A GENETIC INTERACTION STUDY OF THE β-BARREL ASSEMBLY PATHWAY

by

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Abstract

In *Escherichia coli*, the BAM complex is the β-barrel assembly machinery that is essential for the incorporation of proteins (OMPs) into the outer membrane. It consists of two essential components, BamA and BamD and three non-essential components, BamB, BamC and BamE. SurA, Skp and DegP are believed to be chaperones that transport OMPs to the BAM complex. The first aim of this project was to determine whether the non-essential BAM components have distinct physiological roles. A second aim was to determine whether the three putative chaperones are functionally redundant. A third aim was to reveal how OMP biogenesis coordinates with other cell envelope pathways. To address these unknowns, transposon-directed insertion-site sequencing (TraDIS) was utilised to identify genes that are essential in mutants defective in *bamB, bamC, bamE, surA, skp* and *degP* but not in the parent *E. coli* K-12 TraDIS library. In the Δ*bamB,* Δ*bamC* and Δ*bamE* TraDIS libraries, 29, 39 and 17 conditionally essential genes were identified, respectively. There were clear differences between these three datasets. Thus, the functions of BamB, BamC and BamE do not overlap. In the Δ*surA*, Δ*skp* and Δ*degP* TraDIS libraries, 54, 9 and 44 conditionally essential genes were identified, respectively. Seventeen genes were conditionally essential in both the Δ*surA* and Δ*degP* TraDIS libraries, which suggests that the function of these two proteins partially overlap. In contrast, there were differences between the conditionally essential genes in the Δ*surA* and Δ*skp* datasets. Thus, the functions of SurA and Skp are not redundant. The TraDIS data also demonstrated that loss of genes involved in the synthesis and incorporation of heptose into lipopolysaccharide, combined with loss of *bamB*, *surA* or *degP*, is lethal to the cell. These LPS defects increased membrane fluidity, which decreased BAM activity. Cell death occurs due to a lack of OMP insertion into the OM. This study also identified synthetic lethality between *surA* and genes involved in the synthesis of enterobacterial common antigen and between members of the BAM pathway and the gene *dapF*, which is involved in peptidoglycan

synthesis. Thus, OMP biogenesis requires a network of components and impairments in OMP biogenesis has negative consequences on other pathways.

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Table of contents

List of figures

List of tables

List of appendices

List of supplementary tables

Abbreviations

A. baumannii	*Acinetobacter baumanii*
Ala	Alanine
Amp^R	Ampicillin-resistance
ATP	Adenosine triphosphate
BAM	β-barrel assembly machinery
bp	Base pair
CDS	Coding sequence
CIP	Calf alkaline phosphatase
CL	Cardiolipin
D-Glu	Dextrorotatory glucose
DIC	Differential interference contrast
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
E. coli	*Escherichia coli*
ECA	Enterobacterial common antigen
ECA_{CYC}	Cyclic enterobacterial common antigen
ECA_{LPS}	Enterobacterial common antigen linked to lipopolysaccharide
ECA_{PG}	Enterobacterial common antigen covalently linked to the lipid phosphatidylglycerol
EDTA	Ethylenediaminetetraacetic Acid
FRT	Flp recombination target
Fuc4NAc	4-acetamido-4,6-dideoxy-d-galactose

Gal	Galactose
GCMS	Gas chromatography-mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
Glc	Glucose
GlcNAc	N-acetylglucosamine
GO	Gene Ontology
Hep	Heptose
HITS	High-throughput insertion track by deep sequencing
^{1}H NMR	Proton nuclear magnetic resonance
IFR	Insertion-free region
IIS	Insertion index score
IM	Inner membrane
IMP	Inner membrane proteins
INSeq	Insertion Sequencing
Kan^{R}	Kanamycin-resistance
kb	Kilo-base pair
kDa	Kilodalton
Kdo	2-keto-3-deoxyoctulosonic acid
LB	Lysogeny broth
LL-DAP	LL-diaminopimelate
Lol	Localization of lipoproteins
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
Lpt	Lipopolysaccharide transport
LTs	Lytic transglycosylases

Meso-DAP	*Meso*-diaminopimelic acid
MIC	Minimum inhibitory concentration
Mla	Maintenance of OM lipid asymmetry
mRNA	Messenger ribonucleic acid
MurNAc	N-acetylmuramic acid
NT	Nucleotide
OD	Optical density
OM	Outer membrane
OMP	Outer membrane protein
OPG	Osmoregulated periplasmic glucans
P. aeruginosa	*Pseudomonas aeruginosa*
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCP	Phenol, chloroform and light petroleum
PCR	Polymerase chain reaction
PE	Phosphatidylethanolamine
PEP	Phosphoenolpyruvate
PG	Phosphatidylglycerol
PG	Peptidoglycan
PL	Phospholipid
PMA	Phosphomolybdic acid
POTRA	Polypeptide translocation associated
PPIase	Peptidyl-prolyl isomerase
PTS	Carbohydrate phosphotransferase system
qPCR	Quantitative PCR

Rcs	Regulator of capsular synthesis
Rep	Replicate
Rha	Rhamnose
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sec	General secretory
SOC	Super Optimal broth with Catabolite repression
sRNA	Small ribonucleic acid
Tat	Twin-arginine translocation
TCA	Tricarboxylic acid cycle or krebs cycle
TLC	Thin layer chromatography
Tn	Transposon
Tn-seq	Transposon insertion sequencing
TPR	Tetratricopeptide repeat
TraDIS	Transposon-directed insertion-site sequencing
tRNA	Transfer ribonucleic acid
UDP	Uridine diphosphate
Und-P	Undecaprenyl-phosphate
Und-PP	Undecaprenyl-pyrophosphate
σ^E	Sigma-E

CHAPTER 1

Introduction

1.1. Antibiotics and the emergence of resistance

Antibiotics are natural or synthetic molecules that actively kill or inhibit the growth of microorganisms. They are used in the treatment and prevention of bacterial infection in both humans and animals. Antibiotics revolutionized medicine and saved countless lives. However, the emergence of resistant strains threatens these advances. Antimicrobial resistance can be defined as "resistance of a microorganism to an antimicrobial drug that was originally effective for treatment of infections caused by it" (WHO, 2014).

1.1.1. Antibiotics

The discovery of chemical compounds used in the treatment of infections consists of three main periods: the preparation of alkaloid extracts; the development of synthetic agents; and the discovery of antibiotics (Yazdankhah *et al.*, 2013). The first period occurred in the 17th century, where alkaloid extracts from bark of the cinchona tree and the ipecacuanha bush were found to be effective against some infections, including malaria (Greenwood, 2008). The developments of synthetic agents in the second period began with the discovery of salvarson used in the treatment of syphilis and was crowned with the production of sulphonamides by Gerhard Domagk (Greenwood, 2008).

The period of antibiotic discovery began in 1928, when Alexander Fleming recorded that contaminated mould inhibited the growth of staphylococcus, which later lead to the discovery of penicillin. Howard Florey and Ernest Chain followed on from Flemings work, leading to the mass production and successful treatment of individuals with penicillin. In the 1940s, the search for new antibiotics were mostly based on soil microorganisms and marked the beginning of the 'Golden Age' of antibiotics, where several antibiotic classes were discovered including aminoglycosides; tetracyclines; cephalosporins; and macrolides.

Antibiotics are classified based on their mechanism of action and chemical structure. The majority of antibiotics target key bacterial pathways that are required for growth including the bacterial cell wall. However, for each new antibiotic discovered, a corresponding emergence of resistance to that antibiotic occurred (Lewis, 2013). The golden age of antibiotics expired in the 1960s and since 1987 no antibiotic with a new mechanism of action has been made commercially available (Livermore, 2011). The lack of new antibiotics and the overuse of antibiotics has imposed selective pressure on bacteria, resulting in the increased development of resistant infections.

1.1.2. The clinical significance of antibiotic resistance

Antibiotic resistance is "one of the biggest threats to global health, food security and development today" (WHO, 2018). In Europe, 33,000 people die each year because of resistant bacterial infections (ECDC, 2018). Antimicrobial resistance is not new. However, the number of resistant organisms, the geographic locations affected and the extent of resistance in single organisms are rapidly increasing (Levy and Marshall, 2004).

The majority of resistant hospital-acquired infections are caused by the ESKAPE pathogens: *Enterococcus faecium; Staphylococcus aureus; Klebsiella pneumonia; Acinetobacter baumanii; Pseudomonas aeruginosa*; and *Enterobacter* species (Rice, 2009). Therapeutic options for these pathogens are extremely limited. Consequently, previously discarded drugs with significant toxicity are utilised in the treatment of these pathogenic infections (Boucher *et al.*, 2009). Hidron *et al.* (2008) recently demonstrated that 26.4% of *P. aeruginosa* isolates and 36.8% of *A. baumannii* isolates that cause ventilator-associated pneumonia were resistant to carbapenems. Similarly, Souli *et al.* (2008) reported carbapenem resistance rates of up to 85% among ICU isolates. In addition, pandrug-resistant bacteria that are resistant to all existing antibiotics have been reported in several studies (Liu *et al.*, 2015; Paterson and Lipman, 2007;

Valencia *et al.,* 2009). Consequently, searching for new antibiotics, antibiotic targets or new methods of treatments is vital. Understanding mechanisms of resistance and bacterial cell physiology is crucial to identify pathways or proteins that can act as targets for new antibacterials. The first barrier to an antimicrobial compound is the cell envelope making it an ideal target for new antibacterials.

1.2. The cell envelope of bacteria

The bacterial cell envelope is a macromolecular organelle that surrounds the cytoplasm and provides a rigid exoskeleton that protects the cell from mechanical and osmotic stress. The cell envelope acts as a barrier keeping toxic compounds out of the cell, while allowing the selective passage of nutrients into the cell. In 1884, Christian Gram developed the Gram strain, which classifies bacteria based on the structure of their cell envelope into two main groups: Gram-negative bacteria and Gram-positive bacteria (Silhavy *et al.,* 2010). The Gram-positive cell envelope consists of a cytoplasmic membrane lipid bilayer and a complex peptidoglycan cell wall, while the Gram-negative cell envelope is comprised of an asymmetric outer membrane (OM) lipid bilayer and a cytoplasmic inner membrane (IM) phospholipid bilayer, separated by the periplasm. The periplasm contains a peptidoglycan cell wall, which maintains cell shape and provides mechanical strength.

