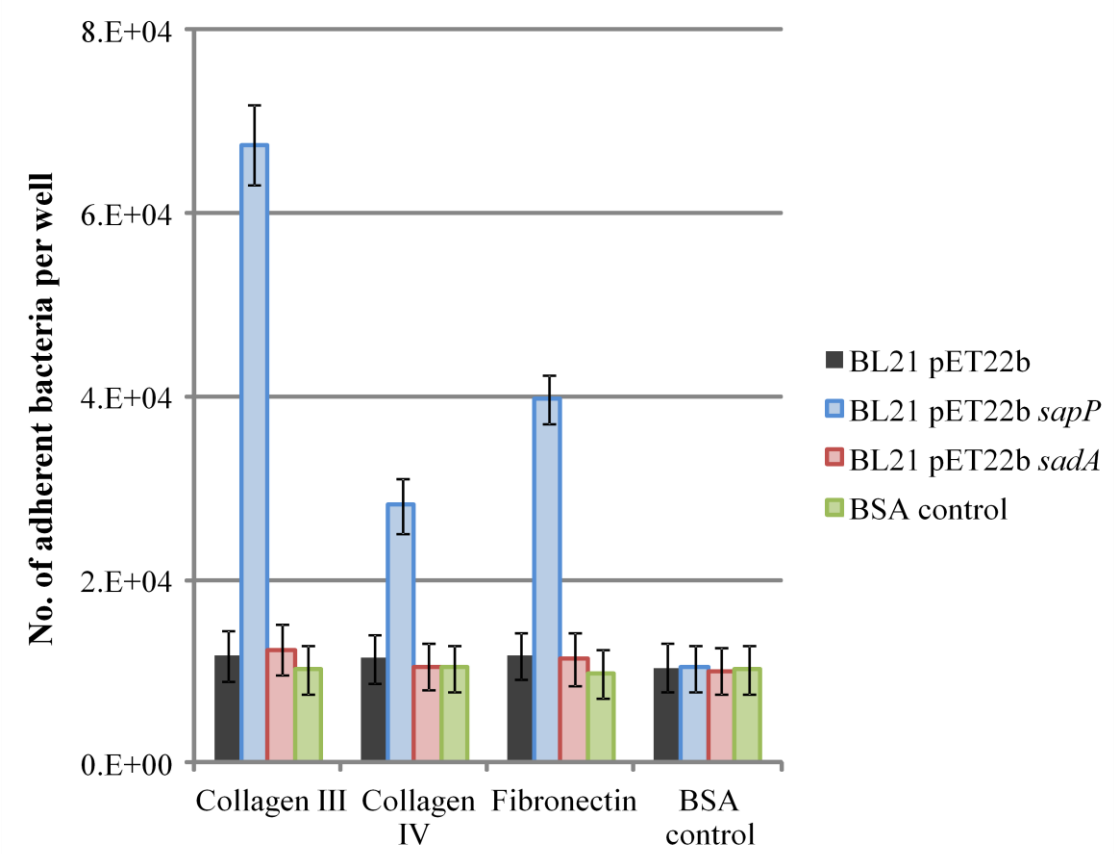


Figure 6.14. SapP promotes binding to ECM molecules
Binding of *E. coli* BL21 strains expressing either SapP or SadA, indicates SapP can promote binding to Collagen III, IV and fibronectin. In contrast SadA does not promote binding to any of the ECM molecules tested

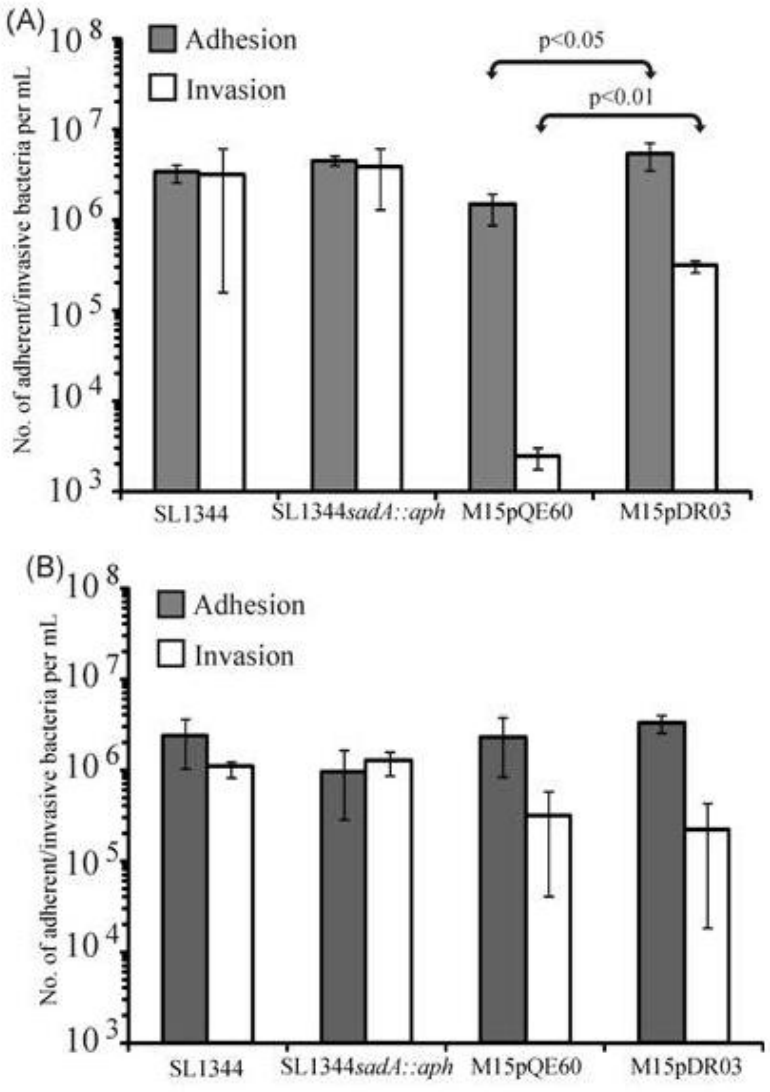


Figure 6.15. Adhesion and invasion of CaCo-2 intestinal cells and J774 macrophages
Adhesion and Invasion of *S.* Typhimurium SL1344 and SL1344 *sadA::aph* and *E. coli* M15 pQE60, or M15 pQE60*sadA*, to **A).** CaCo-2 intestinal cells or **B).** J774 macrophages. *E. coli* expressing SadA, demonstrated a significant increase in its ability to both adhere to and invade CaCo-2 cells, compared with the empty vector, where P=<0.05 and P=<0.01, respectively. No differences were observed between the ability of any strain to adhere or invade J774 macrophages. Figure adapted from Raghunathan *et al* (174).

As *S.* Typhimurium is capable of systemic infection, whereby adhesion and invasion of macrophages is critical for dissemination throughout the lymphatic system, we next sought to examine whether SadA could also promote adhesion and invasion of the murine macrophage cell line, J774. Using the same strain set as described previously we confirmed that SadA does not promote adhesion or invasion in this particular cell type (Fig. 6.15).

Although we had conclusively shown SadA was not required for adhesion or invasion to murine macrophages, we questioned whether the other type V proteins of *S.* Typhimurium may be associated with this phenotype. In order to test this hypothesis we examined the ability of *S.* Typhimurium multiple deletion strains to adhere to and invade three separate macrophage cell lines, the murine macrophages; J774 and RAW 264.7 and human differentiated monocytes, THP1, to confirm the results were not cell type specific. Fig. 6.16 indicates no significant differences were observed in the ability of any strain to adhere to or invade macrophages.

6.2.6.3. SadA does promote autoaggregation and biofilm formation

Several TAAs are associated with biofilm formation and autoaggregation, including the SadA homologue UpaG (119, 138, 143). To determine whether SadA was associated with these phenotypes, we examined the ability of *S.* Typhimurium strains SL1344, *sadA* mutant, complemented mutant and SadA expressing *E. coli* to undergo aggregation and biofilm formation (Fig. 6.17). No statistically significant differences were observed in the ability of *S.* Typhimurium strains to undertake either phenotype. In contrast, SadA expressed in an *E. coli* K12 background (defective for O-antigen) significantly enhanced aggregation and biofilm formation ($p=<0.001$). Previous reports have highlighted the importance of self to self recognition between Type V proteins to promote these phenotypes, whereby OM features

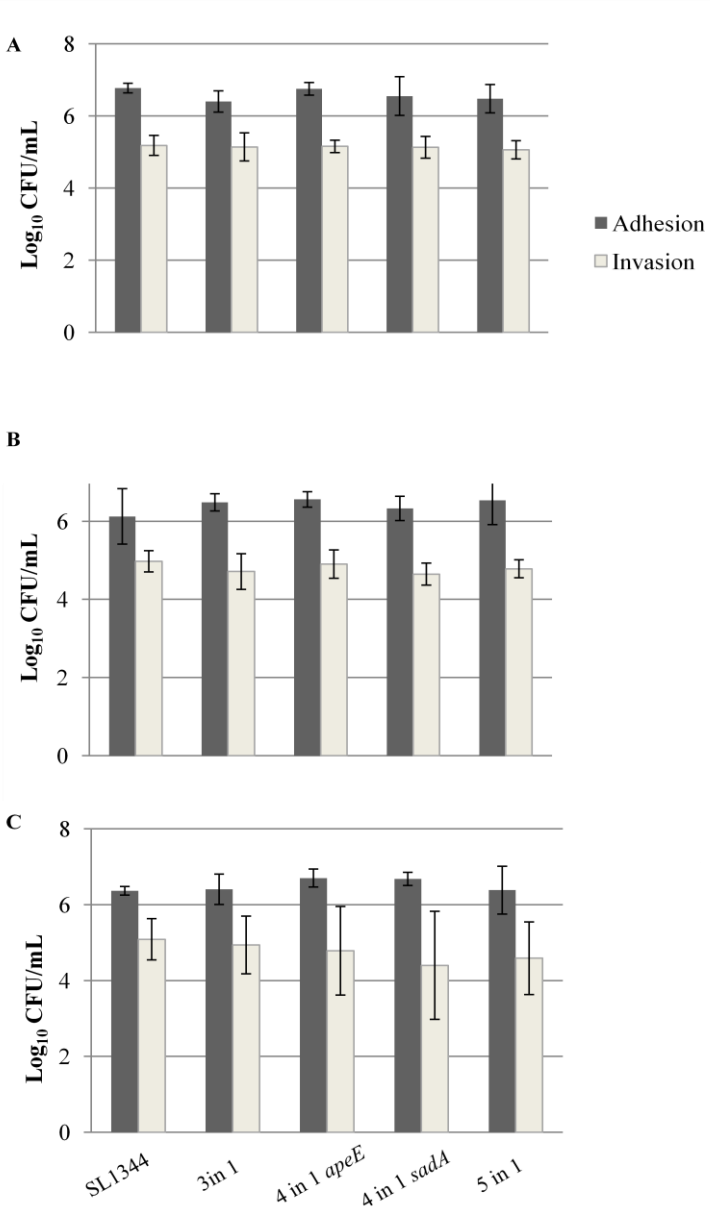


Figure 6.16. Adhesion and invasion of macrophage cell lines with *S.* Typhimurium AT multiple knockouts

A). J774 murine macrophages, **B).** RAW 264.7 murine macrophages, **C).** THP1, human monocytes differentiated into macrophages. Results are the average of three separate experiments, with a minimum of three biological replicates for each. Data expressed as $\log_{10}$ CFU/mL of adherent or intracellular bacterial. The results indicate that deletion of the ATs does not affect either adhesion or invasion of *S.* Typhimurium on any macrophage cell lines, and this result is not cell line specific.

including; capsular polysaccride, O and K antigens and Type 1 fimbriae are capable of blocking these interactions through steric hindrance (273). Therefore, we examined whether O-antigen in *S.* Typhimurium was potentially blocking the self to self interaction of SadA. To achieve this we constructed the double mutant SL3261 Δ*sadA, galE::aph*, using P22 transduction. Disruption of *galE* results in a rough LPS phenotype, lacking O-antigen, due to the inability to catalysing the conversion of UDP-glucose to UDP-galactose during cell wall synthesis (274). By comparing the double Δ*sadA, galE::aph* mutant with the same strain over expressing SadA, we revealed SadA significantly increases in the ability to undertake both phenotypes, as shown in Fig. 6.17 confirming SadA can promote both phenotypes but is susceptible to steric hindrance by other OM structures.