1.2.1. The cell envelope of Gram-positive bacteria

Gram-positive bacteria are comprised of three distinct cellular compartments: the cytosol, a single cytoplasmic membrane and a surrounding cell wall (Giesbrecht *et al*., 1976). The cell envelope can vary greatly depending on/within species. A number of Gram-positive bacteria divide without separating their cell walls and as a result continue to grow as strings of cells (streptococci) or as clusters (staphylococci). Gram-positive bacteria lack an OM but instead are surrounded by a thick peptidoglycan mesh. This mesh is comprised of glycan strands,

which consists of repeating disaccharide N-acetylmuramic acid-(β1-4)-N-acetylglucosamine (MurNAc-GlcNAc) subunits (Ghuysen and Strominger, 1963). Threading through the peptidoglycan layers are secondary long anionic polymers such as teichoic acids. Teichoic acids are composed largely of glucosyl phosphate, glycerol phosphate, or ribitol phosphate repeats.

Between the cell wall and the lipid bilayer is a narrow space called the periplasm (Graham *et al*., 1991). Numerous periplasmic proteins that occur in Gram-negative bacteria are lipid modified in Gram-positive bacteria (Gilson *et al*., 1988). These proteins capture imported substrates, for example carbohydrates and transport them to the cytoplasmic membrane. Thus, the periplasm acts as a space for the transport of substances in and out of the cell. The additional OM in Gram-negative bacteria acts as a selective barrier and renders the bacteria more resistant to antibiotics than Gram-positive bacteria (Wu *et al.,* 2014). Consequently, this study will focus on the Gram-negative bacterial cell envelope.

1.2.2. The Gram-negative bacterial cell envelope

Glauert and Thornley (1969) identified the three principle layers that make up the Gram-negative bacterial cell envelope: the IM, the peptidoglycan (PG) cell wall and the OM (fig. 1.1). The periplasm was later defined as the aqueous compartment separated by the two membrane layers (Mitchell, 1961). The major difference between the Gram-positive and Gram-negative PG layer is the thickness. The PG of Gram-positive bacteria contains many layers and is 30-100 nanometers thick, while the PG of Gram-negative bacteria is only a few nanometers thick (Silhavy *et al.,* 2010). The IM is a symmetrical phospholipid bilayer, which separates the cytoplasm from the periplasm. Membrane-embedded proteins transport specific molecules into the periplasm. The OM is an asymmetric bilayer that consists of an inner leaflet of phospholipids and an outer leaflet of lipopolysaccharide (LPS). The OM also

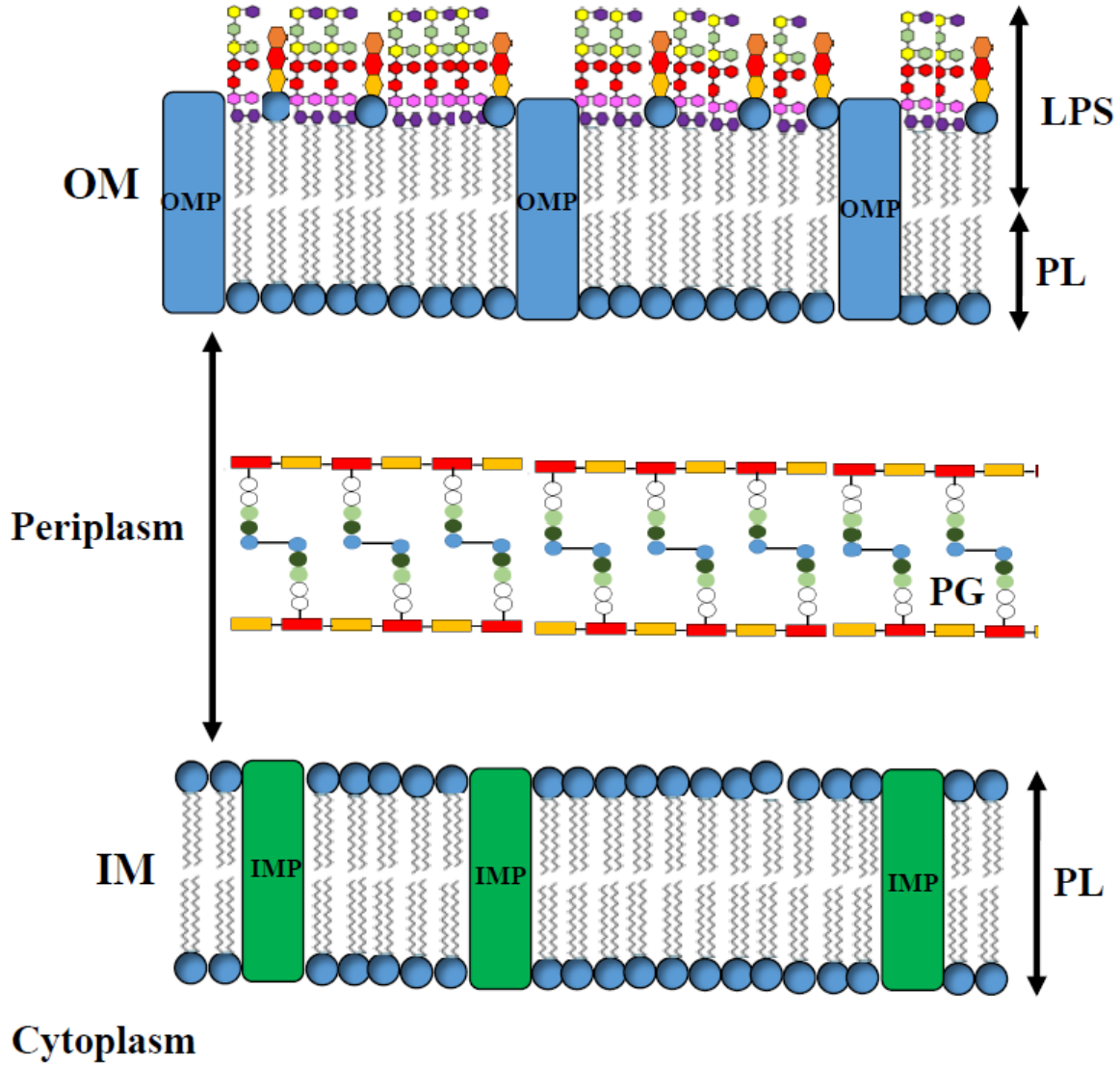


Fig. 1.1 The Gram-negative bacterial cell envelope. The IM is comprised of phospholipids with embedded membrane proteins (IMPs). The periplasm contains a peptidoglycan (PG) layer, which maintains cell shape and mechanical strength. The OM consists of an inner leaflet of phospholipids and an external leaflet of LPS. The external leaflet of the OM contains an additional carbohydrate-derived molecule referred to as enterobacterial carbohydrate antigen (ECA).

contains β-barrel folded proteins commonly referred to as outer membrane proteins (OMPs) (Knowles *et al.*, 2009).

1.2.2.1. Inner membrane. The IM is a symmetrical phospholipid bilayer comprised of three principal phospholipids: phosphatidylethanolamine, phosphatidylglycerol and cardiolipin (Raetz and Dowhan, 1990). The IM also contains proteins that can be divided into three main groups: integral proteins, lipoproteins and peripherally-associated proteins. Lipoproteins refer to lipid-anchored proteins that are embedded into the IM via three acyl chains. The peripherally-associated proteins directly interact with the membrane surface or other IM proteins (Papanastasiou *et al.*, 2013). The majority of the integral proteins are α-helical bundles with α-helical membrane-spanning regions. They elicit a diverse set of functions in the cell ranging from cell signalling to metabolic exchange.

Newly synthesised cytoplasmic preproteins that carry out functions in the periplasm or the OM require translocation across the IM. Translocation can occur via three different methods: twin-arginine translocation (Tat), general secretory (Sec) translocation or a specialised delivery method, which is utilised by specific proteins to the outermost surface of the cell (Manting and Driessen, 2002; Berks *et al*., 2000; Thanassi and Hultgren, 2000).

The Tat system transports folded proteins across the cytoplasmic membrane, independent of the Sec pathway. The precursors are targeted via a signal peptide, which contains a characteristic sequence motif consisting of two consecutive arginine residues (Berks *et al*., 2000). The Tat system consists of TatA, TatB and TatC. TatB and TatC form a complex that binds to substrate proteins, recruiting TatA, which facilitates the transport of the substrate (Palmer and Berks, 2012). The Tat system is utilised by certain preproteins for a number of reasons, examples include: the requirement of cytoplasmic factors for correct protein folding; the maintenance of the protein in an unfolded state is difficult; and the protein requires folding prior to transport due to the insertion of redox cofactors (Palmer and Berks, 2012).

The majority of proteins are exported out of the cytoplasm by the Sec pathway. The Sec machinery can also integrate some membrane proteins into the IM (Mori and Ito, 2001). The Sec machinery consists of SecA, an ATP-hydrolysing protein that interacts with a membrane-embedded translocation complex comprised of SecY, SecE and SecG (fig. 1.2). The SecYEG complex creates the channel for protein movement, while an accessory complex formed from SecD, SecF, YajC and YidC stabilizes this complex, which facilitates the transport of the SecA bound preprotein across the IM (Mori and Ito, 2001; Manting and Driessen, 2002). Another component of the SEC machinery is SecB, which is a cytoplasmic chaperone. Chaperones maintain precursor proteins in their unfolded state and/or assist in the conformational folding of proteins. Certain proteins require SecB to cross the IM, including: DegP; OmpT; OmpX; OppA; FhuA; FkpA; TolB; TolC; YcgK; YgiW; YbgF; and YncE (Baars *et al*., 2006).

1.2.2.2. Periplasm. The periplasm is a multipurpose compartment that is functionally distinct from the cytoplasm. The oxidizing environment of the periplasm allows for more efficient and diverse mechanisms of protein oxidation, folding and quality control including disulphide bond formation (Missiakas and Raina, 1997; Miller and Salama, 2018). Functions like protein transport, signalling and cell division regulation occur in the periplasm. The periplasm is densely packed with proteins including: outer membrane proteins; periplasmic binding proteins; osmoregulatory periplasmic glucans; modules involved in environmental sensing; and chaperone-like molecules. Complex machineries also span the periplasm, for example the Lpt system, which transports LPS to the OM. In addition, the periplasm contains the peptidoglycan cell wall, which is comprised of repeating units of disaccharide *N*-acetylglucosamine-*N*-actylmuramic acid cross-linked by pentapeptide side chains (Vollmer *et al.,* 2008). The rigid PG sacculus determines cell shape and protects the cell from turgor pressure. A lipoprotein called Lpp connects the PG to the OM and dictates the distance between the IM and the OM (Braun, 1975).

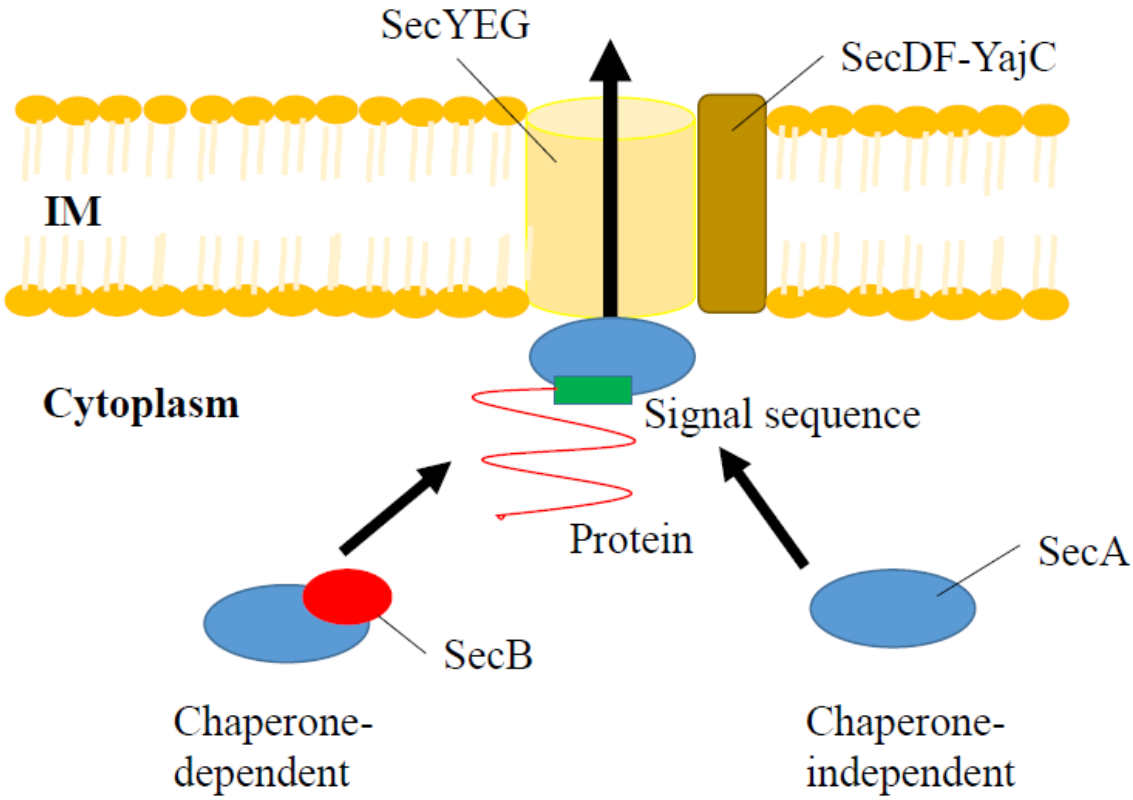


Fig. 1.2 The Sec machinery. The Sec machinery consists of a membrane embedded SecYEG translocase, a SecDSecFYajCYidC complex that stabilizes the SecYEG complex, a peripheral SecA subunit and a SecB cytoplasmic chaperone. Periplasmic and OM proteins that utilize the Sec machinery are either SecB chaperone dependent or SecB chaperone independent.

1.2.2.3. Outer membrane. The OM consist of an inner leaflet of phospholipids and an outer leaflet mainly comprised of LPS (Nikaido, 2003). In addition, around 50% of the mass of the OM is protein, either in the form of lipoproteins or integral membrane proteins (Koebnik *et al.,* 2002). Lipoproteins contain lipid moieties that are embedded into the inner leaflet of the OM (Sankaran and Wu, 1994; Silhavy *et al.,* 2010). The majority of the lipoproteins (~100) identified in *E. coli* possess unknown functions (Miyadai *et al.,* 2004). Lipoproteins are synthesised in the cytoplasm and transported across the IM via the SEC machinery (Okuda and Tokuda, 2011). The Lol system transports the bulk of lipoproteins to the OM, where they exhibit key roles in cell wall synthesis, antibiotic efflux pumps and diverse secretion systems. In addition, several essential OM machineries require one or more lipoproteins, for example the LPS machinery.

Integral transmembrane proteins, also referred to as outer membrane proteins (OMPs) reside in the OM and adopt a β-barrel conformation consisting of β-sheets wrapped into cylinders. OMPs are synthesised in the periplasm and transported across the IM via the Sec machinery. In the periplasm, three known chaperones SurA, Skp and DegP transport the OMPs to the β-barrel assembly machinery (BAM) complex, which folds and inserts these proteins into the OM. The OM also contains a small number of enzymes, including a protease OmpT, a phospholipase PldA and an LPS modification enzyme PagP (Hwang *et al.,* 2002; Snijder *et al.,* 1999; Vandeputte-Rutten *et al.,* 2001). The active sites of these enzyme either faces externally to the cell (OmpT) or are located in the outer leaflet.

The external leaflet contains an essential glycolipid component called LPS. LPS is a glucosamine disaccharide with six or seven acyl chains, a polysaccharide core and an O-antigen polysaccharide chain (Raetz and Whitfield, 2002). LPS provides structural integrity, stabilization and protection from the external environment. In addition, the LPS phosphates confer a negative charge, which creates a specific Donnan potential across the OM into the

periplasm (Stock *et al.*, 1977). In some Gram-negative bacteria, the external leaflet of the OM also contain an additional carbohydrate-derived molecule called enterobacterial common antigen (ECA). ECA exists in two forms in the OM, covalently linked to LPS (ECA_{LPS}) or covalently linked to the phospholipid phosphatidylglycerol (ECA_{PG}).

1.3. Trans-envelope complexes and transport across the periplasm

Numerous molecules require transport across the periplasm including LPS, phospholipids, lipoproteins and OMPs. Some pathways utilise chaperones, while others rely on trans-envelope complexes, which span from the IM to the OM. For example, the Lol system transports lipoproteins from the IM to the OM, in a chaperone-dependent manner.

1.3.1. Transport of lipoproteins

The Lol system consists of five proteins: LolA; LolB; LolC; LolD; and LolE (Okuda and Tokuda, 2011). Lipoproteins are synthesised in the cytoplasm and transported across the IM via the Sec machinery. In the periplasm, lipoproteins are processed into a mature form and a lipid moiety is attached to the N-terminus to anchor it to the membrane surface. Lipoproteins can remain in the IM, depending on the residue at position two. The ABC transporter LolCDE detaches the appropriate lipoproteins from the IM and facilitates binding of the acyl group by LolA (Polissi and Sperandeo, 2014). LolA then transports lipoproteins across the periplasm to LolB, which inserts them into the OM (fig. 1.3). LolA and LolB are key in the prevention of lethal accumulation of mislocalized lipoproteins (Grabowicx and Silhavy, 2017).