6.2.6.4. SadA does not promote serum resistance

As *sadA* is the only gene encoding a TAA in *S.* Typhimurium we choose to examine whether this protein contributed to serum resistance, as observed for the prototypical TAA YadA (143, 174). Thus, we initially examined whether deletion of *sadA* resulted in elevated sensitivity to complement-mediated killing in healthy human serum. By examining the survival of *S.* Typhimurium parental strain SL1344 and *sadA* mutant, we confirmed that deletion of *sadA* does not affect the ability of *S.* Typhimurium to resist complement-mediated killing *in vitro* (Fig. 6.18). Therefore, this result raised the possibility that other AT proteins in *S.* Typhimurium may be capable of promoting serum resistance. To investigate this, we compared the survival of the *S.* Typhimurium parental SL3261 strain with the various multiple deletion strains. As this assay was performed using the *S.* Typhimurium SL3261 mutants, all strains showed some level of sensitivity to complement-mediated killing, (as determined by decreasing CFUs post 45 minutes), though the 4 in 1 *sadA* and 5 in 1 mutants,

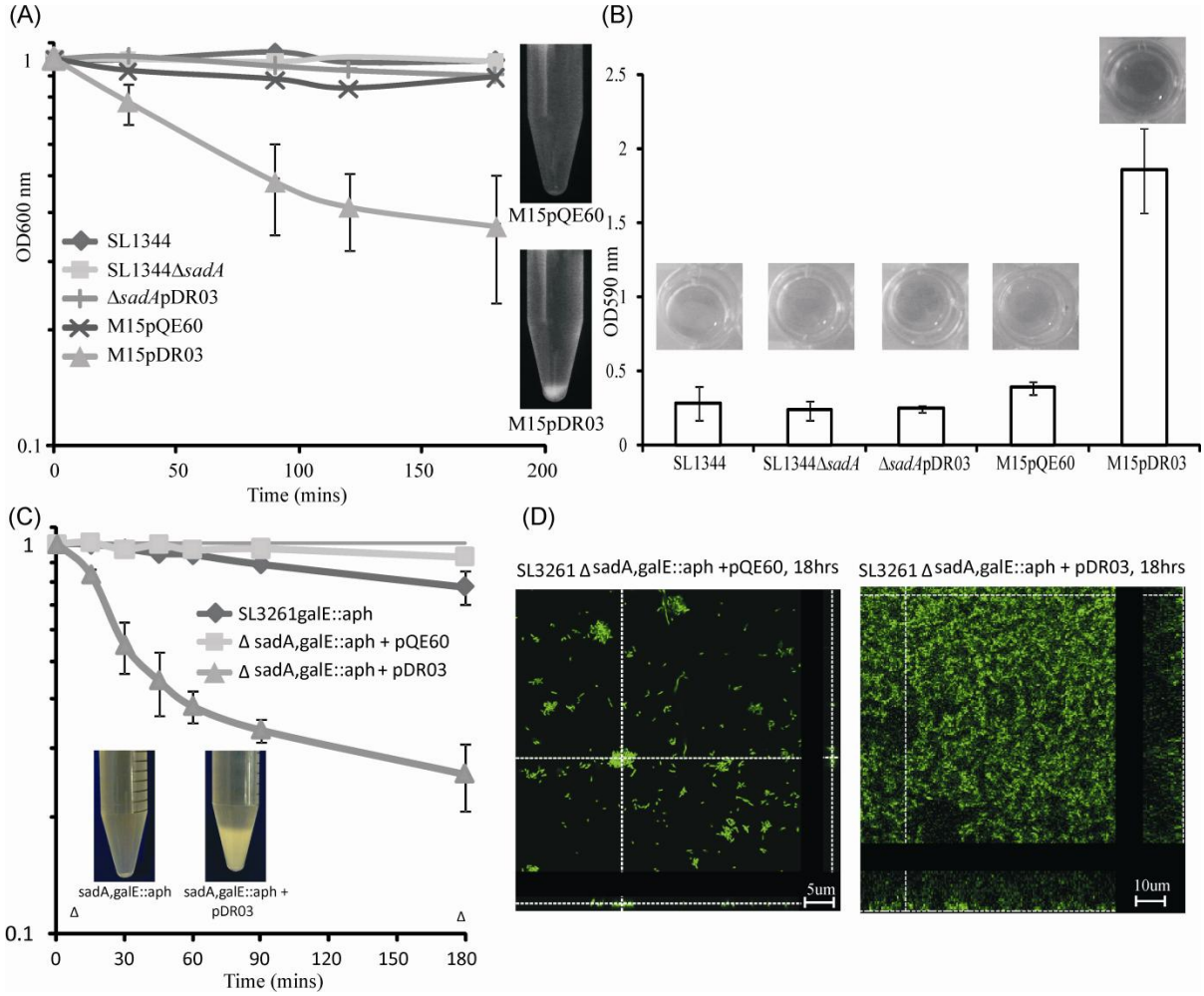


Figure 6.17. Autoaggregation and biofilm formation
A). Settling profiles of STm and *E. coli* expressing SadA strains, images show settling from static liquid suspensions in the SadA expressing *E. coli* M15 strain.where by only *E. coli* M15 expressing SadA (pDR3) shows significant aggregation compared to the parental STm strain or M15 empty vector. **B).** Biofilm formation of the same strains in polystyrene plates. The data is the average of eight replicate samples, performed on three separate occasions **C).** Settling profiles for STm *galE::aph* and Δ*sadA galE::aph* double mutants, with either the empty vector or complemented pDR3, only over expression of SadA in a *galE* deletion background enhances aggregation of the bacteria. **D).** Analysis of biofilm formation using flow cell chambers, for the Δ*sadA galE::aph* double mutant, with either the empty vector or complement, pDR3 post 18hours growth. Figure taken from Raghunathan *et al* (174).

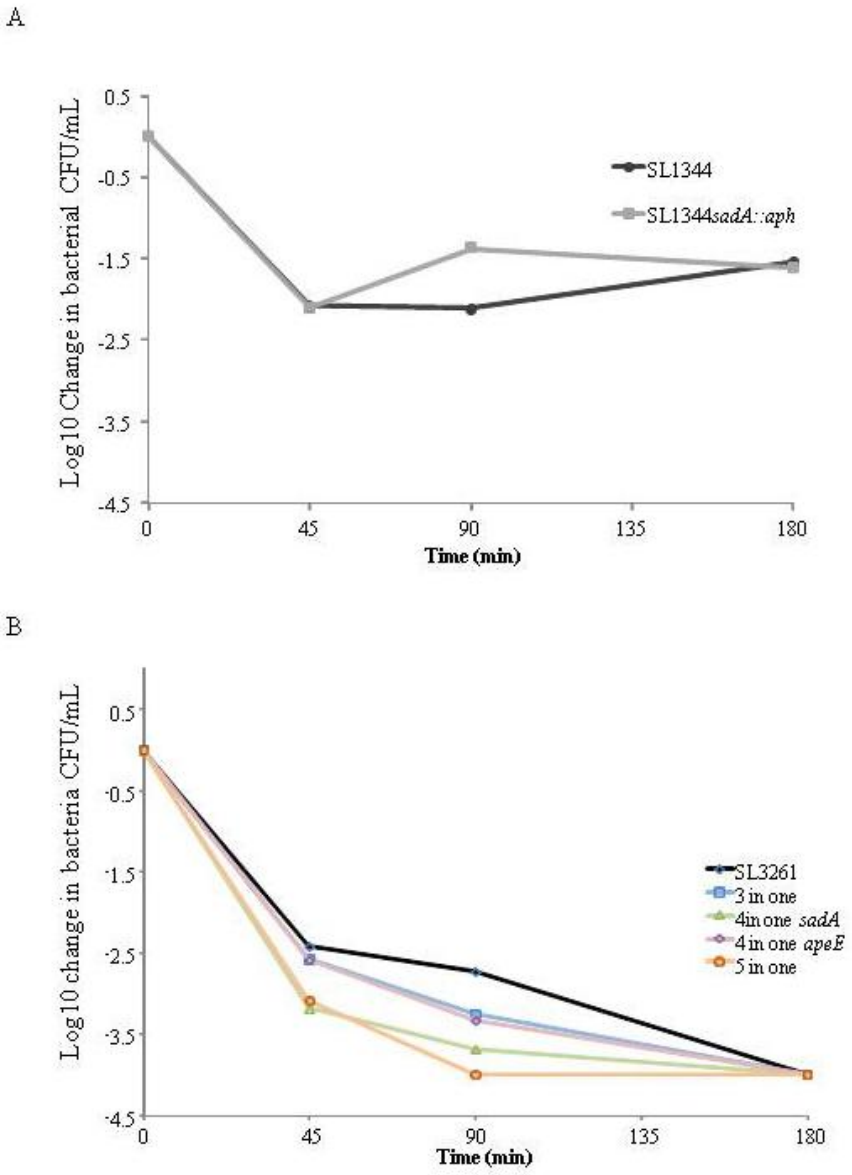


Figure 6.18. Serum resistance of *S.* Typhimurium in healthy human serum
A. Resistance of *S.* Typhimurium SL1344 parental strain and *sadA* mutant. **B.** Serum resistance of *S.* Typhimurium SL3261 parental strain and multiple deletion strains in healthy human serum, as determined by reducing $\log_{10}$ changes in bacterial CFU/mL over time. All strains show sensitivity to serum, though in the absence of other classical ATs, deletion of *sadA* (i.e. the 4 in 1 *sadA* and 5 in 1 mutant) show slightly enhanced sensitivity to serum compared with the other mutants, particularly the 5 in 1 mutant which is killed by 90 minutes, compared to all other strains which are killed by 180 minutes.

showed the most significant decrease, while the 3 in 1 and 4 in 1 *apeE* mutant were more comparable with the parental strain. The 5 in 1 mutant did display slightly increased susceptibility compared to the other strains, with complete killing by 90 min, in contrast to the other mutants, which were killed by 180 min (Fig. 6.18), indicating that ATs may contribute to serum resistance of *S.* Typhimurium.

6.2.7. Immunomodulatory effects of *S.* Typhimurium SadA

Despite the fact that SadA is not critical for virulence in the murine model, this protein forms a large surface exposed structure, accessible to the immune system during the course of infection, we therefore chose to examine whether SadA was immunogenic and a suitable vaccine candidate. In order to achieve this SadA was purified, refolded as determined by tryptophan fluorescence, shown to be endotoxin free, within the limits of detection using a Sigma E-toxate kit and protein concentration determined using the Tannin assay (174). Using purified SadA we aimed to answer two hypotheses; i). was SadA immunogenic and ii). could a SadA AB response protect against infection. The challenge study protocol is shown in Fig. 6.19. Mice were imunised with either purified SadA, SadA bound to Alum (known adjuvant) or PBS at days 0 and 30, prior to challenge with virulent *S.* Typhimurium SL1344 at day 44 and sacrificed at day 46, with bacterial burdens assessed in the liver and spleen. ELISAs were performed on blood samples taken prior to challenge (day 44) and sacrifice (day 46) to determine the relative amounts of SadA specific antibodies.

ELISAs using the pre and post challenge serum demonstrated SadA is immunogenic, stimulating an IgG and IgM response, though the relative antibody titres are enhanced by combination with alum. We were able to show SadA is expressed during infection, with a SadA specific IgM responses induced in the PBS control group 48 hours post infection. AB

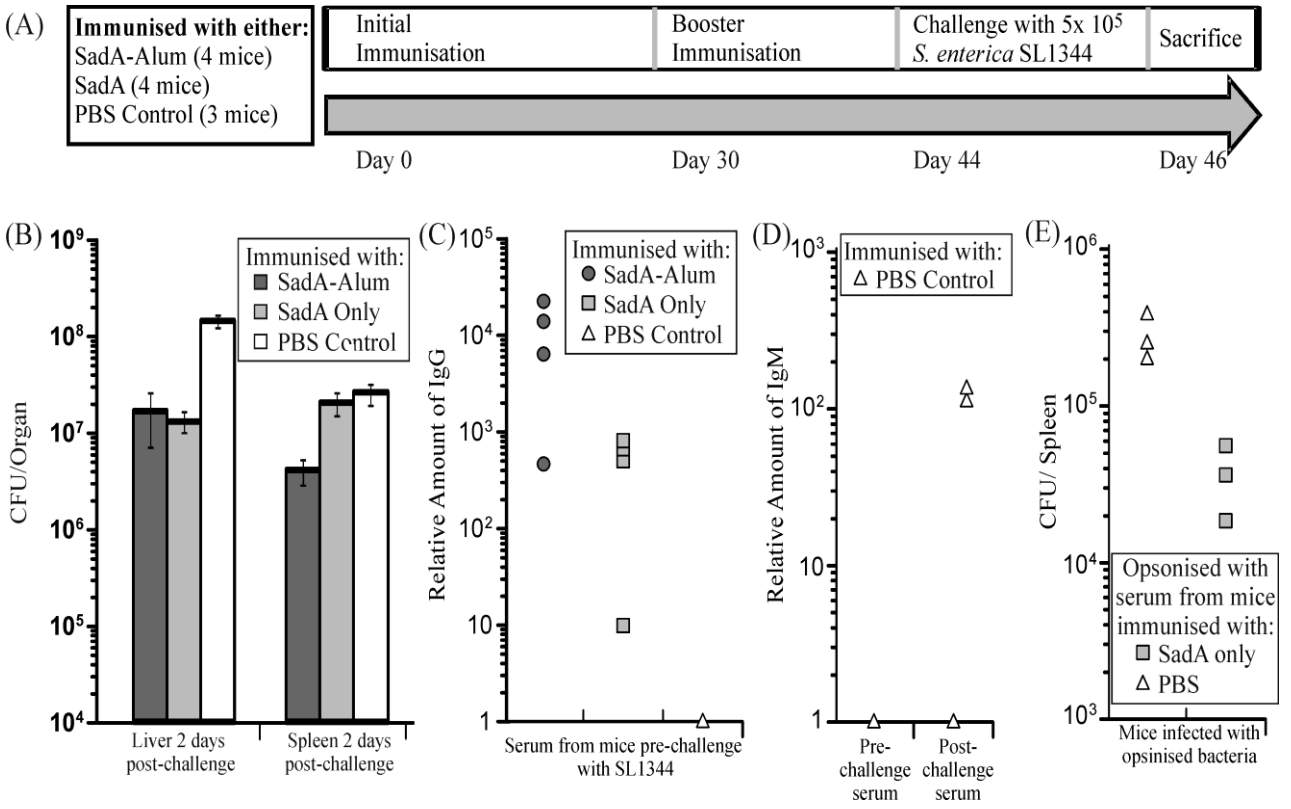


Figure 6.19. SadA is immunogenic and provides limited protection against infection
A). The experimental design of the challenge study. C57BL6 mice were immunised with either PBS only (control) or 10 μg purified SadA with or without Alum on two separate occasions prior to challenge with virulent SL1344. Blood samples taken prior to challenge (day 44) and immediately prior to sacrifice (day 46). **B).** Bacterial burdens determined as CFU/organ, for liver and spleen two days post challenge. Animals having received pre-exposure to SadA, demonstrate limited protection against infection, compared to the PBS treated control group. **C).** IgG response to purified SadA from pre-challenge serum, expressed as relative amounts. SadA is clearly immunogenic and stimulates and IgG response, when administered alone, though this is enhanced by precipitation with alum. Control (PBS) group do not have an IgG response to SadA pre-challenge. **D).** IgM response to SadA is observed in the control (PBS) group 48 hours post challenge, but not pre-challenge, indicating SadA is expressed during infection. **E).** Bacterial burden per spleen from C57BL6 mice post five days infection with STm; SL3261 opsonised with heat-inactivated pre-challenge serum taken from animals exposed to either purified SadA or PBS only, to confirm the limited protection observed in the challenge study is as a result of SadA antibody mediated response. Figure taken from Raghunathan *et al* (174).

responses stimulated by SadA were mildly protective, as determined by the 6.5 and 10 fold reduction in bacterial burdens in the spleen and liver of SadA-Alum treated mice, when compared to the PBS controls, p= <0.05 and p=<0.01 for spleen and liver respectively. SadA-only treated mice showed a 10 fold reduction in bacterial burden in the liver (p=<0.01), though no differences were observed in the spleens.

To confirm the protective effects were due to SadA mediated AB responses we opsonised *S.* Typhimurium SL3261, with heat inactivated (to destroy complement) pre-challenge sera from either SadA only or PBS treated mice, prior to IP administration. Bacterial burdens were determined after five days and the SadA opsonised group displayed a similar, 10 fold reduction in spleen bacterial burdens when compared to the PBS opsonised group indicating SadA does induce an AB response that provides mild protection.