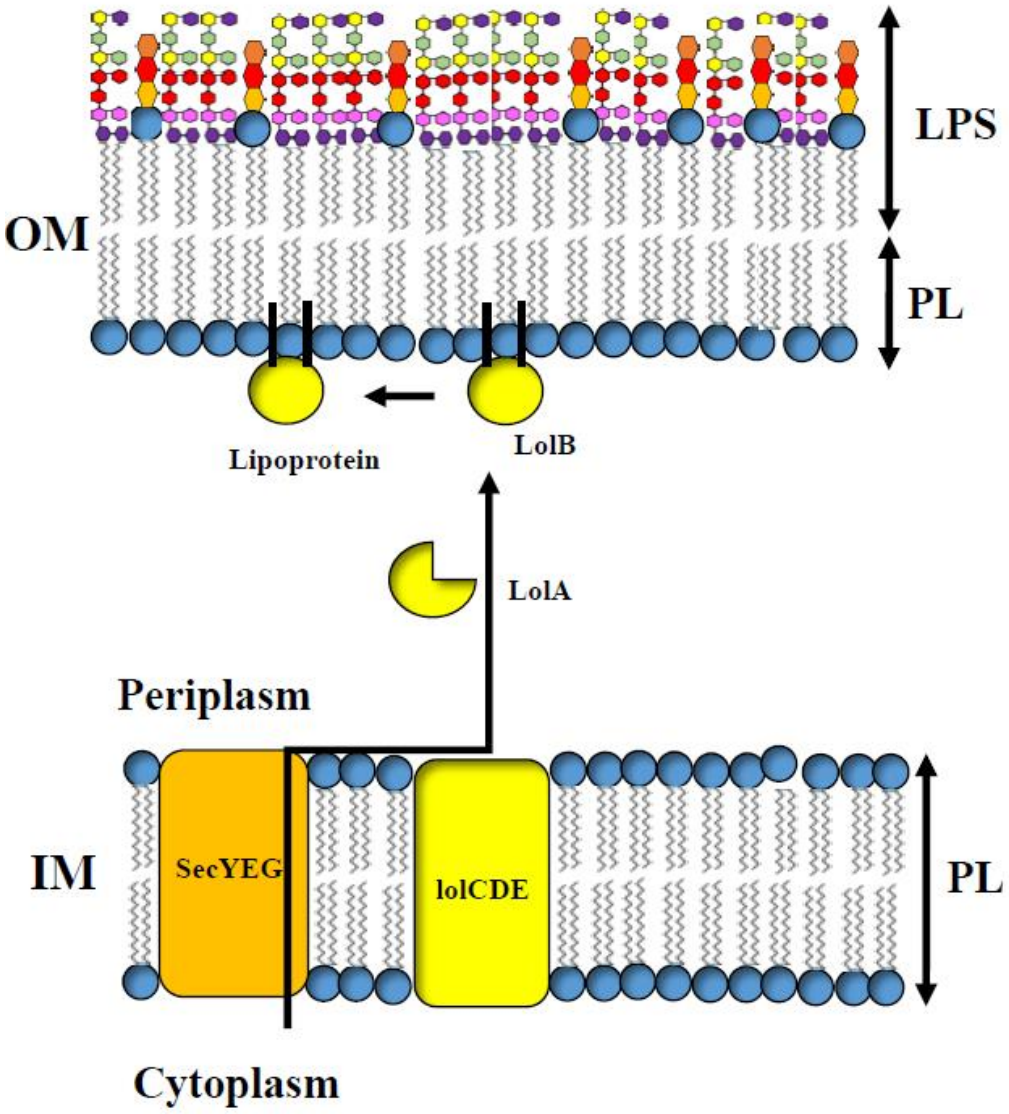


Fig. 1.3 The transport of lipoproteins to the OM. The SEC machinery (orange) transports lipoproteins across the IM. In the periplasm, the lipoproteins anchor themselves to the IM. LolCDE detaches the appropriate lipoproteins from the IM and transports them to the chaperone LolA. LolA then transports lipoproteins across the periplasm to LolB, which inserts them into the OM.

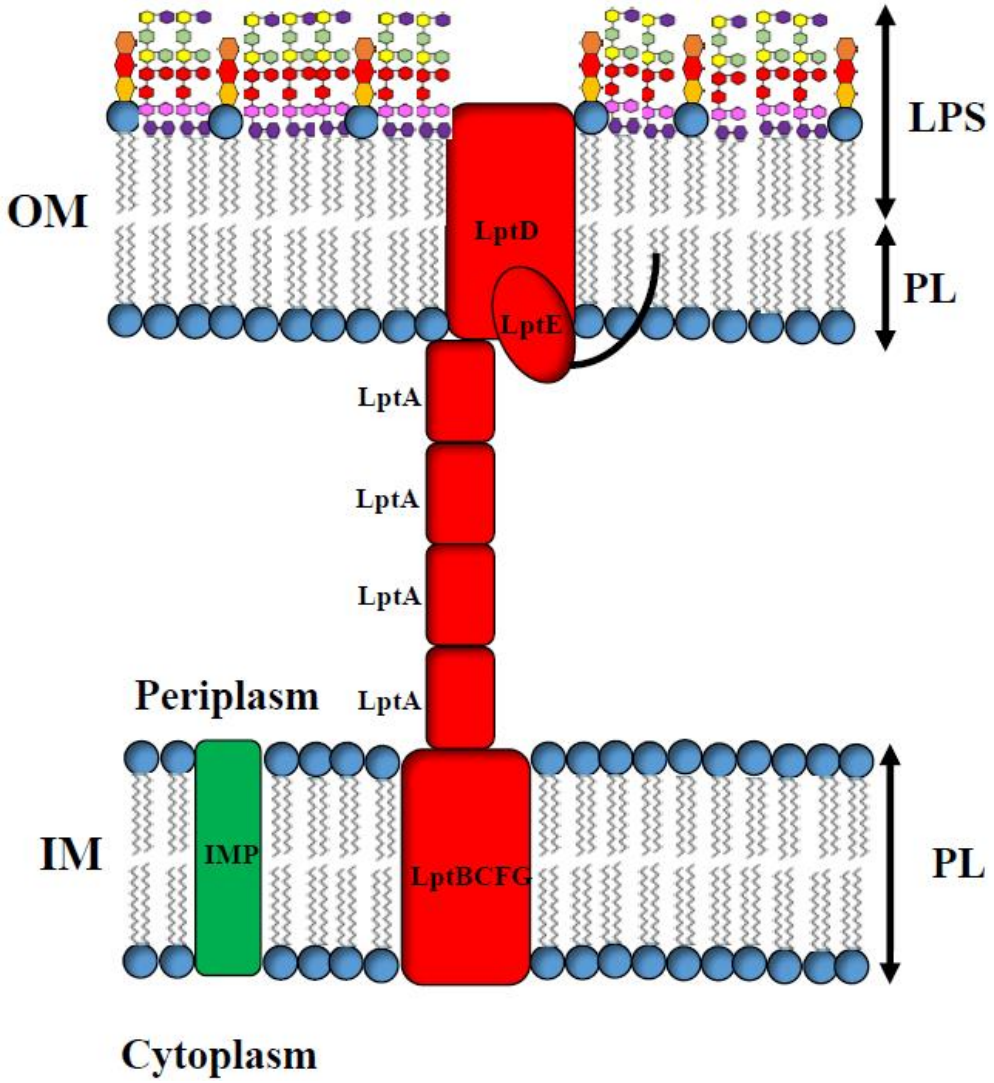


Fig. 1.4 One model of lipopolysaccharide transport across the periplasm to the OM. This model suggests that LPS transportation occurs through a trans-envelope proteinaceous bridge, which spans from the IM to the OM (shown in red). The LptBCFG complex resides in the IM, while a number of LptA monomers form a bridge to the LptDE complex in the OM.

1.3.2. Phospholipid transport

The mechanism of phospholipid (PL) trafficking from the IM to the OM remains largely unresolved. However, it is known that the Mla pathway components help maintain lipid asymmetry in the OM. This transport system prevents PL accumulation at the cell surface, by removing and transporting PLs found in the outer leaflet of the OM to the IM (retrograde transport). This pathway consist of six proteins MlaA-F, which collectively occur in the IM, periplasm and OM (Malinverni and Silhavy, 2009). The OM lipoprotein MlaA is proposed to extract PLs from the OM, while the chaperone MlaC transport the PLs across the periplasm to an ATPase complex that resides in the IM. This complex is formed from MlaB, MlaD, MlaE and MlaF. Hughes *et al*. (2019) suggested that the Mla pathway might elicit an additional role in the transport of lipids away from the IM, where the MlaFEDB machinery facilitates binding of phospholipids by MlaC for subsequent delivery to the OM (anterograde transport).

1.3.3. Lipopolysaccharide transport across the periplasm to the OM

Following synthesis of complete LPS, the LPS molecules interact with the LPS transport (Lpt) machinery. The precise mechanism of transport and assembly of LPS to the external leaflet of the OM remains unresolved. However, an ABC protein complex is proposed to extract LPS from the IM. LptB, LptC, LptF and LptG form this complex, with a respective stoichiometry of 2:1:1:1 (Narita and Tokuda, 2009). Upon release of LPS, LptA transports the LPS molecules across the periplasm to the OM, where a complex formed by LptE and LptD are implicated in the assembly of LPS into the OM.

Two models of LPS transport to the OM exist (Sperandeo *et al*., 2006; Suits *et al*., 2008). The first model suggests that transport of LPS occurs through a trans-envelope complex that physically connects the IM to the OM (fig. 1.4). The linear filament is formed from four LptA monomers that interact in a head to tail fashion. This filament transports the LPS molecules across the periplasm. Suits *et al*. (2008) provided evidence to support this model,

where formation of an LptA filament occurred during crystallization of LptA in the presence of LPS. The second model suggests that LPS transport is chaperone mediated (Polissi and Sperandeo, 2014). This model is based on the similarities between the LPS export pathway and the Lol-mediated lipoprotein transport pathway. LptBFG would be the functional equivalent of LolCDE, while LptA would be the functional equivalent of LolA. However, no component of the Lol pathway is analogous to LptC.

1.4. Biogenesis of peptidoglycan

The peptidoglycan sacculus is a complex heteropolymer that contains long glycan chains comprised of alternating *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues, linked by β1→4 bonds (Barreteau *et al*., 2008). The glycan chains are cross-linked by short peptides, which are attached to the MurNAc residues (Glauner and Höltje 1990). The pentapeptide is typically comprised of L-Ala-D-Glu-*meso*-DAP-D-Ala-D-Ala (*meso*-Dap, *meso*-diaminopimelic acid; Liu and Breukink, 2016; Egan *et al.*, 2015).

Peptidoglycan subunits are synthesised in the cytoplasm and flipped across the IM, where they are inserted into the existing wall. The biosynthesis of peptidoglycan requires synthases and hydrolases. Synthases make peptidoglycan and attach it to the existing sacculus, while hydrolases cleave the sacculus, allowing the incorporation of the newly synthesised material into the existing wall (Höltje, 1998).

Peptidoglycan synthesis can be divided into three main stages (fig. 1.5). The first stage occurs in the cytoplasm, where UDP-GlcNAc (uridine diphosphate) is formed from fructose-6-phosphate. Mur enzymes MurA and MurB form UDP-MurNAc from UDP-GlcNAc and Mur enzymes MurC-MurF synthesise the UDP-MurNAc-pentapeptide molecules (Barreteau *et al*., 2008). The second stage occurs at the inner leaflet of the IM, where the MurNAc-pentapeptide molecule is assembled with an undecaprenyl-phosphate (Und-P) molecule to from lipid I.

MurG adds a GlcNac residue to form the lipid-anchored disaccharide-pentapeptide monomer subunit (hereafter referred to as lipid II). Lastly, FtsW-RodA flips lipid II across the IM into the periplasm (Bouhss *et al*., 2008; Mohammadi *et al*., 2011; Typas *et al*., 2011). In the third stage of peptidoglycan synthesis, transglycosylases polymerize lipid II, releasing undecaprenyl pyrophosphate and leading to the formation of glycan chains (Typas *et al*., 2011). The newly synthesised glycan chains are incorporated into the existing sacculus and cross-linked to form a mesh-like structure.

1.5. Phospholipid biogenesis

The IM and the internal leaflet of the OM is comprised of phospholipids (PLs). The phospholipidome consists of up of 70% phosphatidylethanolamine (PE), 20% phosphatidylglycerol (PG) and 5-10% cardiolipin (CL) (Jeucken *et al*., 2018). PLs are comprised of a variable head group; a phosphate group; a glycerol backbone; and two fatty acid chains. The various PL classes exhibit different functions, for example, CL is involved in the organisation of membranes and in cell division (Mileykovskaya and Dowhan, 2009).

PlsB catalyses the first committed step in phospholipid synthesis, by adding an acyl chain to glycerol-3-phosphate to form 1-acyl-*sn*-glycerol-3-phosphate (Larson *et al*., 1980). PlsC catalyses the addition of the second acyl chain to form 1,2-diacyl-*sn*-glycerol-3-phosphate. The product 1,2-diacyl-*sn*-glycerol-3-phosphate can also be formed by an alternative pathway via phosphorylation of diacylglycerol. CdsA converts 1,2-diacyl-*sn*-glycerol-3-phosphate to its active form CDP-Diacylglycerol, which is an intermediate in the biosynthesis of all membrane phospholipids: PE; PG; and CL.

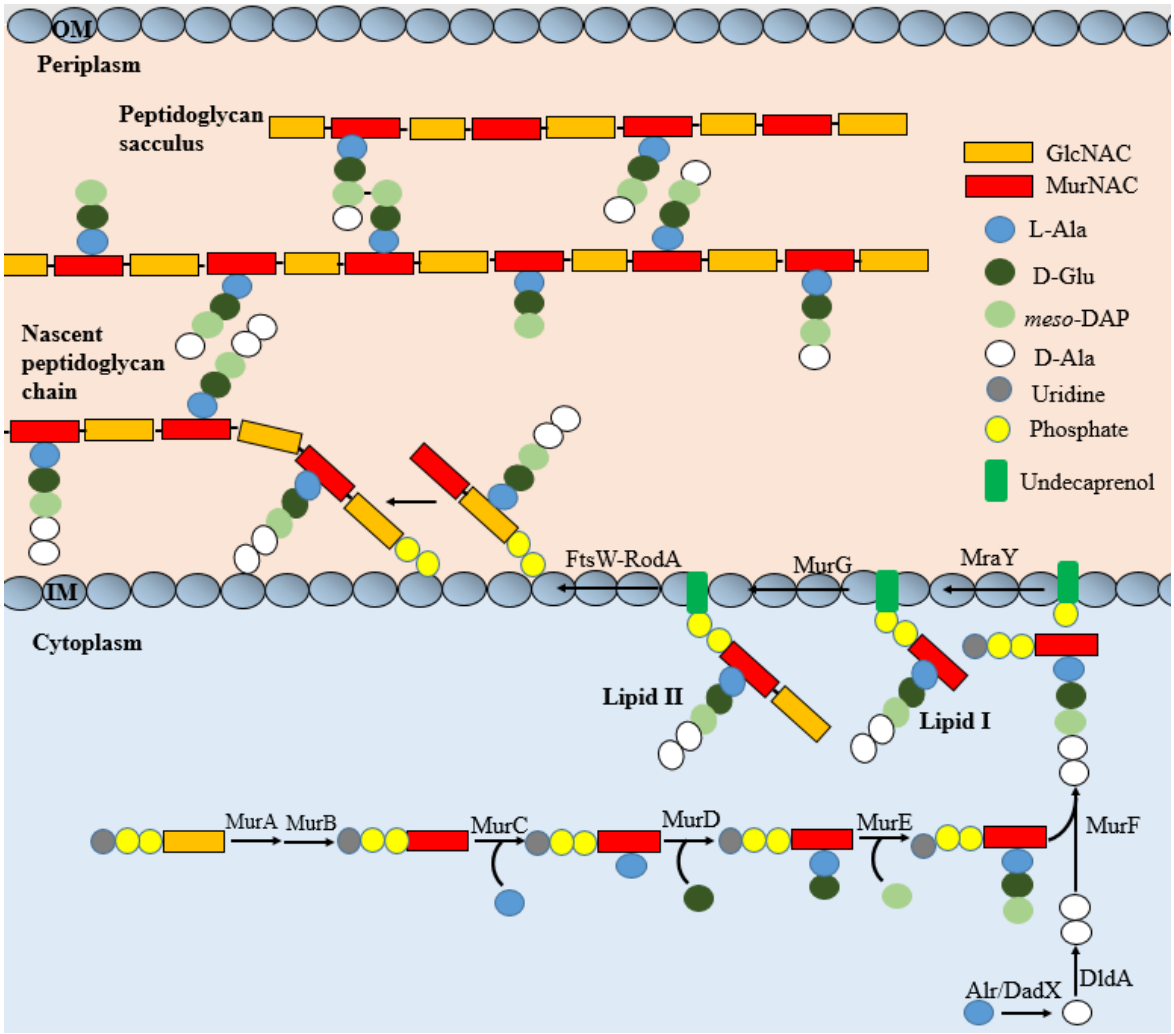


Fig. 1.5 The synthesis of the peptidoglycan sacculus. In the cytoplasm, the genes MurA-F synthesis the peptidoglycan precursors, which are then attached to Und-P to form lipid I. MurG adds a GlcNac residue to lipid I to form lipid II, which is then flipped across the IM by FtsW–RodA. In the periplasm, polymerization of lipid II occurs, leading to the formation of glycan chains, which are incorporated into the existing sacculus. Abbreviations: GlcNAc, *N*-acetylglucosamine; *meso*-Dap, *meso*-diaminopimelic acid; L-Ala, L-Alanine; D-Glu, D-glutamic acid; MraY, UDP-MurNAc-pentapeptide phosphotransferase; MurA, UDP-GlcNAc enolpyruvyl transferase; MurB, UDP-MurNAc dehydrogenase; MurC, UDP-MurNAc–L-Ala ligase; MurD, UDP-MurNAc-L-Ala–D-Glu ligase; MurE, UDP-MurNAc-L-Ala-D-Glu–*meso*-Dap ligase; MurF, UDP-MurNAc-tripeptide–D-alanyl-D-Ala ligase; and MurG, UDP-GlcNAc-undecaprenoyl-pyrophosphoryl-MurNAc-pentapeptide transferase.

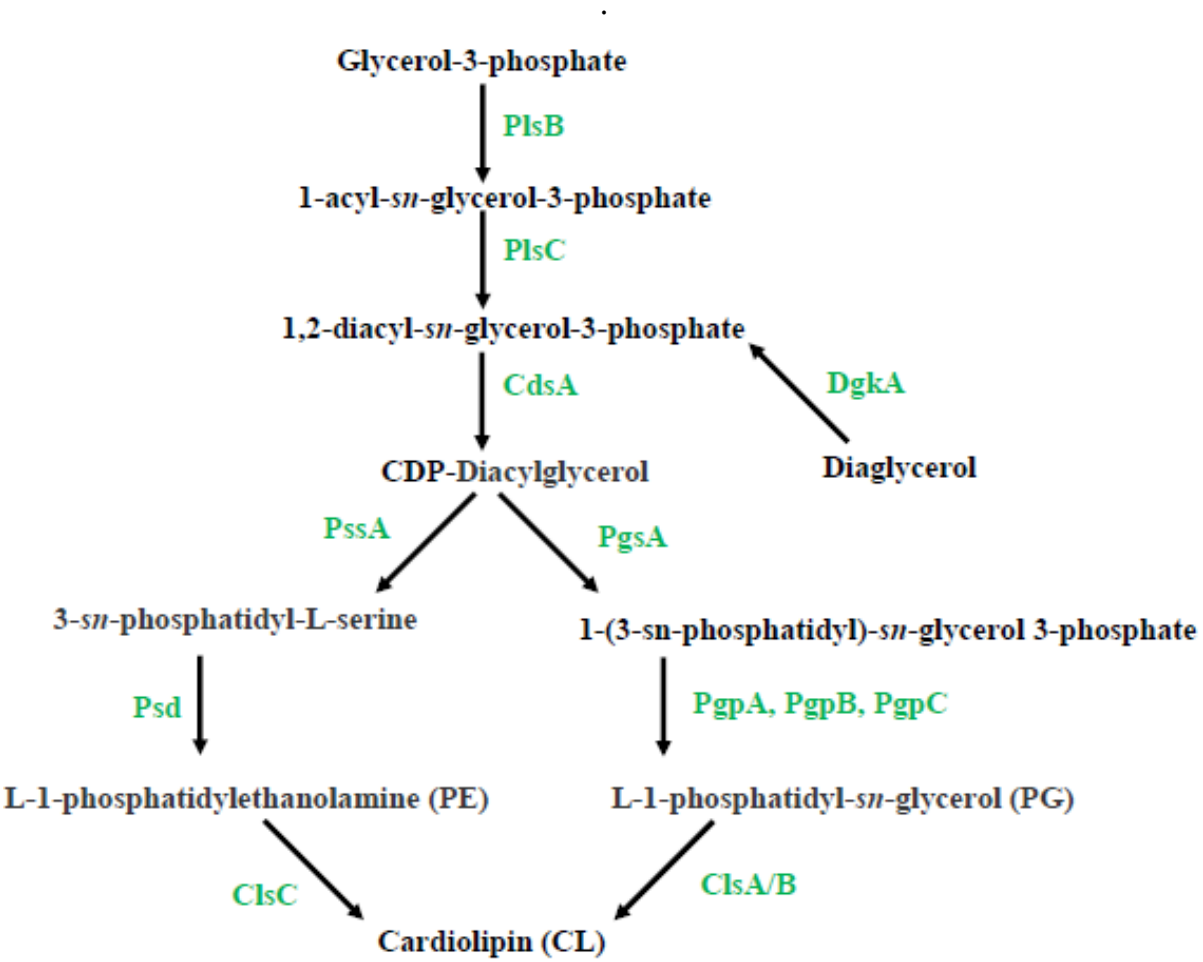


Fig. 1.6 Phospholipid biosynthetic pathways. The synthesis of PG, PE and CL. CDP-Diacylglycerol is an intermediate in the biosynthesis of the three known membrane phospholipids. Enzymes are shown in green next to the process they mediate (Abbreviations: CDP-Diacylglycerol: cytidine diphosphatediacylglycerol).