6.3 Discussion

Characterised type V proteins are typically virulence factors associated with wide ranging functions, including mediating serum resistance, binding to ECM molecules and adhesion and invasion of eukaryotic cells, including animal and plant cells (119, 138, 144, 275, 276). Despite previous investigations into ApeE, MisL and ShdA from *S.* Typhimurium, the context of previous findings regarding substrate specificity of ApeE has not been linked to pathogenesis in any host, nor has the potential for functional redundancy been investigated despite the significant functional overlap observed for MisL and ShdA (123, 124, 135, 257, 258). During this investigation we therefore aimed to characterise the functions of *S.* Typhimurium ATs proteins, with regards to pathogenesis in known hosts and vehicles of *S.* Typhimurium transmission. The investigations presented as part of this study have enhanced our understanding of several aspects; the role of *S.* Typhimurium AT proteins in murine

pathogenesis and their functional redundancy, the biological context of ApeE substrate specificity and how *Salmonella* exploit this to use fruits as a vehicle of transmission and we have provided the first peer-reviewed characterisation of a TAA from *Salmonella* (174).

Previous characterisations of MisL and ShdA have shown both of these ATs are required for intestinal colonisation and faecal shedding (123, 135). Therefore, we chose to examine whether other uncharacterised proteins also contributed to virulence in the murine model. By examining bacterial burdens post oral and IP infection, we have confirmed SapP is required for full virulence. In contrast, SadA is not required to mediate infection in this host. These results partially conflict the recently published TRADIS data examining the role of non-essential genes in *S.* Typhimurium, whereby mutations in both *sadA* and *sapP* (annotated as *yaiU*) resulted in attenuated virulence in chickens, cattle and pigs but not mice, when administered orally (220). Our subsequent investigations of SadA are consistent with this hypothesis, in that we observed *sadA* expression was enhanced at higher temperatures, which parallel the normal body temperature of poultry, birds and livestock, which ranges between 38.5-43.8^{o}C, between resting and high activity states for birds respectively, while pigs and cattle range between 37.8–39.9°C (277-279). Suggesting this virulence factor maybe more critical for infection in other hosts. The differences between the literature and the results presented during this study for SapP cannot be easily explained and therefore require more extensive characterisation of this protein to determine its role in other aspects of pathogenesis.

Despite reports of overlapping function between the previously characterised MisL and ShdA, this is the first instance where these genes in addition to the other 'classical AT' *sapP* (STM0373) have been deleted simultaneously, with the additional deletion of either *apeE* and/or *sadA*. This offers the unique opportunity to study the degree of functional redundancy among the type V proteins of *S.* Typhimurium and the contributions of ApeE and

SadA in absence of other Va proteins. Using these multiple deletion strains; we have been able to identify unique events previously unobserved in single deletion strains. For example, the persistent infection observed in the liver for strains retaining only ApeE may reside from ApeE's functions as an esterase, potentially hydrolysing fatty acid substrates, including liver triglycerides for utilisation as a carbon source. This is consistent with our investigations showing ApeE is required for the hydrolysis of fatty acids and the characterised substrate specificity of thioesterase proteins from *P. aeruginosa,* responsible for the degradation triglycerides, including glycerophosphocholine (280). Unfortunately, due to the time constraints of the project, this phenotype has yet to be explored fully, and requires significant further investigation, though if found to be correct provides new insights as to the role of ApeE in persistent infection and would be the first report of ApeE functioning as a key virulence factor for the long term survival and persistence of *S.* Typhimurium infections in the mouse liver.

Our investigations into the substrate specificity of ApeE has provided biological context to this previously known phenotype. During this study, we have shown ApeE is required for degradation of the tomato cuticle and identified the component, oleic acid, which ApeE most likely uses as a carbon source. Degradation of the cuticle allows the bacteria to both penetrate to the underlying sugar rich, fleshy tissues, but also provide a carbon source for growth during nutrient limiting conditions. This enhances our understanding of the role of this specific protein in the pathogenesis of numerous *Salmonella* serovars, but also expands our understanding of how bacteria uses fruit and vegetables as a vehicle for transmission to other hosts. In contrast to public perception, a leading cause of gastroenteritis caused by *S.* Typhimurium in developed countries, such as the USA and Canada, is as a result of consuming contained fruit and vegetables, with outbreaks in the USA now more commonly

attributed to this than poultry (152-154). A wide range of fresh produce has been attributed to outbreaks of *Salmonellosis* including melons, serrano peppers, lettuce and basil, but most frequently to the consumption of contaminated tomatoes (281-287). Though previous investigations into the mechanisms by which *Salmonella* adhere to fruit and vegetables has largely focused on OM features such as flagella, curli, cellulose and O antigen, with mechanisms shown to be serovar specific in most instances (288). In contrast, little investigation has involved the role of type V proteins, with MisL being the first reported *S.* Typhimurium AT to be involved in adhesion to lettuce leaves (276). This study has uniquely provided evidence for a mechanism which is not serovar specific, in addition to characterizing the molecular pathways involved. We have confirmed the role of the catalytic serine, as with other GSDL ATs and the requirement for a function FAD system, for fatty acid uptake. Unfortunately, we have yet to confirm this substrate specificity with more physiologically relevant fatty acids, therefore future experiments are planned using cutin, to confirm both the role of ApeE in hydrolysis and also the utilisation of the fatty acid molecule as a carbon source for growth.

Furthermore, our investigations characterising the specific roles of SadA and in part SapP, have shown the functions of this particular TAA and AT are unique from their respective homologues. TAAs for example are commonly associated with functional roles including regarding adhesion to eukaryotic cells and ECM molecules, biofilm formation and autoaggregation and serum resistance. Although we have been able to confirm roles for SadA in mediating phenotypes such as autoaggregation, biofilm and adherence to epithelial cells, we have shown the functions of this protein are susceptible to steric hindrance as these phenotypes could only be observed when using *S.* Typhimurium strains with rough LPS and with rough *E. coli* K12 strains. In contrast to the characterised role of EhaG and UpaG (SadA

homologues) from EHEC and UPEC, respectively, we were unable to identify binding of SadA to any ECM molecules. As described previously, we were also unable to clarify the role of SadA in mediating serum resistance.

Although our investigations into the role of SapP are preliminary, we have determined key phenotypes for which SapP is associated and these may contribute to virulence of *S.* Typhimurium. In contrast to the homologue EhaB from EHEC, we have shown SapP is uniquely capable of binding collagen III, but also collagen IV and fibronectin, similar to that previously described for MisL and ShdA (123-125). This may in part contribute to the reduced virulence observed when *sapP* single mutants are administered orally. However, this hypothesis requires confirmation, by examining bacterial colonisation at specific gastrointestinal sites, such as the Peyers patches and MEL, in addition to *in vitro* assessments examining the adhesion of *sapP* mutants various epithelial cell lines. Unfortunately due to the time constraints these assays have yet to be performed, though the outcomes will significantly contribute to our understanding of the role of this protein in pathogenesis.

As type V proteins contribute large surface structures which in the cases of MisL, ShdA and SadA, are known to be expressed during *in vivo* infection, we wanted to examine their potential as suitable vaccine candidates for future investigations. Unfortunately, we have only performed these investigations for SadA; this protein is immunogenic, promoting an IgG and IgM AB response that is mildly protective against infection. Unfortunately, limited IgA responses were observed, however this maybe relate of the route of administration (this study IP) as it has been shown previously that oral administration induces a more significant secretory IgA response (289). However, the challenges associated with oral administration (i.e., protein stability and folding on transit through the stomach) make this an unfavourable route for the assessment of vaccine efficacy.

Although alone SadA would not be suitable as a vaccine candidate, this does raise the possibility that it may provide a suitable component towards a multivalent vaccine against *S.* Typhimurium. Within our group, current investigations are underway to express all *S.* Typhimurium AT proteins using the Pet AT platform of secretion (106), to provide a means of investigating the immunogenicity of all these proteins and their potential as vaccine candidates, examining their ability to induce specific AB and memory mediated T-cell responses.

As described above, due to the time constraints many aspects of the project are still at the preliminary stages, though provides numerous avenues for further investigation; including i). the further characterisation of SapP, to confirm how this protein contributes to specific phenotypes associated with virulence. ii). How regulation of ATs and the TAA SadA is affected by single or multiple deletions of other type V proteins in these strains, i.e. could increases in protein expression account for alterations in phenotype? iii). how does deletion of these ATs affect adhesion and invasion of epithelial cell lines and does this correlate with an effect during oral infection and gut colonisation; do proteins like ApeE and SadA contribute to virulence and colonisation in the absence of known factors such as MisL and ShdA? iv). How does removal of these large surface structures affect AB and T cell responses against *S.* Typhimurium, for example is there an elevated LPS specific AB response? Studies are planned to investigate these avenues further to enhance our understanding of this family of proteins in *S.* Typhimurium virulence and survival in various hosts, and further explore their potential as multivalent vaccine candidates of the future.

CHAPTER 7

FINAL DISCUSSION

The investigations presented as part of this study have enhanced our understanding of OM homeostasis in two separate Gram-negative bacterial strains, the evolved laboratory strain *E. coli* K12 and the broad host range pathogen *S.* Typhimurium providing insight into three main features; the roles of nonessential genes in maintaining membrane barrier function, the mechanism by which OM phospholipids are localised and the roles of *S.* Typhimurium ATs in virulence. The impacts of these findings are discussed below.

Membrane homeostasis and virulence

The role of nonessential genes in maintaining cell envelope homeostasis has been investigated previously by numerous groups. Early investigations charactered the roles of periplasmic chaperones and OM localised components commonly noting their association with maintaining membrane integrity (19, 69). However, no large scale high throughput screen had been performed in order to determine all the nonessential genes required for OM homeostasis. Shortly after commencing our investigations a series of publications were released utilising the same collection with alternative approaches, to answer similar question (199-201). In the midst of these publications we chose to continue our screen, but provide further insightful characterisation of the mutants identified. We have therefore characterised the mutations identified to provide mechanistic evidence for the means by which these genes contributed to barrier function. Our investigations predominantly focused around nonessential genes in the *E. coli* K12, strain BW25113, though we have also investigated a selection of these mutants in *S.* Typhimurium, to further explore the roles of these genes in a physiologically relevant human pathogen. Our investigations in *E. coli* K12 have highlighted potential secondary roles for genes of predicted function, with the identification of efflux defects in the *yibP* and *yciM* mutants. We have highlighted a series of mutations resulting in

the excessive production of membrane vesicles and potential periplasmic leakage, phenotypes which are of significant interest due to their wide ranging biological applications, in respect of recombinant protein production and vaccine development.

Due to the nature of *E. coli* K12, we felt that further investigation in a physiologically relevant strain was required to confirm both the phenotypes and examine the roles of these genes in pathogenesis. By recreating these mutants in *S.* Typhimurium we have been able to demonstrate that in some instances the defects are consistent, highlighting the similar functions and mechanisms of OM homeostasis across different organisms. Investigations using the murine model of *S.* Typhimurium infection have provided further insight into how these proteins contribute to virulence, particularly highlighting how the route of administration can significantly affect the observed results. Our data is relatively consistent with that of orally dosed *S.* Enteritis *bamB* mutants (242). However, this study also indicated how complex *in vivo* infection experiments require careful interpretation; mild attenuation in bacterial burdens can have a significant effect on lethality and the outcome of infection. Importantly, our investigations give promise for the potential use of these proteins as targets for future drug development.

Biogenesis of membrane components

Despite the significant advances in our understanding of the biogenesis of key outer membrane components, including the identification of the BAM complex, Lpt and Lol pathways, the OM phospholipid transport pathway has remained recalcitrant to identification. The investigations presented as part of this study have provided significant insight into key questions regarding phospholipid transport.

Phospholipid transport

Previous investigations on phospholipid transport have predominantly focused on the pathways and proteins responsible for retrograde trafficking and recycling of OM phospholipids. The identification of the Mla pathway, PldA and PagP proteins are responsible for recycling and degrading phospholipids. By either transporting these back to the IM or catalysing their degradation, through the removal of fatty acids and palamite, respectively (71-73). In contrast our investigations and characterisation of *plpA* has provided the first experimental evidence for the mechanisms of phospholipid transport to the OM, with specific regard to PG and CL are trafficked across the periplasm for insertion into the inner leaflet. Consistent with previous hypotheses, we have shown for the first time an inner leaflet localised lipoprotein, PlpA, is responsible for this function. Through lipidomic analysis we have confirmed the altered lipid composition of the OM in *plpA* mutants, is caused by the absence of PG and consequentially resulting in elevated levels of PE.

Our collaborators have determined the NMR structure for PlpA, providing the first structure for a dual BON domain containing protein. Using NMR analyses and mutagenesis, we have been able to identify and confirm the roles of particular residues, critical to both stability of the protein and its' function in binding PG. We confirmed PlpA binds PG and CL at one face of α helix 3. Site directed mutagenesis and random insertion sequences at these sites confirm these results with the abolition of protein function whilst maintaining structural integrity of the protein. In addition, we have confirmed the importance of other residues located in the BON1 and BON2 domains.

This data provides the first experimental evidence for the role of BON domains in binding phospholipids emphasizing the need for further investigation into the functions of other BON domain containing proteins. Despite enhancing our understanding of phospholipid

transport, key issues remain unresolved, including how PlpA identifies phospholipids ready for transport from the IM and subsequently releases these into the inner leaflet of the OM? Does BON1 play a role in this aspect of function? We have shown several residues in this domain are critical to protein function, though none of these are required for substrate binding questioning how the interaction of BON1 and BON2 enables functionality, i.e. does the interaction of these domains initiate substrate binding or release at the membrane? Further investigations into the biochemical mechanism of PlpA function will aid our understanding of this process.

Importantly, the identification of PlpA has highlighted the fact that the mechanism by which PE is translocated and inserted into the OM remains unknown, as PlpA was shown to only bind PG and CL. Therfore, an alternative pathway must exist for the transport of PE to the OM.

Despite our investigations, with double mutations in *plpA* and *mlaA-F, pagP* and *pldA* providing an element of confirmation for the role of PlpA in maintaining inner leaflet phospholipid content, these studies have further emphasized that maintaining membrane stability is a highly complex process. These mutations could only counteract sensitivity to vancomycin but not to SDS. In the absence of the SDS suppressor mutant sequencing data it is impossible to draw conclusions as to the means by which resistance to this compound is restored. However, this data will provide further key insights as to the functions of this protein and the mechanisms by which Gram-negative bacteria maintain a selectively permeable OM.

The identification of the TAM complex

Previous investigations have highlighted an array of proteins involved in the transport and biogenesis of AT proteins, including the role of periplasmic chaperones (including HlpA, SurA, DegP and FkpA) and the BAM complex components BamA and BamD (16, 100, 244). Though these investigations have failed to address the mechanisms by which AT passenger domains become surface localised. Until recently several theories have existed regarding the route of passenger doamn transport to the bacterial cell surface. These included BamA enabling the AT barrel to be held in a semi-folded conformation to allow an extended pore diameter through which the passenger domain could be transported and the hairpin model, requiring passenger domains to be extruded as a linear polypeptide before folding on the surface (90). Through collaboration with other groups we have identified and characterised a new OM secretion complex, termed the TAM complex (103).

Our investigations into the components of this complex in *S.* Typhimurium contributed indirect supporting evidence for the role of this complex in AT passenger domain surface display, particularly regarding the heightened serum sensitivity of these mutants. However, our *in vivo* investigations failed to show significant defects in systemic infection for *tamB* mutants. Although our investigations did not clarify the mechanisms by which TAM associates with BAM and AT precursors to aid in there secretion, these new findings need to be reconciled with the previous theories of AT secretion. Additionally, the role of *ytfP* (transcribed in the same operon as *tamAB)* in this complex has yet to be clarified. Interestingly, our investigations suggest a potential role for this gene product in resistance to innate immune responses, however this requires additional investigation to both confirm this hypothesis and determine the means by which this is mediated. Further investigation of this complex may provide key insights as to the fundamental aspects of AT biosynthesis.

Roles of *S.* Typhimurium autotransporters in virulence

Numerous studies have investigated the role of AT proteins in virulence and the specific phenotypes they are responsible for. However, previous investigations on the ATs of *S.* Typhimurium have largely focused on only two of the five proteins encoded by *S.* Typhimurium viz. MisL and ShdA. While one study examined the potential for functional overlap between these two proteins, the study failed to account for the other type V proteins, namely ApeE, SadA and SapP. To address this issue, we have created a series of deletion mutants removing all ATs from *S.* Typhimurium. By examining the ability of these mutants to colonise and cause persistent infection *in vivo,* we have identified unique events that were otherwise unobservable in the single mutants, including the role of ApeE as factor for persistent colonisation of the liver.

This investigation has highlighted a number of additional questions for further investigation, including how multiple deletions of AT proteins affects the transcriptional and translational regulation of the remaining ATs. Do ATs including SadA and ApeE have a more pronounced role in intestinal colonisation, in the absence of factors such as MisL and ShdA. How does the removal of these large surface structures affect immune recognition and responses to the bacteria? Future investigations directed at answering these questions will enhance our understanding of the role of *S.* Typhimurium AT proteins in virulence and their specific phenotypes. Additionally, experiments are planned to further investigate the substrate specificity of ApeE, to address its potential role in liver colonisation.

In addition to ApeE's potential role in liver colonisation we have confirmed a role for this protein in the survival of *Salmonella enterica* serovars on tomato's, providing biological significance to the previously known phenotype of Tween-80 degradation and utilisation as a carbon source. Until recently this was the first description of a *S.* Typhimurium AT protein

participating in virulence outside of an animal model. Further investigations are planned to assess other potential substrates also highlighted during the BioLog screen of 2000 carbon sources. These studies will enhance the results presented here, and provide novel insights into the role of this largely ignored AT protein.

Furthermore our investigations into the specific roles of SadA, have provided the first peer reviewed characterisation of a TAA from *Salmonella* (174). We have shown in contrast to other TAAs SadA is not required for serum resistance, though in the absence of other Va proteins, SadA is more critical. Despite *sadA* single mutants not being attenuated in the murine model, our *in vitro* investigations have suggested that this particular virulence factor, maybe associated with *S.* Typhimurium infection in other hosts, particular birds, cattle and pigs which demonstrate higher natural body temperatures consistent with temperatures shown to increase SadA expression, emphasizing the need for more investigation using alternative models.

Key to our investigations, we have shown SadA is immunogenic and can afford limited protection against infection. We plan to further investigate the immunogenicity of other Type V proteins from *S.* Typhimurium, with the view to identifying suitable contributors to a multivalent vaccine. Whilst these proteins only induce a mild attenuation upon deletion, as shown with OmpD from *S.* Typhimurium which is a protective antigen (131), it remains possible they will induce a suitable protective memory based immune response against infection.

APPENDIX

Appendix Table 1 – Primers used in this study

Gene Disruption		
Name	Sequence (5'→3')	Description
Fwd apeE DW	TGGTGCCAAACGTACCGGATTTAGTGCGACGCCAATGCGTGTAGGCTGGAGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *apeE*
Rvs *apeE* DW	ACGGCACCATGACAAAATCATACCGCTGGTTAACCTCGCGGGAATTAGCCATGGTCCAT	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *apeE*
Fwd *bamB* DW	GCTTTCCGTTACCCTGCTCAGCGGCTGTTCACTGTTTAGCGTGTAGGCTGGAGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *bamB*
Rvs *bamB* DW	GGGCCACAAAACGCCCGTCATCGACATTAATCCAGTGCAGATGGGAATTAGCCATGGTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *bamB*
Fwd *bamC* DW	GCTTACTCAGTACAAAAGYCGCGCCTGGCGAAGGTTGCGGGTGTAGGCTGGAGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *bamC*
Rvs *bamC* DW	TTACTTGTTGAACGCTGCCTGGAAGAACGGCAACCAGCGCGTCGGGGAATTAGCCATGGTCCAT	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *bamC*
Fwd *bamE* DW	CGCTGTAAAACGCTGACTGCTGCCGCAGCAGTACTCCTGAGTGTAGGCTGGAGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *bamE*
Rvs *bamE* DW	CTTCGTCAACGCCGGTTTATCAATATTGGTTAACACGATGGGAATTAGCCATGGTCC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *bamE*
Fwd *degP* DW	CTGGCTCTGAGTTTAGGTTTGGCATTGTCGCCTCTGTCTGCGTGTAGGCTGGAGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *degP*
Rvs *degP* DW	CACCACGCTGAATATTGAGCGCCAGAACCGACGGCTTGCATGGGAATTAGCCATGGTCC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *degP*
Fwd *fkpA* DW	ACTACGATGGCCGTTGCTATGCACGCACCGATCACTTTTGCGTGTAGGCTGGAGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *fkpA*

Name	Sequence (5'→3')	Description
Rvs *fkpA* DW	AGCCGCATCGGCAGGTTTAG CATCCGCTTTCGGCGCTGGA ATGGGAATTAGCCATGGTCC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *fkpA*
Fwd *hlpA* DW	GCTGCAGGTCTTGGTTTGGC GATGGTAACGTCCGCACAGG GTGTAGGCTGGAGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *hlpA*
Rvs *hlpA* DW	GCGGTGATGTCTTTCACATC GCTGCTGTTGTAAGCAACGA TGGGAATTAGCCATGGTCC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *hlpA*
Fwd *misL* DW	GCATCTACGTGGATGGTCAG GATGGTGACGGTATTAACGC GTGTAGGCTGGAGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *misL*
Rvs *misL* DW	CAATGGTTTGCGCCCCGGCT CCTCCAATATTATTTACTGC GGGAATTAGCCATGGTCCAT	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *misL*
Fwd *plpA* DW	GCGTTGCTATTGCAAGGTTG CGTCGCCGCCGCGGTAGTGG GTGTAGGCTGGAGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *plpA*
Rvs *plpA* DW	CTGCCGCTTTACCTTCACGTT CAGTCACCAGGCCCAGCAG ATGGGAATTAGCCATGGTCC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *plpA*
Fwd *sadA* DW	TCGGTCTTAGCAGCATTATG GTTTCTGCGGA TGCACTGGCGTGTAGGCTGG AGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *sadA*
Rvs *sadA* DW	GTCGTATTGGCGGCGATATC GGTTGTGTTCT GGTTAATGCGGGAATTAGCC ATGGTCCA	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *sadA*
Fwd *sapP* DW	TACGATACCTGGACTTATTA CGACAATCCTACCACCGCGC GTGTAGGCTGGAGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *sapP*
Rvs *sapP* DW	ACACCAACGTTTGCAGACCA GTCTTGATCAACATCACCGC GGGAATTAGCCATGGTCCAT	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *sapP*
Fwd *surA* DW	GTATCGCCATGATCGCGAAT ACCAGTTTCGCTGCCCCCCG TGTAGGCTGGAGCTGCTTC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *surA*

Name	Sequence (5'→3')	Description
Rvs *surA* DW	GGCGCGCTGTTCTTGCATCC AGGTCGCCGCTTCTTCTGAC ATGGGAATTAGCCATGGTCC	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *surA*
Fwd *tamB* DW	AGCCTCGGCGTGTTGATTTT CATTGTGGTGTTACTGGCGA GGTGTAGGCTGGAGCTGCTT C	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *tamB*
Rvs *tamB* DW	GCAAATCAAGTGCCTGATCT ACGCCAGACACTGCTTCCAG GGGAATTAGCCATGGTCCAT	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *tamB*
Fwd *ytfP* DW	AGTTTACGACAGAAACAAG GCAACAGTCACTGGATGACC AGTGTAGGCTGGAGCTGCTT C	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *ytfP*
Rvs *ytfP* DW	CCAGTCACCGCTTTCAATCA GAGTTAACCCATGAACAGG ACGGGGAATTAGCCATGGTC CAT	For amplification of kanamycin resistance cassette (from pKD4, homologous region in blue) with 40 bases homology to *S.* Typhimurium *ytfP*
Fwd pDOC *tamA*	TAGGGATAACAGGGTAATTC CAGGGGTGGGAATAGGGGA TATTCAGGAGAAAATGTGGA CCGGTCAATTGGCTGGAG	For amplification of kanamycin resistance cassette (from pDOC-K, homologous region in Blue) with 40 bases homology to *tamA* (black) and I-SceI site (Green)
Rvs pDOC *tamA*	ATTACCCTGTTATCCCTATC ATAATTCAGGCCCCAGACCG ATATAAAACTGTAAACCAAT ATCCTCCTTAGTTCC	For amplification of kanamycin resistance cassette (from pDOC-K, homologous region in Blue) with 40 bases homology to *tamA* (black) and I-SceI site (Green)
Cloning Primers		
Name	Sequence (5'→3')	Description
Fwd *apeE* pQE60	CGCGCGAGATCTCATATGAC CCAAAAGCGTACCCTGC	For amplification of *apeE*, containing an NdeI site (Blue), for cloning into pQE60
Rvs *apeE* pQE60	GCGCGCGCTAGCAAGCTTCA AAATCGGGCGCTAAACCCA ACG	For amplification of *apeE*, containing an HindIII site (Blue), for cloning into pQE60
Fwd *apeE* frag MP	GGGCATATGACCCAAAAGC GTACCCTGC	For amplification of partial *apeE* gene to produce a fragment containing NdeI site (Blue) for use in mega primer reactions
Rvs *apeE* frag MP	GGGCCATGGTTGGTTGCGCG ATGGCCGCCGCC	For amplification of partial *apeE* gene to produce a fragment containing NcoI site (Blue) for use in mega primer reactions
Fwd $apeE_{S10A}$ pQE60	ATTACCGGTATCGCTAAGGG CATCGCCAATCACCG	Fwd primer for mega primer reaction to mutagenise $apeE_{S10A,}$ with alterations shown in blue
Fwd *apeE* pET22b	TCCATATGACCCAAAAGCGT AC	For amplification of *apeE*, containing an NdeI site (Blue), for cloning into pET22b
Rvs *apeE* pET22b	TCGAATTCCTGTTAGTGAAC ATCCGG	For amplification of *apeE*, containing an EcoRI site (Blue) for cloning into pET22b

Name	Sequence (5'→3')	Description
Fwd *E. coli plpA* pJH10	TATAGAATTCATGAAGGCATTATCGCCAATCGC	For amplification of *E. coli plpA*, containing an EcoRI site (Blue), for cloning into pJH10
Rvs *E. coli plpA* pJH10	CGGGGAGCTCCTATTTAATAAACGTAAACGCCGTAG	For amplification of *E. coli plpA*, containing an SacI site (Blue), for cloning into pJH10
Fwd STm *plpA* pET17b and pQE60	TATACATATGAAGGCATTTTCGCCGCTCG	For amplification of *S.* Typhimurium *plpA*, containing an NdeI site (Blue), for cloning into pET17b and pQE60
Rvs STm *plpA* pET17b and pQE60	GCCGAAGCTTTTACTTAATATACGTAAAGGCGG	For amplification of *S.* Typhimurium *plpA*, containing an HindIII site (Blue), for cloning into pET17b and pQE60
Fwd *plpA* pET17b and pET20b	GGGAATTCCATATGAAGGCATTATCGCCAATCG	For amplification of *E. coli plpA*, containing an NdeI site (Blue), for cloning into pET17b and pET20b
Rvs *plpA* PET17b	CCGGAATTCCTATTTAATAAACGTAAACGC	For amplification of *E. coli plpA*, containing a EcoRI site (Blue), for cloning into pET17b
Rvs *plpA* pET20b	CCGCTCGAGTTTAATAAACGTAAACGCCGT	For amplification of *E. coli plpA*, containing a XhoI site (Blue), for cloning into pET20b
Fwd *sadA* pQE60 and pET22b	GGAAATTACTACTCACATATGAATAGAATATTTAAAGTC	For amplification of *sadA*, containing an NdeI site (Blue), for cloning into pQE60 and pET22b
Rvs *sadA* pQE60	GCTACTAAGCTTTTACCACTGGAAGCCCGC	For amplification of *sadA*, containing an HindIII site (Blue), for cloning into pQE60
Rvs *sadA* pET22b	AGAGTTAATTTAGAATTCTGTGAGTAGGCTGTGG	For amplification of *sadA*, containing an EcoR1 site (Blue), for cloning into pET22b
Fwd *sapP* pQE60	CGCGCGAGATCTCATATGCACTCCTGGAAAAAGAAAC	For amplification of *sapP*, containing an NdeI site (Blue), for cloning into pQE60
Rvs *sapP* pQE60	GCGCGCGCTAGCAAGCTTACCAGGTATATTTAACACC	For amplification of *sapP*, containing an HindIII site (Blue), for cloning into pQE60
Fwd *sapP* pET22b	ACCCATGGACTCCTGGAAAAAGAAACTT	For amplification of *sapP*, containing an NcoI site (Blue), for cloning into pET22b
Rvs *sapP* pET22b	GAATTCCTGTAGTGGCTTGC	For amplification of *sapP*, containing an EcoRI site (Blue), for cloning into pET22b