PE is synthesised in a two-step mechanism, where PssA converts CDP-Diacylglycerol to 3-*sn*-phosphatidyl-L-serine and Psd catalyses the formation of L-1-phosphatidylethanolamine (PE) from 3-*sn*-phosphatidyl-L-serine. The other branch in the PL biosynthesis pathway forms PG (fig. 1.6), where PgsA converts CDP-diacylglycerol to 1-(3-*sn*-phosphatidyl)-*sn*-glycerol 3-phosphate (PGP) and one of the three phosphatidylglycerolphosphatases PgpA, PgpB or PgpC then dephosphorylates PGP to form L-1-phosphatidyl-*sn*-glycerol (PG) (Lu *et al*., 2011). PG and PE act as substrates in the formation of CL. ClsA and ClsB are synthases that condense two PG molecules to form CL. Alternatively, ClsC condenses one molecule of PG and one molecule of PE to form CL (Tan *et al*., 2012).

1.6. Lipopolysaccharide biogenesis

LPS consists of three main subunits: lipid A, core oligosaccharide and O-antigen (fig. 1.7). Lipid A is an endotoxin that provokes a strong immune response in humans and exhibits a significant role in bacterial pathogenicity and immune evasion (Walker *et al*., 2004). Lipid A anchors the structure into the membrane and consists of six hydrophobic acyl chains connected by a glucosamine and a phosphate head group. In *E. coli*, the phosphate head group is connected to a pair of Kdo sugar residues, which forms part of the core oligosaccharide. Attached to the outer core oligosaccharide is the O-antigen, which is often absent from laboratory strains, including *E. coli* K-12 strains (Osawa *et al*., 2013).

LPS synthesis occurs at the inner leaflet of the IM, where lipid A is synthesised through nine enzyme-catalysed steps. LpxA acylates UDP-N-acetylglucosamine (UDP-GlcNAc) with β-hydroxymyristoyl-ACP. LpxC then deacetylates UDP-3-*O*-(acyl)-GlcNAc, catalysing the first committed step of this pathway (Emiola *et al*., 2015). LpxD then incorporates a second hydroxymyristate moiety into the lipid A precursor and the peripheral membrane proteins LpxH and LpxB catalyse the fourth and fifth steps to produce a lipid A disaccharide.

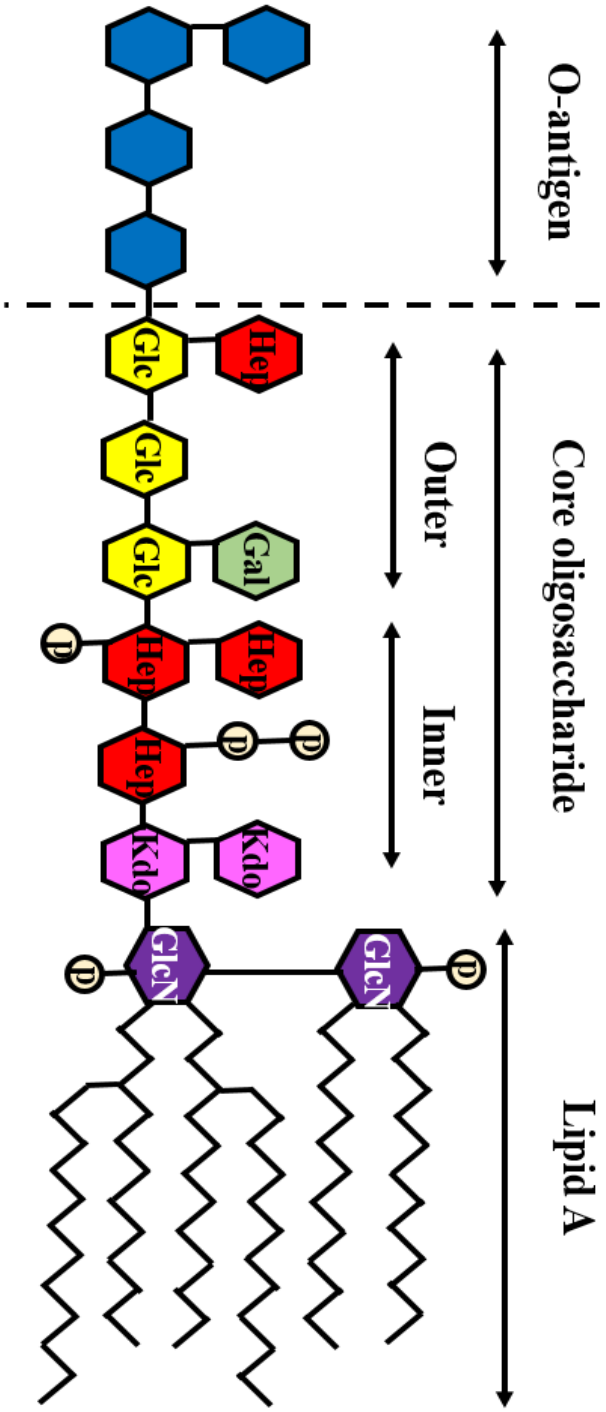


Fig. 1.7 The structure of lipopolysaccharide. LPS consists of lipid A, a core oligosaccharide and O-antigen (blue). However, O-antigen is absent from LPS in *E. coli* K-12 strains (dashed line). The core-oligosaccharide can be further divided into an inner and outer core. Abbreviations: GlcN, glucosamine; Kdo, 2-keto-3-deoxyoctulosonic acid; p, phosphate; Hep, heptose; Glc, glucose, Gal, galactose.

(Babinski *et al*., 2002; Radika *et al*., 1988). The remaining lipid A biosynthesis steps are catalysed by integral membrane enzymes, where LpxK phosphorylates lipid A to produce lipid IV_A and WaaA transfers two Kdo sugar residues to the product to produce Kdo_2-lipid IV_A (Emiola *et al*., 2015). The late acyltransferases LpxL and LpxM sequentially acylate the complex to form Kdo_2-lipid A (Brozek *et al*., 1990 Clementz *et al*., 1996). In *E. coli*, the Kdo_2-lipid A complex is the minimum form of LPS required for viability of the cell. LpxM is the only non-essential enzyme utilised in the synthesis of the Kdo_2-lipid A complex.

Subsequently, three heptoses are added into the inner core of LPS by WaaC, WaaF and WaaQ, while WaaP and WaaY add a phosphate to heptose I and heptose II, respectively. The inner core is highly conserved within species. However, the outer core exhibits minor variations. The synthesis of the outer core consists of the sequential addition of glucose I, galactose, glucose II, glucose III and heptose IV by WaaG, WaaB, WaaO, WaaJ and WaaU, respectively (Parker *et al.*, 1992; Shibayama *et al.*, 1999; Qian *et al.*, 2014).

The ABC transporter MsbA transfers the LPS complex from the inner leaflet to the outer leaflet of the IM (Zhou *et al*., 1988). The O-antigen is independently synthesised from the other LPS components. WaaL ligates the O-antigen onto the core oligosaccharide at the periplasmic side of the IM (McGrath and Osborn, 1991). However, O-antigen is not synthesised in *E. coli* K-12 strains due to a mutation in the *rfb* operon (Stevenson *et al*., 1994). LPS that lacks O-antigen is often referred to as lipooligosaccharide (LOS).

1.7. Forms and synthesis of enterobacterial common antigen

Enterobacterial common antigen (ECA) is a carbohydrate-derived molecule found on the external leaflet of the OM and in the periplasm. ECA occurs in almost all of the members of the *Enterobacteriaceae* family (Kuhn *et al.*, 1988; Kunin, 1963). However, this surface antigen is absent in all other Gram-negative and Gram-positive bacteria. ECA exists in three forms:

covalently linked to lipopolysaccharide (ECA_{LPS}); covalently linked to the phospholipid phosphatidylglycerol (ECA_{PG}); and cyclic ECA (ECA_{CYC}). ECA_{LPS} and ECA_{PG} both occur in the OM, while ECA_{CYC} is periplasmic. All three forms share the same biosynthetic pathway (Mitchell *et al*., 2018; Westfall, 2018). ECA is composed of three main subunits: GlcNAc (*N*-acetylglucosamine); ManNAcA (*N*-acetyl-D-mannosaminuronic acid); and Fuc4NAc (4-acetamido-4,6-dideoxy-D-galactose) (Lugowski *et al.,* 1983; Männel and Mayer, 1978).

WecA catalyses the first step in this pathway, with the transfer of GlcNAc-1-phosphate to undecaprenyl-phosphate (Und-P) (Al-Dabbagh *et al*., 2008). WecB and WecC synthesize ManNAcA, while WecG attaches ManNAcA to Und-P-P-GlcNAc. RffH, RffG, WecE and WecD together synthesis Fuc4NAc, while WecF attaches Fuc4NAc to Und-P-P-GlcNAc-ManNAcA (fig. 1.8). WzxE flips the ECA molecule across the IM into the periplasm (Rick *et al.,* 2003). In the periplasm, WzyE polymerizes the ECA, while WzzE controls the length of the ECA chains (Brade, 1999; Barr *et al*., 1999). ECA can reside in the periplasm as the cyclic form or it is transported through an unknown mechanism to the external leaflet of the OM to produce the LPS and PG form.