Salmonella Typhimurium Check Primers

Name	Sequence (5'→3')	Description
Fw *apeE* Check	CAGGATAATACCGCGG	Flanking (approx. 200 bases upstream) to

Flank	ATCA	*apeE*, to confirm the excision of *apeE*
Rvs *apeE* Check Flank	CTGTTAGTGAACATCCGGCT	Flanking (approx. 200 bases downstream) to *apeE*, to confirm the excision of *apeE*
Fwd *bamB* Check Flank	AGTTGCAGCTTAAGCAAGCC	Flanking (approx. 200 bases upstream) to STm *bamB*, to confirm the excision of *bamB*
Rvs *bamB* Check Flank	CAGACGGTTAAATAGCGTGG	Flanking (approx. 200 bases downstream) to *bamB*, to confirm the excision of *bamB*
Fwd *bamC* Check Flank	GTCGAACCCAATCCTATCCC	Flanking (approx. 200 bases upstream) to *bamC*, to confirm the excision of *bamC*
Rvs *bamC* Check Flank	CATTTCCTGCTACGCCTGCC	Flanking (approx. 200 bases downstream) to STm *bamC*, to confirm the excision of *bamC*
Fwd *bamE* Check Flank	GAACTGCTGGCGGCGTAAAC	Flanking (approx. 200 bases upstream) to *bamE*, to confirm the excision of *bamE*
Rvs *bamE* Check Flank	CCAGCGCGCCGAAAAATCGG	Flanking (approx. 200 bases downstream) to *bamE*, to confirm the excision of *bamE*
Fwd *degP* Check Flank	CTTTATTCCGGAACTTCGCG	Flanking (approx. 200 bases upstream) to *degP*, to confirm the excision of *degP*
Fwd *fkpA* Check Flank	ATGGTAATCTCCTGGAACGC	Flanking (approx. 200 bases upstream) to *fkpA*, to confirm the excision of *fkpA*
Rvs *fkpA* Check Flank	CAGTGAGGGTTCATCTTTCC	Flanking (approx. 200 bases downstream) to *fkpA*, to confirm the excision of *fkpA*
Fwd *hlpA* Check Flank	AGATGTACCGGATTACAGCG	Flanking (approx. 200 bases upstream) to *hlpA*, to confirm the excision of *hlpA*
Rvs *hlpA* Check Flank	CACGGTACTTAGGATTCACC	Flanking (approx. 200 bases downstream) to *hlpA*, to confirm the excision of *hlpA*
Fwd *misL* Check Flank	CATAGCGGTATCAGCAGCAT	Flanking (approx. 200 bases upstream) to *misL*, to confirm the excision of *misL*
Rvs *misL* Check Flank	CGGCTCTGTTGTTACCTGAA	Flanking (approx. 200 bases downstream) to *misL*, to confirm the excision of *misL*
Fwd *plpA* Check Flank	ACACCACAGCGCGCGCATTC	Flanking (approx. 200 bases upstream) to STm *plpA*, to confirm the excision of *plpA*
Rvs *plpA* Check Flank	CCATGCTGGCGGAAGCGCTG	Flanking (approx. 200 bases downstream) to STm *plpA*, to confirm the excision of *plpA*
Fwd *sadA* Check Flank	AGTCCTCTGGAATGCCGCTA	Flanking (approx. 200 bases upstream) to *sadA*, to confirm the excision of *sadA*
Rvs *sadA* Check Flank	TTGTGCTTAGCGCTGAATGC	Flanking (approx. 200 bases downstream) to *sadA*, to confirm the excision of *sadA*
Fwd *sadA* Check Internal	TGTGGGATGAAAGCATCAGC	Internal approx. 400 bases inside the start of *sadA* gene
Rvs *sadA* Check Internal	ATCGCGGAGGCTTTATAACC	Internal approx. 200 bases upstream of gene disruption end

Salmonella Typhimurium Check Primers

Name	Sequence (5'→3')	Description
Fwd *sapP* Check	GCACTCTGGCAATCACT	Flanking (approx. 200 bases upstream) to

Flank	TCT	*sapP*, to confirm the excision of *sapP*
Rvs *sapP* Check Flank	GAATTCCTGTAGTGGCTTGC	Flanking (approx. 200 bases downstream) to *sapP*, to confirm the excision of *sapP*
Fwd *surA* Check Flank	GCGCCGTCCGCACAGACGAC	Flanking (approx. 200 bases upstream) to STm *surA*, to confirm the excision of *surA*
Rvs *surA* Check Flank	CTACGGCCTCGGCACGCAAG	Flanking (approx. 200 bases downstream) to STm *surA*, to confirm the excision of *surA*
Fwd *tamA* Check Internal	CGAGTTGATGATGCGATTCG	Internal to *tamA*, to check for complete excision/duplications of *tamA*
Rvs *tamA* Check Internal	GCGAAGAAACGTAAATCCGG	Internal to *tamA*, to check for complete excision/duplications of *tamA*
Fwd *tamA* Check Flank	TGTTAAGCCGTTTAACGGCG	Flanking (approx. 200 bases upstream) to *tamA*, to confirm the excision of *tamA*
Rvs *tamA* Check Flank	GACCTGAATGTCTTTCAGCG	Flanking (approx. 200 bases downstream) to *tamA*, to confirm the excision of *tamA*
Fwd *tamB* Check Flank	CGAAGTTGATAACCGGATCG	Flanking (approx. 200 bases upstream) to *tamB*, to confirm the excision of *tamB*
Rvs *tamB* Check Flank	AACGTACATCCATGCACTCC	Flanking (approx. 200 bases downstream) to *tamB*, to confirm the excision of *tamB*
Fwd *ytfP* Check Flank	TGGCGTAAGCAATTTAGCGC	Flanking (approx. 200 bases upstream) to STm *ytfP*, to confirm the excision of *ytfP*
Rvs *ytfP* Check Flank	TAATACGCATCTGCACCTCC	Flanking (approx. 200 bases downstream) to STm *ytfP*, to confirm the excision of *ytfP*

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse
aceE	ACAGAAGTCGTGAAGAGAGC	TCTCTTTAACGATACCCGCC
aceF	TATCGCTCAGGTGATGAACG	GTAATACCCTAACCACCACC
ackA	GGTGTCATCATGCGCTACGC	CAGCCGACGCTGGTTCCGG
acrA	AATGCCAGTAGATTGCACCG	GCAATCGTAGGATATTGCGC
acrB	CGGATGACAAAGTGGAAACC	CGTATGAGATCCTGAGTTGG
ada	TCACCAAAGAAGGCGATAGC	AGAGATAACCTTGCCGATGG
aer	GATGATTGCTGCGCAAATGC	CAGAGCGATATCAGTGATGC
afuB	GCATAACCTGAATCTGAGGC	ACACCATACAGATATCGCGC
agaD	CTGCGCGTAATGTTCAAAGC	ATACGTCAGTAATGGCGTGG
ampD	CTGTTCCGCCTAAATCTTCC	CGTTGAACAATACTCCCTGC
aslA	GCCTTACATATCAACTGCGC	TATGCATGCGCATTTGCTGG
asmA	TGCTGGAGTTCTACAACTCC	CTTGAGCGTTCCATAGTAGC
aspC	TGCTGTGGGTATCGTTTACC	TGGATTTCTGGCAAAGTGCG
asr	ATTGCCAGCTCAAACCAACG	AATACTATAGACGGGACGGG
astB	GTTCGATCAACTGTTGCTGG	GTATTCCCGCTGAAATCACC

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse

atoA	TAATTCGCGCTCATCGTTGC	ATAAGCGCCATCTGCATACC
atpA	ACTGAGTGAACAACAGCTCG	ATGCGATCCTGCGATTTACG
atpB	TAAAGGCGGTATTCTTGCCG	AAAGCAACGCTTACTACGCG
atpC	CATCATGGAAGGCGAATACG	CATCGTGAAATGGACAGAGG
atpD	ATCTCAGGTTTATCAGGGCG	TAAACTCTTCGTGACCGTGC
atpE	GTTCGGTAACATGTATGCCG	TCGATGGCTGCCATTAATGG
atpF	CGTCAACCYGATCTGATTCC	AGTTGCTCACCACAAACTGC
atpG	AAATTGGCAGCTTCGAAGCC	TTTGCACCTCAAGAGCATCG
atpH	ATTCTGGACGAAGCGAAAGC	TTCAGTGCGATAGCGTAACG
bglA	TTTCAGTCAGCGTATCCAGC	TCGCGTTAAGTCGAACATCC
bglF	AAATTACCCGCAAGCATGGC	ATAACGCGATATCTTCCGGG
bglJ	TGCATGGATAAATAGCGCGC	TGAGATGAAGCAACTGCTCG
caiF	TAGCTCACACTTATCGACGG	GACGAGTTACACTGGAAACG
celC	TGGGAGCCTTCTTTAACACC	ATCTCCTGGAAATAACGCCC
chaB	TAAGCGAAGTCTCCTTGTGG	TTCAATGGCACCAAATGCCG
cheA	AAAGTGTTACGTGTCGTCGG	ACGCCTTTGATAAACGCTGG
cheB	TACTGGCGAAACAGTACACC	ACAAATCCATAACCGCCTGC
cheY	TGTTGTTCCATTCTGTCGCC	TCCATTTGATCCTGATGCGG
chpA	ACAGTAGCGTAAAGCGTTGG	TTCTTCAAGGGTGTAAGGCG
cmk	GTCCATGTTTGACTCTCACC	ATAGCAACAACAACGCCACG
coaE	TGGCTATAAGGGTAAGAGCG	TCTTTCAACAGCTCAGTGCG
cpxA	TACCCTGCTCTATTTGCTGG	TATCACCGTTTACTCTCCGC
cpxR	TAATGAACTGACTGCCAGCG	CCACATTAAATCGTTGGGCG
csiE	ACTAACCCATGATCGCTAGC	GGTGCAAATTCTGCATTGCG
cueR	TATCTACAGTCTGTGGTCCC	AGTCATTTAAACCAGCGCGG
cusA	TTTGTACCGAAACGCGTTGC	CCAAGAAACAGACCAGTACC
cusB	AGGGTATCGATCTGGAAAGC	TGGTTAGCGGATAAGTCACC
cusS	TATCTGCTGCGTTAACCAGG	TCAGGGTGATTTAGTACCCG
cvpA	AATACGGTCTTGCCTGATGC	AAATACATCGCTCACCAGCC
cydB	TGGTTCGTGGCTGAATATGG	TTCCAGTTCACTGGTCTTCG
cysB	GATGGCAAATGGGTTGAAGG	GCAAGACGTTGAACGATTGG
cysE	GCAATACGAAAGAAGTCCGC	GCGATTATCTGATGTTCGGC
cysE	GCAATACGAAAGAAGTCCGC	GCGATTATCTGATGTTCGGC
cysI	TGACACGTATCGATCTTGCC	TTGATTCACCAGATGCAGGC
dacB	TGATAGGGCAAGTCTTCACC	TGATGAAGACTGGGATGACG
dapF	GAAAGGTCCGCTCTATTTCC	GAATATGATTACGTGCGCGG
dcuR	AACATTGCACTACCGTCACG	CTGATCTACCCATTTGTGGG
dedD	TGATGACTGGTATTGTGCGC	TTTATGCCTTTGCGGCATCG
degP	GGAACTTCAGGCTATAAAAC	GAAGATGTATGGAGTTGTGG

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse

degQ	GGCGCAGCCATTGCGCCCTC	GGAACGGCAGCAGCATACC
deoB	TGACACCATCGATTACAGCG	TTGTAAGTACCGGTGAAGCC
dgoK	TAGCATTGCGATCAGTTCGC	ATGCTTTGCTCCCATTGTGG
dksA	CGGAAATTTCTGCTGAAGGC	CGGGTCGATATCTTCTATGC
dnaJ	TGAAGACAAAGCCGCTATCG	TCAGGCGTAATACCACAACC
dpiA	GATGTGGTGATTGAAGTCGC	TTTGACTATCCCGATGCTGC
dpiB	TCCTAAGGAGTATTTCGGCG	TTAATCCCTCTACCGTCAGG
dppD	TATGGCTGCTCTTGGTTTCC	TCAATCATCGTCAGCAACCG
dsbA	CTGATTGAACCTTTACGCGC	ACACCTGTAGCTGACTTACG
dsbB	GGTTTATGCAAATGGGCTGG	CACTGAATGCTAAAGAGGGC
dsbD	GGATAATCCACACGTTCAGC	GGATAATCCACACGTTCAGC
eda	ATCCACGTAACACCAGAAGC	TGACCAGTCAGAATGTCACG
ego	TCCCTGTTCTGAAATTGCCG	CACATTCCGGTAATCGAACC
elaD	ACTGGCGAATGATTTCACCG	GTTACGCGGTTCTGAATACC
emrA	GCAAATCACCCGCAAATTGC	CCCGAAAGAAGTGATTACCC
emrY	AAATCGTTCAGCGTGTACCG	TTTCAGCGCAGAACAACTGC
envZ	TGAGTTTGCGGTACTGAAGG	ACACGAAGATTCTCGATCCG
exbD	TCAAAGACCAGCGAAGAAGC	GTTCACCTTCACATCTACCG
fabH	CAAGTTCCTCAGCGAATTGC	CAACAGATGCAGTCAACAGC
fadD	ACGTTGGCATTTAACCCAGC	TTCAGTATCCAGGGCTATCG
fadL	ACTTGCAACATTCCAGCTGG	GTGTATGAGCGCACAATAGC
fadR	GATCTCCATGATGGTTTCCC	AGAAGTCCTGAAGCATGTGC
fbp	TACTTCATGGCCTAACTGG	TTGGAACGGTATTAGGGACG
fcl	TGATTATCGCTGTTGACCCG	AAACTGGCCTGCTGTTATCG
fdhE	GATCCGATTCGCGTTAATGC	CTTGTCATCAGTCTCATGCC
fdx	AGAACGTCAGGTCATTGACG	GAAATTATTCGGCCTCGTCC
fepA	TTGCAATAGCGTAATGGCGC	TCGAGACTGATGACAAACGC
fepB	TGATGGATTCGCATAAGCGC	GATGTTAGGGCGGATTAACG
fepC	TATTGCTTTAGTCGCACCGC	CGGTGAATTACGTAATCGGC
fepD	TTGAGATGAAACGAGCGAGG	TAAAGCCCATTACGTCAGGG
fepE	TCTATCGTCAGGATTGTGCG	TGTTAAGCGAACTGAACCGC
fepG	TTATTGGCCTGATGATGCCG	GCTTAAGGTACGCAGTAACG
fhuA	AAGCGTGAAATACCGGATGG	AATCAGACCGGTCACTTTCC
fimB	ATGGCGTTTGTATGGCAACG	TGGCCTGAACTTCTTTACCG
fimD	GTAACAGAGTTGAATGCCGG	TGTTAAATTGCTTCGCCGCG
fkpA	GACAACGCTTTATAGTACCC	CTAATTTAATACAGCGGAGG
fkpB	TTCAACCTTGCCGATACTGC	CCACATAGCGGTTATGTACC
flgB	TTGCGATGTGAGATTGCTCG	AACATCGGCAACCTTTACGC
flgC	ACCTCAACGCAACACATTCC	GCTGTTATCAATCTGTCCGG

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse

flgM	ACAACGCGTCAATGTGATCG	ACAGTAATTTCCTGCCAGCG
fliG	GAAAGCTGTACAGCAACAGG	CTGATAACCTTGCTCATGGG
frdA	TACGCAGGAATCAAACAGCG	CGTTGTTAACCATCATGCCG
frdB	AATGGCACGTAAAGAGTCCC	TTTCGGTGCCAGTTCAAACC
frdC	AACTGTGGTTTGTGCTACGC	GAATACGCGACCAATGAAGC
frdD	TTAACCTGATCACTCTGGCG	TGATAAATTACCGCCACCGC
frvR	CGCACATAACGATTTACCGC	AAGGCGGCATGAATTTCTCG
fsaA	TAAGAAATACGACCGTGCGG	AAATGCTGGCAGGTTATGGC
fumA	ATTCTGCACTCGTGAACTGC	AAATGCGGAAATGCTCCAGC
fur	TGTACCTGTACAATGTCCCG	ACAACATCAGCAGTTTGCCG
gadW	CATAAGCTATACGCTGTGCG	CAATACTGACCCACATTCCC
galU	ATGAGCAAACGATAACGCGG	TGAGCAAGGTAAATCCCTCG
garP	TGCAATATTCTCCAGCCAGG	AACCGATATCCAGAAGACGC
gcl	GATGTCGGTAGTGTTGTTGC	CGAGTTTATTACTCGCCAGC
glcB	GAAGAGATGGTGAACAACGG	AACGCAATTGCTACCTCTGG
glgC	CAAATTCTGCTCTCGGATCG	ACAACAGCGTGATATGTCCG
glnA	AGGTCATTGCACCAACATGG	AAAGCAGTCTCCTGAACAGG
glnL	GACGCAAATGCGTCTTATCC	ATGATGACCGGAAGCATTGG
glyA	AGAAATCCGTTTCCGGTTGC	TACGACAGATTTGATGGCGC
gnd	TATTGACCTGTGCTTGAGGC	AACGCTACGTTGTTCGATGG
gpmB	GATGCAATATGGCAGGATCC	TGCAGGAGTTTATCCTGACG
gpmI	CAGGAAAGGAGTATACCTGC	TTTCAATTGTGCGAGCAGGC
guaB	GACGAACCGTTTGATTCAGG	CACAGTTCGCAGTAAACACC
hcaD	CGATAATGCAGTCTCTGACG	CCATATATTGGACCGTACCG
hcp	TCAATGGATTTCACCCTGCC	AAATGGTGTAAGCACGCAGC
hfq	TGTGGTCTTACCTTGAAGGC	TAGATGTGTACCAGTACCGC
hisQ	GCACTGAACAAAGCCTTTGC	CACCAGTAAAGCGATAACCG
hlpA	GGAATGTAGTGGTAGTGTAG	CCGGTGATGACGATATCGCC
hofD	CATGGTTTACTCGGTCATCC	TTCATGATTACGTCAGCCGC
hrpA	ATGTTTGAATGGGCGATGCC	TGGTTCCTCCTTATCGTTCC
hscA	GAACAGGCGAAAGATGAAGC	GCGTGTTCAATCTCGATACC
hscB	TGCAATTTCTGGACGGTACG	AGTGAACAACAGATGGCAGC
hslR	AAGAGGATCAGCGGTTATGG	CCATCAAACTTCAGCGTAGC
hslR	AAGAGGATCAGCGGTTATGG	CCATCAAACTTCAGCGTAGC
htrB	ATGCGGTTGTGTAACACTGG	ACATAGCAATCCGCTGTTGG
hupA	TTAGCTGAGTGCGAAGAACG	TCTTCAGCAAATCCACTGGC
hyfB	GCCAACACAACATGCTTTGC	ATCAGACCTGAAGATGTCGG
hypD	AGCGCGATGTCGATTTAACG	AGAACAGCGGGTCAATAACG
idnD	TTACCTGCTGCGATACTACG	TTCTACAGCAAGTTCTGCGC

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse

ihfB	GCAAACTTCTCCAACAACGC	GTGTGCAACTTTGTTGAGCG
intB	CACATCGCTGTATGCAATGC	TGGTTGTCTGGAGATTCAGG
iscS	GCGATCGACGTTAAGTTACG	ACTTCCCTTTCACCCATTCG
ispZ	TTCCGGGAATTATCATGGCC	CAACCACCAAAGATGTCACC
JW0258	AGTAAGTTGGCAGCATCACC	TGTCACACTGTACCGTAACG
JW0530	TGTCACACTGTACCGTAACG	ACCAACAGATGGCCATAACC
JW5326	ACGACGGATATGTTCAGTCG	GACTTGAACCGAAGAACACG
JW5460	TCCTGCGGACTAAATTCAGC	GGTTCCTGATGTACTAAGGG
JW5675	ACCTTCGCGAACAAACTTGC	AAGGAATGCTCTGTTCCTGC
kch	GCAACCGTGTTCTTGTTTGG	CGCATATGGATTCATCTGGC
kdpD	TAAACTAATAGCCGCACGCG	GTCGCGGATAAACTCAATCC
kdpE	TCTACAACTGGATGTCTGGG	AGTTGTCCGCCTTATATGGG
kilR	TTGAAGTTCGGCTGATGTCC	TCGGTAGTTACAACAGTGGC
lamB	TCCATATCCAGATCCCTTCC	CTGCTGATAAACAGAGGACG
ldcA	TATTGGCATCCACTGCATCG	ATTGTTGTGGCATCCAACGG
ldcC	ATCGTTGAAAGCGCAACTGC	GATAGACTTCGCTTTGCAGC
lhr	CCGTATTGTCAAAGGCTTGC	ATACAAGTCTGAAGAGCCGG
lipA	TGTTACAGCCAGATCACAGC	CTCATAAAGAGTGACGTGGC
lipB	AAGCAGCAAAGGCAACTACC	GTCTAATTGGAACGGAACGC
lon	TGGTCAAAGCAAACCGTTGC	AGCAGTTATATCAGGCCAGC
lpcA	TACTTGTAGGAGGTCTGACC	CAATTCGCACATGGGTAACC
lpp	TGAATCCGATGGAAGCATCC	TAAAGCGTTGTATCGTCGGG
lysR	TGCCGACGAATAATTTGCGC	ATTCCTGAAGCGGATGAACG
macA	CTGCTGAGAGATTCAGATGG	GCCGAGAATATTCATCAGGG
macB	TGCGCAAGTGCATATTCAGC	TCCACCATTCAGATGGATGG
maeB	CCGTCTAACTCGTTCATAGG	TGGAGAGATATTCGCTGTGG
malG	TCAACTGTTAACCAACGGCG	GGGATAACGTAAGTTGAGGG
manB	TGTAGAGGAATGACAGTCCG	TTAATTGAAGTGCGCTCCGG
manC	TTCCCGCGAATATCATAGGC	CAGCTTTGCGAAACCTTTCC
maoC	TGAACTGTGACGGATTTCGC	TGTTCTTGTTAGCAACCGCG
mcrB	AAGGCTGTATTGTCCTCACC	TGATGCCAGGAATGATCTCG
mdh	CGGTAGGGTATATTGTCACC	TTCGGTGGATAACAAACCGG
menA	TTACACACTGTTCTGGAGCG	TATTTGTCCGCCAAACGACG
menD	AATTAGCGGCAATGTCGTGC	AATCCATCGACGCTAATCGC
menG	ATTACTGGTGAAGCAAGCCC	AAACAACTCACTGTCGCAGG
metE	TGAAAGTCCTTCACTTCGGC	GCAGAGATTATCGTGTACCC
mioC	GATCATGCGTACCATCAAGC	CTTATTAGGATCGCACTGCC
mipA	AATGTAAAGACCAGGCTCGC	TAAACCGAGTTCAATGCCCG
mltC	AGATCTCCAAAGAAGCCTGG	AAAGCACATTTGACCGTGCG

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse

mltD	TTCTTCCGCACGATTTGTCC	TCTCACGTACGAAACTACGC
moaA	CATAGCGAAAGTGTGGATGG	TACCACTTGTACATCGTCGC
mobA	ATGTCATCCGTCAAATCCGC	TGGTGTTTCCGTCATCAAGG
mqo	CCGGACGGCATAATATTACG	TATCAGCATACGCCACATCC
mrcA	GATAATGTCGTTCTCCAGCG	ATGGATTTGCTGGAAGACCC
msbB	TATGGATAAACCAGCAGGCC	AATCTGCTGCGCGATAAAGG
mscL	AATCTGCGCATTGAGCAAGG	TGATCCCTTATTCCGACAGC
mtlD	TGGCAGGTCGTAAGTAATCC	TCTACAGCATACTTCACCGC
mtlR	TACGCTGGAATATGGTCTGC	AATACCCGATGAAAGCGTGC
nagZ	TCAATGACTATGCCACTCGC	TAGGGTTGAAGATCACCTGG
nanE	TTTACCTACAACGTTGGCGC	GATTATGCGGATTAAGCGCC
nanR	CAAGGCTATTTATGGTGCGG	ACGTATAAACCGTCGATGCC
ndh	TGATGCGCTTCTTATCAGGC	TTATTCACGGGAACACCTCC
ndk	TGGTTTCTGAATGGCGAACC	TCCACTTCATCACCTGATCG
nifU	TCGCAGTTTCTTCAGGTTCC	CAGAAATTGCAGGCTCTTGC
ninE	TGAATACCTCATCAGCAGGG	ATGCAATGGTGGCAATCAGG
nlpB	GGTCTTGTGGCGACCGATAC	CCTTATCCGAACTACGTCCG
nlpC	GATTGTGATGAGCAGTCACG	ATCTTCATGGTAACCGTCCC
nohA	TCACTTTACGGGTCCTTTCC	ATAAATGCCGGAGCATGAGC
nudH	GCTTATTCAGAATGCTCCGG	ACAACGTCGATCATGATCGG
nuoA	TTATCCTGGAGTCGTCAAGG	ACGAAGTCACCATCTCAACG
nuoB	TATCTTCGACGTTGAAGCGC	TGAACAGTAAAGGCATCCGG
nuoC	TCTATCGGCAAAGAACGTCG	TCTGACTGTAGAACGTTGCC
nuoE	GCCTGCCAATGAATCTTTCC	GCATTACACAGCAGGTAACG
nuoF	GCTGCTGCCAACTTGCTGCC	GTCTACATGAATTGTAGCCA
nuoG	GCGCCATCAAATATTTCCGC	TAACGGTTCTGGAACAGACC
nuoH	GCCGTACATCAAACTCAACC	ACGATACGACCACGATAACG
nuoI	TCTCTGCATTGATGGTGACG	CACGAACAGCACCATAATGG
nuoJ	TTCCGCATCAACTTCTCACG	GAATGTACATCACCTGACCG
nuoK	TAACGATCAGGGTATCGACG	GATGTTAAAGTCGCCTACCG
nuoL	ATCATGATTAACGCCTCCGC	TAGCCAGGGTAGTAACATGG
nuoM	TGCTGTTAAGTGAGAACGGC	TAAGCCCAATAACCGAGAGC
nuoN	TTACATCGCGCTTACTTCGG	ACGACTGCATTTCAGGAACG
ompA	GTAGACTTTACATCGCCAGG	TATTGTCTGGCAACGTCTGG
ompR	TGCGCACATTGGGTATAACG	TTGGAGTAGAGAGAGATCCC
osmY	GTGATGACATTTCTGACGGC	CCATGAACAAACTCACCAGG
oxyR	ATTTGAGCCTGGCTTATCGC	ACAAATCGTCGGCATGAACG
paaC	GTTGGCAGTTTACATGCTGC	ATCAAATGTTCCGTTGCCGG
pal	CAGATGGGCGTTTCAAAGCG	CCTGAGCAAAAGCGGCCCAG

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse

parC	CTGCTTAAACCCTACGTTGG	ACCTGAAATCAGGTTAGGGC
pcm	TCAAAGGTATTCGCGTGACG	AGCGAAACCAGTGACAAAGC
pdxH	CTACTGCATCTCAAACAGGG	ATAACGACTGCACCTTTGCC
pepB	TTTGTTAGTCTGGCTGGACG	TGGATTCCAGTAGAGTACGG
pfkA	GAAATGGAAGACTCTCTGCC	TTGCGGGTATATGTTGAGGG
pfkB	TCAAAGATTAGCGTCCCTGG	CATCATCCGTCATAGTGAGC
pflC	AGCAGGATTACGCAAACTGG	GCAATGTTCAAATCGCTCGG
pgi	AATGCTTCACTGCGCTAAGG	ACCTCACGGTATGATTTCCG
phnG	GAAATCCCGAATATGTCGCC	TTCGTTCTTCGGCAATACCC
phoQ	CGAAGTGATCAAACTGACCG	TCAACTTCGCTTTCCATCGC
potF	CCGAGCTATAGTCTCAAACC	CATCGTAGGATTTGGTCAGG
ppdC	TGGAAAGTCAGTCATTGCGC	TTAAAGGCGCTCTCTTTGGG
ppiA	GGCATAGCGGGATATGCCGC	GACGCCTTCAAGACGGCGAC
ppiB	GATTCCACACACGTACATGC	CCATGCTTCAAACAGATCGC
ppk	ATTAGCTGGTTTGCCGATGG	TTTAAACCGCGCGTCATTGC
ppsA	GCCGCATCATTCATTATCGC	GTCGAAGAGAGCAGATTTGC
priA	CATTACGTTACCGCAAGAGC	TCATTAATGCTCGGGAGACG
priB	GATGAATGTTGAAGCTCCGC	GTATTAATGGACCGTGACCG
proW	GTGGTCGACGAGGACCAACA	CAGTAATGCCTTTGCCCGGC
pstS	GTGACAAACCGTCATCTTCG	GAGAGACAATAATGCCACCC
purA	GGAAGAAGATGATCTCTGCG	TAACACTGTGTCGGATAGCG
purD	CGTGATGACGAAGTGATTGC	TCAGGATATTCAGCCGTTGG
purE	GCATACGGAAGGTTCAATGG	CTATCTCAGCGGTAATCACG
pykA	TTGAAGCGGGTCAAAGAAGC	CGAAATCCAGCCGTATAAGC
pykF	TTTCAGCGTATAATGCGCGC	AAAGAAGCATCGAACGCTGG
pyrC	TAACCAGCAACCTGCTTACG	CATAACGTACCGATACGTGG
pyrI	AAGCGCAGTTTGTTCTTCGC	TAAGTGGCGTTTACGGTTGC
qor	AGCCTGCTGTTTGTTGAAGG	TTGTGCGGATGATCACAACC
rbfA	TCATCGAGATCCAACGTACC	CATCAGAAGTATCGGTACGC
rcsA	ATCTTCCAGGATACTCCTGC	CGATGAAGGTGAATGATCGG
rcsB	AGTGACTTTGCTGCGTTAGC	TCAGTGCAAATGCCAGATGC
rdgC	TCGTTTCAATAGTCTGCCGG	TCGAACAACCCGATCTTTCG
recA	ATGCATTGCAGACCTTGTGG	GTTTACGTCGCAGTTCTTGC
recB	AAGGCATGGCTATTCTGTCG	GATTTCACTGACACAGGTCG
recC	CAGAAACCAGTACCAACAGC	GTTTCTGCCTGACTTAAGGG
rep	TGGATTCACGATGAACTCCG	GCCAGAGATGACATTACAGG
rfaC	GTGATCCGTTTGATTACCGG	GTAAGTAGCACGAAATGGCG
rfaD	GCAATTAGCATCCTTGCACC	GAGGCATAGGAATAGCTTCG
rfaE	TATTCTGGAACTACTGGCGC	GGGCCTTGTATAAGGAAAGG