1.8. OMPs and their assembly

OMPs elicit a range of functions that are crucial to the cell. OmpC and OmpF are among the most abundant OMPs in the cell. These porins allow the diffusion of ions and other small molecules (Koebnik *et al.,* 2002). Some OMPs help maintain the structural integrity of the OM, for example OmpA, which acts as a physical linkage between the OM and the peptidoglycan layer (Dermot and Vanderleyden, 1994; Koebnik, 1995). Other OMPs are involved in the efflux of compounds, for example TolC, which pumps out toxic exogenous compounds (antibiotics, detergents, organic solvents) and some intracellular metabolites (enterobactin).

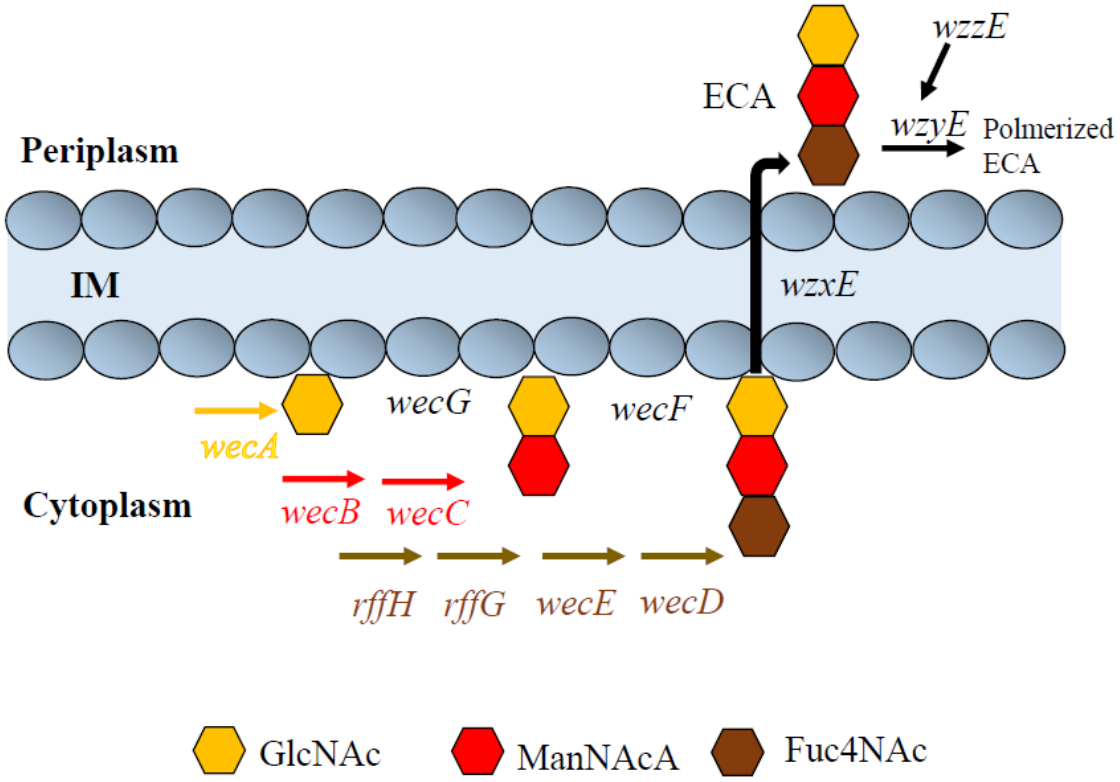


Fig. 1.8 The synthesis of enterobacterial common antigen. In the cytoplasm, the GlcNAc, ManNAcA and Fuc4NAc subunits are synthesised to form Und-P-P-GlcNAc-ManNAcA-Fuc4NAc. WzxE flips the ECA molecules across the IM into the periplasm. In the periplasm, WzyE polymerizes the ECA molecules. ECA can reside in the periplasm or it can be transported to the external leaflet of the OM.

Due to the high biological importance of OMPs, understanding the transport and insertion of OMPs is vital. Nascent OMPs are synthesized in the cytoplasm and are targeted via a signal sequence to the Sec machinery. The cytoplasmic chaperone SecB maintains the OMP in an unfolded conformation until the OMP is trafficked from the cytoplasm via the Sec translocon (Bechtluft *et al.,* 2010; Driessen and Nouwen, 2008). During translocation, the OMP signal sequence is cleaved, releasing the OMP into the periplasm.

OMPs are prone to aggregation and misfolding in aqueous environments like the periplasm. Thus, OMPs must be maintained in a folding-competent state during their translocation through the periplasm to the BAM complex (Ruiz *et al.,* 2006). The three quality control factors that chaperone OMPs across the periplasm are SurA, Skp and DegP. Single mutants Δ*surA*, Δ*skp* and Δ*degP* are viable. However, double mutants Δ*surA*Δ*skp* and Δ*surA*Δ*degP* are synthetically lethal, which suggests functional redundancy exists between SurA and Skp and between SurA and DegP. Consequently, Sklar *et al.* (2007) proposed that these three chaperones make up two parallel pathways of OMP transport, where SurA functions in one pathway and the combination of Skp and DegP functions in another pathway. However, despite this genetic evidence, there is no molecular evidence to suggest that roles of SurA, Skp and DegP are redundant.

Thus, the synthetic lethality between SurA and Skp is still unclear. Consequently, the precise contributions of SurA, Skp and DegP to OMP biogenesis requires identification. There are three potential outcomes to the simultaneous deletion of SurA and Skp. (1) SurA and Skp make up two parallel pathways, where SurA is main chaperone to the BAM complex and Skp is functionally redundant (fig. 1.9A). (2) SurA and Skp act in sequence, where one functions in early OMP biogenesis at the IM and the other acts in late OMP biogenesis at the OM. Loss of one component of the pathway is kinetically unfavourable but loss of both destabilizes OMP biogenesis. This leads to a lack of OMP insertion into the OM, which results in cell death (fig. 1.9B). (3) SurA and Skp act in separate pathways and/or have separate distinct functions. Loss

of SurA and Skp in combination elicits downstream negative effects that are lethal to the cell (fig. 1.9C).

1.8.1. SurA, a periplasmic peptidyl-prolyl isomerase

The gene *surA* was first identified as a gene required for the survival of *E. coli* during stationary phase (Tormo *et al.,* 1990). SurA was later described as a peptidyl-prolyl isomerase (PPIase) that facilitates the assembly of OMPs (Missiakas *et al.,* 1996; Rouviére and Gross, 1996). A PPIase catalyses the cis-trans isomerization of peptide bonds involving a proline residue. In *E.coli*, a mutant SurA protein lacking both PPIase domains still binds to β-barrel substrates and exhibits chaperone-like activity (Behrens *et al.,* 2001). In addition, one or two PPIase domains are missing from whole classes of bacteria, for example alphaproteobacteria (Gatsos *et al*., 2008). Thus, the PPIase domains are not fundamental for the function of SurA. The sequence of *surA* consists of a 20-residue leader sequence; an amino-terminal segment (N domain); two ~100-residue PPIase domains referred to as P1 and P2; and a carboxyl-terminal segment (C domain) (Bitto and McKay, 2002; Rahfeld *et al.,* 1994). The crystal structure of SurA reveals an asymmetric dumbbell-like protein, where the N, C and P1 domains form the core structural module, which is tethered to a second module comprised of the P2 domain (Bitto and McKay, 2002).

SurA can assemble all of the OMPs efficiently. However, some OMPs, for example LptD prefer the SurA pathway, while no OMPs prefer the Skp/DegP pathway (Vertommen *et al.,* 2009; Ruiz *et al.,* 2010). Loss of SurA reduces the OM levels of eight of the 23 identified β-barrel proteins, including OmpA; OmpX; OmpF; and LamB (Lazar and Kolter, 1996; Rouviere and Gross, 1996; Vertommen *et al*., 2009). A Δ*surA* mutant is defective in converting unfolded LamB into the folded LamB monomer and activates the σ^E and the Rcs stress response systems. In addition, an increase in permeability and sensitivity to a wide range of conditions occur (Castanie-Cornet *et al*., 2006; Ureta *et al*., 2007).

1.8.2. The importance of DegP and Skp in OMP biogenesis

Apart from SurA, the seventeen kilodalton protein (Skp) is a predominant periplasmic chaperone involved in the biogenesis of OMPs. Chen and Henning (1996) demonstrated that Skp only binds to denatured OMPs and does not bind to denatured periplasmic or cytosolic proteins. Skp might function in early OMP biogenesis as Skp interacts with unfolded OMPs as they emerge from the SEC machinery (Harms *et al.,* 2001; Schafer *et al.,* 1999). Loss of Skp activates the σ^E stress response, which suggests that the accumulation of unfolded periplasmic proteins occur. Loss of Skp also results in a reduction in OMP levels in the OM, for example LamB; OmpA; OmpC; and OmpF (Chen and Henning, 1996). In addition, Skp is involved in the assembly of the essential OMP LptD (Schwalm *et al.,* 2013). Skp interacts directly with membrane lipids and LPS. This interaction is required for efficient Skp-assisted folding of membrane proteins (Cock *et al.,* 1999; Walton and Sousa, 2004). The crystal structure of the Skp trimer revealed a jellyfish-like molecule that consists of α-helical tentacles protruding from a central cavity, which is occupied by the substrate (Walton and Sousa, 2004).