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse
rfaF	GGCTTATCACAAGAAAGGCC	TTGCCACAGGAATAACTCGC
rfaG	ATTTGGTGCAACGGATCACG	TCACTCAGGCGATGAATAGC
rfaH	TGATTTCCAGATGCGGATCC	ACTGATCGAAACTGATGCGC
rfaP	GGGTACGCGCATTATATTGC	CAGCCATGCATTATCCATC
rfaQ	AGCACGTCAAAGTAAGTGCG	TGGTATGGGACTTAACTGGC
rfaY	CTAAACCGTGGCACAAATGG	GGTATAACGACTTCTCTGGC
rfbB	ACAACGAGTTAGCAGTAGGG	AACTCCGGTTCTGATTCTGC
rfbD	CTTATGTTGCTGATCGTCCG	AGCGGGTAATAGATCATCGG
rffA	AACTCAATGCGACAGATGCG	GTACTGGGCAACGTATTTGG
rffC	CTCCATCAACCAGATGTACC	TCGGCATGATCACTTCATCG
rffH	TGACCTGATCACCTTTGTCG	CAACTTCACCTTCTACCAGG
rhaR	TTCTCTTTCACTGCGTACGC	TGTGTTACTCTCGACACTGG
rhaT	AAATGCCCGAGATGTGAAGC	TGCGCAATATCTTCTCCAGC
rimK	GCAACTGGCGGAATATTACC	AGACACCGTCGAAATTCTGC
rimM	TTCTACCAGGTTGTTGTCGC	TTGATTCGTTGCCAGTTCGC
rmf	AGATGGTTAAAGTGCTGGCC	ATTCACTGGTCAGGTACTGC
rnfC	AATGTATTCAGGCGTGTCCG	GCTTGCGTAGTTTGAGTACG
rnk	CGTCTTCCATAAACCAAGGC	GCCGCCATAATCAGAAATCC
rnt	TACGGTTATCGCGTTTGTGG	GAAGGGTCTGGATTATCTGC
rpe	TGAACAACAAGCGCATCTGG	TACGTACCTGTTCTTCTGCC
rpiA	ATACCCAGACCGTTGTATGC	CGGCAATATGAACGTTTGCG
rpiB	TTGTGAACAATGGCGAGTGG	ATCGCCTGCTGAATATTGCC
rplA	CTGGTATCAAGTCTGGTTCC	TCTATCCGTCGCTATTCAGC
rplI	AACTACATCACCGAAAGCGG	TCCTTATCAAGTTCAGCCGC
rpmE	AGTCAAAGGTACGAGGAAGC	TGAAAGAGTTCGACCTGAGC
rpmE2	GGAGATAACCTCCATTCTCG	GTTGGCCTCAAATGAACAGG
rpmJ	ACCTTTATCTGCCTGATCCC	ACGGCATGCTTATGATCAGG
rpoN	CGTTAAGCGTGTATACCTTGGC	GTAACAAATTCGCGCAGTGCCG
rpoS	CCATAACGACACAATGCTGG	GTGTTAACGACCATTCTCGG
rpoZ	CAGAAATGAGCCATTACGCC	TAGTCGAGTTTCATCTCGGC
rrmJ	TCGGTCAAACCATTACTGCC	AGAGTAATCCACCTTACGGC
rsd	ATTCGCCGATTTGTAGTGCG	ATGAAGCAAGAGACGATCGC
rseA	ACGCATGGCAATAACCTTGC	ACAATTGTGCAAGAGGACGG
rstB	TGGGAATTAGCTACCCATGC	CTTGCGAAAGGTTATCGACC
rtn	TCAGCCGCAGCTAATAAACC	GAAATTAAACGCGTACCGGG
rusA	ATCCCTGGCGTATGTAATGG	CCAGAACGCCAATTACAAGC
sbcD	GAGCTTCATCTTCTACGACC	ATACAGCGCCAGACAAATGG
sbm	TCACAAAGTCCTTCGTCAGG	TCAACAACATGCCAAAGGCC

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse
secB	AGAGATGATCAAACGCAGCG	CGAGATCGCTTTCAAGATGG
secG	GTGACTGAATTTGCACACCG	TGACAACCATTCCTCAGACC
sfsB	TCAACGCGATCGTTAATCCC	CTTTATGCATGTGCCAGAGC
slyA	TGTAAGGGCAATCCTGTTGC	TGTTCTTGGTGGTTTCCTGG
smpA	GCTTCACGGTCAGAGTAAAC	GAGCTTCGCAGGCAACGAGC
smpB	ACTGATACATTTGCTCCGCG	GATTTATGAGCGCCATGACC
soxR	ACATAACCCAGGTCCATTGC	GTATACCGTTCAGGATTGCG
sprT	AACACCTTCTGGGTGTATGC	GATTGCAGATCAACAACGCC
srlR	GCAATTACCGGTATGCATGC	TAAACAGCATCACATCGCGG
stpA	TCGGTGTATGTAGTAGCTGG	TCAACCCTTGCCGTTAATGC
sucB	GAAGTGTTGCAGCAGTTTGC	CGTTAAACGTCACATCAGGC
sucD	TCTGGTTAACATCTTCGGCG	ATTTACCCTGATAGCGCAG
sufI	CAAAGATCAGGTTCGTGAGC	TTGGATCAGGGAGATAGAGC
surA	GCGGTATATGACAACGCAATC	CGGAGGGTGAGCGGCAAAC
surE	ATTCGAACAAGCAGCTGTCG	ATTCGCGCCACCATATATGG
talA	ACGTGGCGCACATTATTACC	CAAACTGTAGAGCAGCATCG
tap	AACAGGCGAGTCGTTTAACG	CGCTGTGCTGATTAGATTCC
tatA	ATGTACGTCAGGGACAATCG	TCAGACTGTCCTGAAACTCC
tatB	GCAATGAGCGATGATGAACC	ATCATCGTTGAACCTTGCGG
tatC	TAATGAAGCTGCGCATGAGG	CAACAGGACGAATACTGACG
tatD	ATCGGTGTCTTCTTCTCACG	CGAAAGACATTGTCGATCCG
tatE	TCATTATGGCGGAAGTGACG	GTTCGACGAAATGAAAGCCG
tbpA	TAAATCAGCGTGGTTTGCGC	AAGGTCATTGCACACAGACG
tfaD	TATCTCCCGAAAGAATCCGC	GTTGTGCACAATAGCTTCGG
tig	AGTGATGCCGCTATAATGCC	CTGCTGTACCGATTGTAACC
tktA	CGCTCAGTCTCAGTATAAGG	AGCAATTGAGCAGGGAAACC
tolB	CAGGCACCGGTTGCCACGGG	GCAATTGCCATAACAGGCAG
tolC	CCGCGATAAAGTGTTTCTCG	TTCAGCGCATTGTTGTACGC
tolQ	CAACGCCGAGAATACTTTGC	GCAACAGCACCAGCAGTACG
tolR	GCAACACTGCAAATGGTTGC	AAGATGACATGCAGCACTGC
tonB	TTGAAAGGGCGAAGATCTGC	AATGACTTTCTTACGGCCGG
tpiA	TGCACTGCGTTATGTTGTCG	ATGCTGGCAGAAGAAGTACC
treA	TCTTCGCTAATCACGAACCC	CTTACCCGTAGAAATGACGC
trmU	GTGTGTAGCGGGTTAATTCG	TATGCATCAGTGGATTGCGC
tsx	ACTCTGCTTACATCACCTGG	CAGGCCTACGTTATTTCAGC
tufA	TGGTATGCTCAAAGGTCAGG	CATGCTTCCATTGCCAATGC
tufB	ATTACCTCAGCCTTCCAAGC	GGCAAACCAAATCGAAACGC
ubiC	CCACTTCATGCGATTGTTGC	CACAACGCCCATAATGTTGG
ubiE	AGCCAGGATGAAGAGTATCG	ATTCGCCCAGTACATCAACC

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse
ubiF	GTACTCGTTCATCTGACAGC	GATTCTGATTCCGGCATTCC
ubiG	CGCATAATCCAGATAGGAGC	ATATCAGTCAGCGAGTGTCC
ubiH	GTGTTTATGGTCAGGATCGC	TACGAGAGAGAATGTCCTGC
ubiX	TGAATGCTCGGTGTTCAACG	GGTCTTATGCCATCTTGACG
ugpA	GATTGTGGATGAAGAGCTGG	AGCTGTTAAGCAACATCCGC
uidB	TATCATCACCGAATACGGCG	TGAAATCGAGCAATCCTCCC
uvrY	GTCACATTTCATCGTAGGGC	TCAATTTGCTGGATCTGGGC
uxuR	CGTTGTAGGCCGGATAAGGC	CCAGGAAGAATGAGTACTAAC
vacJ	CGTATAGCTAAAGCGTAGCG	TACGTCTAGGTCATTGTCGG
wbbJ	TATATGGGATAAAGCCGCCC	TTAGCCACGACCCTTTAACC
wcaD	TTCTCGCGTCGATAACTACC	CGAAGCTGATATCTTCCACC
wza	TCTCATCAACGACTGCATGC	GAGACAGTTGATGTTCTGCG
wzzB	GAAGAGCTTAAGGATGTGGC	AGAAAGTGGGTGAAGAAGGC
wzzE	TTGTGCTGATTACCCTTGCC	GCAGTGACGCAAACTTTAGC
xapR	GTTTACAATCGTGCGCTTGC	CAAAGATTCGAACCTGCAGC
xdhA	CTATTTCCCATCCTGATGCG	TAAATGTCGTTGCAGAGCCG
xylF	AATTCACAAGTGTGCGCTCG	CACACAGCACTTTCATCAGC
yadM	GAGCAAGGCAATGTCATTGG	ATGTAGCGGTACAAGTACCC
yaeD	ATAAGCGTACTCTTACCCGC	GTTAAAGAGCAGTTGCGACG
yafC	CTATTACGTCAACCCTTCCG	TAAACACAATGCGACTCCGG
yafP	TCTACGACGACTCCTTTACC	ACTTATGTAAGCTCCTGGCG
yagE	GTAAGTGTCTACCATGTCCC	TAATCAGCACCTCTTTGCCG
yagT	CCCATCATCATCAACATCCC	CTTCAATCTTATCGAGCCCG
yagV	TCCTTTATTACCCGCAACGG	ATGATTTCCAGCCGTTACCG
yagY	ATGAATATAGAGCGGGACGC	AGGATGGTTTCACCTTCTCC
yagZ	TCCATTGAGATAACCTGCCC	TATACCCATCGGCGTAATGC
yahL	CGCGGTGATGTGAAATATCG	AGAGATGGAATGCCAGTTGG
yahN	TTCAGCGGCATAGACATTGG	GTTTGGCTATTCAGGATGGG
yaiO	AGAGGGAATTATCGCTACCG	TTTCGCACCTCATCATCTGC
yaiP	AATTCTCGGCTACGAAACCC	GGCGTTCGAGCATTATAACG
yaiT	TGTCCACATTCGGACTTAGG	CTACGCAGAGAGATAACTCC
yaiY	CTCACAACGAAGATTCACGC	CTGGCATTCAACTGGAAAGC
ybbK	ATCCTTCGCAGGAATAAGGG	CAGGATGGCAAACATTACCC
ybcI	TACAGGTTGTTGCCTGATCC	TCACCTGCGTAAACTTTCCG
ybcN	CGGCATAATTATGTCACCGG	GGATTCTTACCTGGCATTCC
ybdF	GACCATGTTCCATCTGATGG	ATGGGCTGTATTGCAGTAGC
ybeB	GAGCGCCATTGATATCAACC	ATATCGAGGGTGACAATGCG
ybeD	TCTGAAAGCCAGCTATGTGC	TATATCACAGCGAGGAGAGG
ybeY	AAATCAGGCTTACGTCACGC	GCGTATCTTCGTCGATAAGG

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse
ybgC	CAGAAGGTTCTTTGGCAAGG	GTCCGCTGGATAATAATGGC
ybgF	CTCCGGAATACAACATCTCC	TACCAACTGAGCTAACGACC
ybgI	ACGTCACGTAATAGTTGCGC	TCAATAAAGCGAGACTCCGG
ybgQ	ATCGTATTAGGTTCAGCCGC	GTTCCAGACGTTGAATAGGG
ybgT	CAATGTGGGATGCAACTTCC	CTTGCCAAAGAACCTTCTGC
ybhD	ATGTTCCGCTAACAGGAACG	TCACCACATCTCGGTATACG
ybiO	ACTTGTAGGACGGATAAGGC	TGAAGAGAGCAAAGGCTTCG
ybiU	TAACAGCTGACCTCTCTACC	AACGCGTGATCGACTTTAGC
ybiW	GCTTTAATGCCGACGAAACC	TGTGAATGATTTAGCCCGGC
ycaI	GATCGCGCCAATATTTACGG	CAAGAAGTGGCTTAAGGAGC
ycbF	TGCAGAGCGAAATGTTGTCC	AACGGCGTTCCTGTAATACG
ycbR	TGAATGATGGTACAACGGCC	TCGAAACGAGACAAATCGGC
yccW	TTCGTACTGTGTGGTGTAGC	TATCGTCCTCCATCACTACG
ycdK	TTTCTTCAATACGCCGCTGG	ACTGCGTAATGCTCAATCCC
ycdP	AGCCTGATGATCGAGAATCG	TCGTCAACGGTGATATCACG
ycdQ	GCATCAGGCTATTTCTTCGC	CGCTTTGCCAGTAATAACCC
ycdS	TGCCGTATTCCTGAAGATCC	AGCCAGGCAAATTGTTCACG
ycdZ	TATCTGTTACCCTGGTGTGG	ACGAACCTGTTATGCTGTGC
yceP	GAATTAATCGCGTATCGCCG	AATTCTCCAGGAAACGTGGG
ycfF	GGTTTATGTTGTCGTCAGGC	TAACGGGTTCTTGCCTTTCG
ycfJ	TTAATCGACGTACACTGGCG	TTATGTCAACGACAAGCGCG
ycfK	TATCTTGCACAGCTTGCTGC	TAGAATCTGGCGGAATGACG
ycfM	AACCTTCAGCATCCTCAACG	GTGATAAAGCCCGATACTGG
ycfS	AGAACGCGATCGCTTAATGG	CGGTACAATCAGTGCTAACG
ycgY	TCCGCTTTCACATCAGAACG	ATGCTGGATTTGATCTGCCC
ychE	CAAAGCTGACACCTTTCAGC	ACATGATGGTGAGGAGAAGC
yciM	TATTTCCACATTGCTGGCGG	GTTAACGTCATGACCAGACC
yciT	CTCTGCGATCCATAATCTCG	ATTCCGTGAAATCTGCTCGC
ycjU	TGTCTTTCCCTCTGTTCTGG	CATTCATTCGTTTGCCGTCG
ycjZ	GGAGGGAAATAGATCACTGC	AACTCCGAACACTACGTTCG
ydaT	TGTCCAGCAATTGAACGTGC	AGGATGGTTCAACATCAGCC
ydcP	CTTGATCTGTGCGTTGATGG	AATGTGGATCATCAGCTGCG
ydcS	CCTTACAGACGGCATAATGC	ATACTGACGCCATCTACTGC
ydcX	GATGGATAAGGGCAAGTTGC	ATCTTCCCGACTTAACTCCG
yddM	TAAAGTGGTGCCGAAAGTCG	AACGTGTAGGCCTGATAAGC
ydeU	ATAACAGCGGTGTGATGTCG	TCCACAGCAAGTTTATCCGC
ydfP	TGTCCCTATAACATTGGCCC	GAAGAAATTAGCCCTTGGGC
ydfQ	GCCTTACAGGACAAAGAACG	TCAGCAGATTTCTGTTGCGC
ydfR	ACAACAGGTGTTGCCTATGG	TACGATCACCAGCATTCAGC

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse
ydgI	AATTCTCTGGCAACTACGGC	AGTACTTCATCGGCAAACGC
ydhM	TCGTCAGACTGGCAAATTCC	TTGCCTGGGCAGAAATTTGC
ydhP	TATCGTCCTCGGTTTGATCG	GTGAACTGGGAATTCGTAGC
ydhQ	ATTTCGACCGTTTGCAGAGC	TCTGGTCGTTGAACCTATGG
ydiK	CGACGAGTAAATCGTTTGCG	ACCGTGAGCTAATAGTAGGC
ydjE	AAGACAATGATCCGTCAGGC	ATTACCGATGGTTGTCGTGG
ydjI	GCTATGCCAATCGTTATCCG	GCGGCATCTTTACCAATACG
yeaY	ACGAATGCAACAGCTTTCCG	GAGATTGATAACGGTCAGGG
yebB	GGATAGATATCCAGCGAAGG	TGCCACGTTAGTCAGAATGC
yebU	AGGCAAACACTTCCTGTTGC	GTTAACGTTCGATCACCTGG
yecT	GGTATTGAGACTGTAGAGCG	CGATTTATCTGGGAAGTGCC
yedV	TATTACACTGACGCGCAAGG	TTAATGACGACATCACCGGG
yedW	GCGGCATTAACCAAAGAAGG	GACTAACATCCATCATCCGG
yeeF	TAGCAAATAGTGCGATCGCC	AGAGCACCACAAATTAGCCC
yeeU	TCTGTACCGGTAAATACCGC	GTGTGTCATTCAGTGTGAGG
yegI	ATAACCTCCCGTCACTAACG	TCAGCAATGAACTGGCTACG
yegN	TTGTCTGGGTGCTGAATAGC	AGGTCATTTCACTGACTCCG
yegP	GCCGAACAGTCATTTATCCG	TCTGATGACAGGTCAGATCC
yegT	CGATTGGATATCGGCTATGG	AGGTATCGTCGGTGAATTCG
yehB	TTGCTGGTAAATCGCAGTGG	CCTGAACAACCAAGTTGTGC
yehK	ATGAGCTTCCGTTCTCTTCG	GCGCATTAGTTGTTTGAGCG
yehM	ATTATGTCCACCGCTGAAGC	TACTGTTGATCCACTCAGGG
yeiA	AAGCATGTACGGTTATCGGG	TCCGCATTCGGTCATAAACG
yeiW	CTTAACCAGCAGCAGTTTGC	CGCTCTATACCAACACTTCG
yfbV	CCCGCTGAGTTGTGAATTTG	GTAACGTTCAGCATTTGCCG
yfcQ	CTCAGTACCAATATCACCGG	GCAACGTTCAAATCCTGACG
yfeU	GTGAAGAAACGGATTGCAGC	AACTGACGTTTGTTCTGGGC
yfgA	AGCCATCGACATTAAAGCGG	TGAGTTTGAACGCTTCTGCC
yfgC	TCGAGCATAAAGACCTTCCC	TTCTGGCGCATCAATTCACG
yfgL	GCGCGTAGTGCATGGGAAGC	CAACGCACGCTATATTCGCG
yfgM	CGTCTGCGTGATGAATTACC	ATAGAAGTTGCCAATGCCGC
yfhG	TTGCCAGGGATTGTATTCGC	GCATACCGTCCATTTCATCC
yfhM	TCCTACAAACCATGTCGTGG	AGAGGCCAGAGTTTATCTGC
yfiM	ACAGCGAGAGAAAGAACTGG	CTATATTTGGTGGTTCGGCG
yfiR	GCCAACTGCATGAAGTATGG	AGTGTTTCAGTTGCTGCAGG
yfjD	CTATATTGTGCTGCTGTGGG	ATACGCTGAATGGTCGTACG
yfjN	AGTATCGAGTTGCTGCTTGG	ATTTCCAGCGTAACGAAGCG
yfjQ	TAGGTGCCATACTCGATACG	GTCATGAGAGATTCTCCAGG
yfjR	GATGTGAAGCTTAACCGTGC	ATCAGACGAACCACTTTGGC

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse
ygaM	CTTTCTGGGTTACCACATCG	GCGCATGATTCGTATTTCCG
ygaT	TGGCTACGAAATGAGCATCG	TCCGTTCCATTTCGAGATCG
ygaU	TCTGGAGAATCGTCATCACC	GTCATCAGGCGTTTCATACG
ygaW	CTTATAGCGTTACCTCACCC	ACCGCTCTGGTAATTCATCC
ygbE	TGAACTGCGCAACTTTACGG	TTAGTTTACCCATCCTGCCC
ygbT	CAACTTCAGGTTGGTGTTCC	ACTACATTGCCTTCTTCCGC
ygcO	CAGCGTTATCAGCATGTTCC	CTGATGGTGCAGATATTGCC
ygdD	GCTGGCAAATAACGTGATGG	CTGTAACATGCCGACAATGG
ygdQ	TTCTGATCCAGTAGCCATCG	GACGTAATCACTGGAACACC
ygeA	TGTGGGCATTACTTTCTGGC	TATTTCGGTGGTTAACCCGC
ygeF	ATCAGAAGCTGCGAATTCGG	TTCCTCTGTGATGTGTCTCG
ygeI	ACTGAGAGTGAAAGCCATCG	TTAGAGCAAGAGTTGCTGGC
ygeP	CAGCAGTAAGACTTCCTTCC	TCCGATTTGACAGCAACTCC
yggC	TTCCCAGTTCTGGCATTACC	CTAGGATAAGCGAAACGTGG
yggD	ACGTTAACCGTGTGTACTCG	GAGATATTCCCAGAAGGTGG
yggL	TAAACGCCGTATCGTTCAGG	ATTGCCGTCACTGAAAGTGC
yghB	TTTAGCGGGACCTTGATTCG	ATTACCGTCTGCATAAGGCG
ygjR	GACCTCAATATGAAGCACGG	GGAATACGTCTGCGTATTCC
yhaC	ACAATGTACTTGCTCAGCCG	TTTATTCATGCCGGATGCGG
yhaK	GATCGATCGCATCCATAACC	TTTACGCGGTAGCTTCATCC
yhbE	CTGTTTGCTAAAGCAGACGG	TAAGCGTGTTCAGGTTCTCG
yhbG	GACGGGTAATGCTTATCTGC	GAGTGTCGATTTGCTCAAGC
yhcB	TAAGCGCCTTCAGGTATTGC	CCGACACTTAACGCTAATGC
yhdP	GTAGCTGAAGCCTTGAAAGG	TTGTAGGCCTGATAAGACGC
yhdV	ATCCGCCGTTGCTTTAAAGG	GAAAGCAGCGAAATAACGCG
yheL	GCATTAACTGACGATCTGGC	ACCAGCTGGTTAACTGTTGC
yheM	ACGAATTTGACCTCGTACGG	ATAAGGTCTTCGTTCAGGGC
yheN	AGATCTGGTTACCCAAACGC	AGTGGCAATGTAATCACGCG
yhfW	TACTATCGTCGTGGTCATGC	TCATCAGGTCGTTCATCTCC
yhiI	TGCGTGTTTCATTTACGGCC	CATTGCCCTGTTCAATGACG
yibP	GTTCCGCTGATTTACGTTGG	TGAATCAACACTTCGTGCCC
yidL	AATAGCCTTGTTGCGATCCG	ATCCCGCAGTTTCAGAAAGG
yihF	TCCGGCGATGTTTGTTAACG	TGTTACGCTGTGTTATCCGG
yjbB	TCCTTACAACAAACAGGGCG	TGAGTAAAGGTCACGCTACG
yjdB	AATGCAGTCCATCTTCGTCG	ATGCGCTGAATTACAACCGG
yjfR	TTCCACATGGATAGAGTGGG	GCTCAACGACTTTCTCAACG
ykfA	TTTAAGGTGTATCACGGCGG	GTCCTCACTGAATACACTGG
ykgE	AACATCGGGCATAAATGGGC	GAGAGCTGGTACAGATAAGC
ykgG	GCCGGTGCTCATGCGGCAAG	CATAACAATGACGGAACGGG

E. coli Checking Primers Sequence (5'→3')

Gene	Forward	Reverse
ylaC	TCATTAACCCTGGTGTGACC	GTGTTCTCACCAGCATATCC
ylcE	GCAAGCCGATTTGATACTCC	ACTACAACGCACCTGAAACC
ylcG	CACTAAAGCAGGTTTCTGGC	ATGATTATTAGCCGCCCACG
ymdC	GGATGCCTATCTCAATAGCC	ACATTACACCGCACATCACC
ymfM	TCGTTCAGCAATCGAAGACG	TGATATTCATCCACCACGGC
ymfP	ATCTGTATCACCTGAACGGC	GCGGTTTATCAGTTCAGTGG
ynbA	TGAACTGGATTTAGTCCGCC	ATCAGAAACCCAGCCAAAGG
ynhG	TCAATTTCGATGTGCGTCCG	TCAGGCTGCTAAAGATGACG
yniC	TGACCGCATTAATGCCTTCC	TTGGCAAATACCGCACAAGC
ynjI	TGATCAAGCCAAACGTACGC	AGGTGAGCAAACCAACTACG
yoaC	GTGTTCGCCCTTTGATTAGC	TTCACTCACCCATGAACAGC
yobG	TTCGTTAGTCCGTGAACTGG	TCCATACCAGTGCTATCAGC
ypdF	ATTCCGTCGTATATGGTCGG	ACCTGTCGATTCATTCAGGC
ypdI	GAGAAATCAGAGAGCAACGG	GGTAATGAACTAGTGACGGG
ypfH	GTGATGCGCAGTTATGTACC	CGAAGAAGTAAGCTGAGTGC
yphH	ATGGGATGTATATCCTGCGG	ATGTGCTCGTTGTTCATGCC
ypjB	GTAACGCTGTTATTGCTGGG	CTTAAGCCAAGTAGTACGCG
ypjC	CGTGGCTATGCTCATTAACC	AGCACCAATTCAGTCACTGC
ypjF	ATGTCCTAAGCTGGATGTGG	TTGGTGTTGTCCTTACACCG
yqaA	GCTTCCTCTTGTTGATGTGG	ATGGTGACCATATGTGCAGG
yqaD	AATTATGATGGGTCCACGCG	ACATGGCGATGCTCATTTCG
yqeJ	CATCCGTGGTTATACTTCCC	TCGACTCATGGATTTGCACC
yqiG	CAAAGCTCGTATGGAATCCC	TATGATACGGGTACGATCCG
yraP	GGCATTGACCGGCTATGACG	TCGGATGCGGCGTAAACGCC
yrbI	GTATTGCCGATGTGATGACG	CAATCGATAGCTTAGTGCCC
yrfD	TCGTTATCTGTAAGCTGCGG	GCGGTATAGGCCATAAATCG
ytfM	TGTTAAGCCGTTTAACGGCG	GACCTGAATGTCTTTCAGCG
ytfN	CGAAGTTGATAACCGGATCG	AACGTACATCCATGCACTCC
ytfP	TGGCGTAAGCAATTTAGCGC	TAATACGCATCTGCACCTCC
znuB	TTCTCACGATCTGCATCTGG	ATCACTTTGGCATAACGCCG
zur	CGACAAGGCAACATAACACC	GAATAAAGATGAAGCCGGCG
zwf	AGATATTACGCCTGTGTGCC	CTGCGCAAGATCATGTTACC

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