Skp is proposed to function alongside another periplasmic chaperone, DegP. DegP is a dual acting protein with both protease and general chaperone activity and the regulation of these activities occur in a temperature-dependent manner (Spiess *et al.,* 1999). A Δ*degP* mutant exhibits temperature sensitive growth and is unable to grow at 42°C (Sklar et al., 2007). In addition, loss of DegP decreases the level of OMPs in the OM, including OmpA, OmpF and OmpC (Krojer *et al.,* 2008). Conversely, significantly less OMPs are inserted into OM in a Δ*surA* mutant than in a Δ*skp*Δ*degP* double mutant (Sklar *et al*., 2007). This suggests that Skp and DegP elicit only a minor role in the assembly of OMPs. However, the role of Skp and DegP might be amplified in stress conditions or they might rescue OMPs that fall off the normal assembly pathway as loss of Skp/DegP leads to the accumulation of misfolded OMPs in the periplasm, such that the σ^E stress response system is activated (Sklar *et al*., 2007).

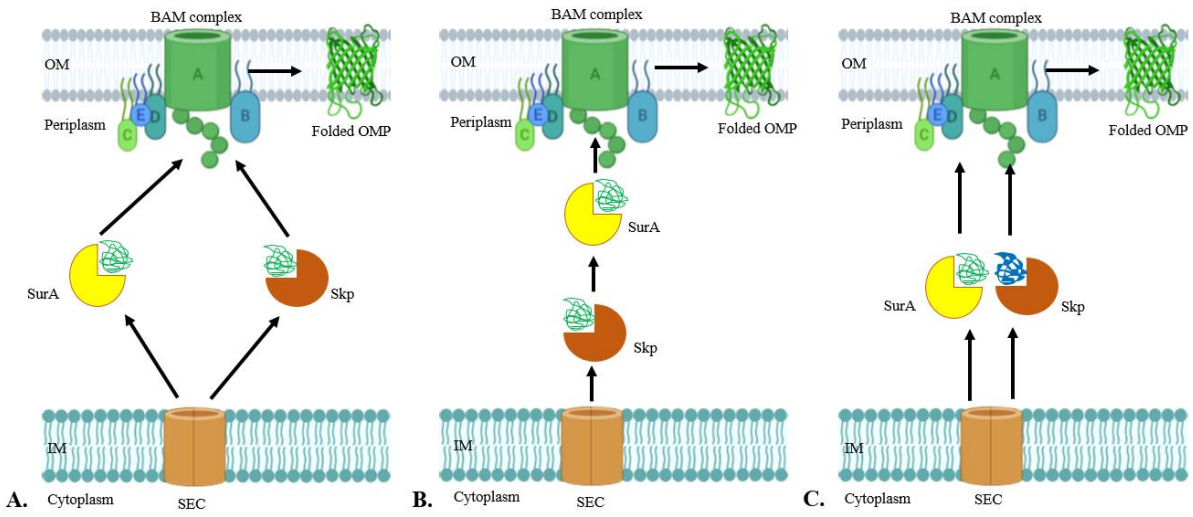


Fig. 1.9 Possible outputs from combined deletion of *surA* and *skp*. (A) Two parallel pathways comprised of SurA and Skp, where SurA is the main chaperone and Skp is a redundant chaperone. (B) SurA and Skp act in sequence, where loss of one component of the pathway is kinetically unfavourable but loss of both SurA and Skp completely destabilizes OMP biogenesis. (C) SurA and Skp are in separate pathways and/or have separate distinct functions, where loss of both SurA and Skp elicits lethal downstream effects.

In summary, understanding the functional redundancy between SurA and Skp is key in understanding OMP biogenesis. Once the OMP is delivered to the OM, the BAM complex folds and inserts the OMP into the OM (Knowles *et al*., 2009). In *E. coli*, the BAM complex consists of five components, the essential core component BamA, which interacts with 4 accessory lipoproteins: BamB; BamC; BamD; and BamE.

1.9. The structure and function of the subunits of the BAM complex

The BAM complex consists of several components that vary by species. In *E. coli*, the BAM complex is a 200-kDa machinery that consists of two essential components, BamA and BamD and three non-essential components: BamB, BamC and BamE (Hagan *et al.,* 2011; Han *et al.,* 2016; Malinverni *et al.,* 2006; Ricci *et al.,* 2012). BamA is comprised of a C-terminal β-barrel that is fully immersed in the OM and five POTRA (polypeptide translocation associated) domains that interact with BamD to form a ring in the periplasm. The POTRA domains also interact with BamB, BamC and BamE, which surround the BamAD core. BamD consists of five tetratricopeptide repeat (TPR) domains, which are important in mediating protein-protein interactions.

Depletion of BamA or BamD produce identical OMP assembly defects, which demonstrates that these proteins play a key role in OMP assembly (Malinverni *et al*., 2006). In *Neisseria gonorrhoeae,* a homolog of BamD is ComL, which associates with peptidoglycan. This suggests that BamD might alter peptidoglycan structure and accommodate the transport of OMPs across peptidoglycan (Fussenegger *et al*., 1996). In addition, BamD might also be involved in the activation of BamA.

1.9.1. The role of BamB in OMP biogenesis

Of the three non-essential BAM components, loss of BamB produces the most severe defect, suggesting it elicits a key role in the folding and insertion of OMPs (Charlson *et al*., 2006).

Loss of BamB results in decreased levels of OMPs in the OM, including OmpF, OmpA and LamB; increased sensitivity to a wide range of conditions including rifampin, SDS and novobiocin; increased levels of TolC; and increased σ^E activity (Ruiz *et al*., 2005; Wu *et al*., 2005). BamB interacts with BamA independently of BamC, BamD and BamE, while BamC/D/E form a sub-complex that interacts with BamA. Thus, the role of BamB in the complex might be separate from BamC and BamE, while the function of BamC and BamE might be connected. BamB adopts an eight-bladed β-propeller fold that contains WD40-like motifs, which suggests that BamB might act as a scaffold to the BAM complex (Noinaj *et al*., 2011). Ureta *et al*. (2007) demonstrated that the same defect in folding LamB monomers occur in Δ*bamB* and Δ*surA* mutants. Thus, BamB might function in the early steps of OMP assembly. Gunasinghe *et al*. (2018) demonstrated that protein assembly precincts that contain multiple BAM complexes form within the membrane. In the absence of BamB, these regions disperse, suggesting that BamB might mediate the interactions between the BAM complexes.

Severe phenotypes occur in Δ*bamB*Δ*bamE*, Δ*bamB*Δ*bamC* and Δ*bamC*Δ*bamE* double mutants. The Δ*bamE*Δ*bamB* mutant is not viable at 37°C, while the Δ*bamB*Δ*bamC* mutant has reduced growth at 30°C. In addition, defects in OMP assembly occur in the Δ*bamE*Δ*bamC* mutant (Sklar *et al*., 2007). This suggests that BamC and BamE also elicit key roles in OMP biogenesis.

1.9.2. The role of BamC in OMP biogenesis

BamC contains an unstructured N-terminal domain followed by a surface exposed C-terminus comprised of two helix-grip domains (Gu *et al.*, 2016; Webb *et al*., 2012). The BAM complex can dissociate into smaller stable modules: BamC:D:E and BamA:B (Hagan *et al.*, 2010; Webb *et al*., 2012). In the absence of BamC these modules are destabilized and BamA and BamD are more susceptible to proteases. Of all of the BAM mutants, loss of BamC confers the weakest phenotypic effect on the cell. In addition, there is only one known synthetic lethal partner of

bamC. Loss of BamC results in only very slight defects in OM permeability and no clear decrease in OMP levels in the OM occur (Wu *et al.,* 2005). Although *bamC* is widely conserved amongst Gram-negative bacteria, the importance of BamC in OMP biogenesis remains unclear.

1.9.3. BamE, the smallest of the BAM subunits

Loss of BamE results in a reduction in OMP levels in the OM such as LamB and OmpA; increased sensitivity to a range of conditions including rifampin, cholate, SDS and SDS EDTA; and increased levels of DegP (Sklar *et al*., 2007). In addition, loss of BamE increases sensitivity of BamA to externally added proteinase K, which suggests that BamE modulates the conformation of BamA through its interactions with BamD (Rigel *et al*., 2012). BamE is also involved in the assembly of RcsF-OMP complexes. The OM lipoprotein RcsF is a component of the Rcs stress response system and forms a complex with OMPs to sense stress in the outer leaflet of the OM. Loss of BamE prevents assembly of these complexes, which implies that BamE might elicit a role in the Rcs stress response system (Konovalova *et al*., 2016). In the absence of BamC or BamE, disruption of BamA–BamD interactions occur to the same degree, which suggests that BamE and BamC have an overlapping role in stabilizing these interactions (Rigel *et al.,* 2012). However, in a Δ*bamE* mutant, additional phenotypic effects occur in the cell that are absent in a Δ*bamC* mutant, suggesting BamE elicits an additional function in the cell.

1.10. The mechanism of OMP insertion into the OM

Previously reported crystal structures of the BAM subunits, BAM sub-complexes and the complete complex suggests that the BAM complex can occupy numerous conformations (Albrecht and Zeth, 2011; Dong *et al.,* 2012; Kim *et al.,* 2011; Chen *et al.,* 2016; Noinaj *et al.,* 2011; Han *et al.,* 2016, Gu *et al.,* 2016). The conformation of the BAM complex depends on the arrangement of the BamA POTRA domains; the positions of BamB–E relative to BamA;

and the structure of the BamA β-barrel (Iadanza *et al.,* 2016). The precise mechanism of OMP folding and insertion into the OM remains unclear.

External to the IM, the cellular compartments are completely devoid of ATP, suggesting that OMP insertion occurs without ATP. Gu *et al*. (2016) reported two novel crystal structures of the *E. coli* BAM complex: BamABCDE and BamACDE. These complexes adopt two distinct conformations: an inward open and a novel lateral open conformation. The addition of BamB stimulates the cap of BamA to close to the extracellular environment, while adopting an open conformation to the periplasmic side. This inward open conformation is proposed to be the resting state of the complex. In the absence of BamB, a lateral open conformation is adapted, where the crown of the BamA barrel opens to the extracellular side, while the periplasmic side remains closed. This conformation might be the post-insertion state of the complex. This model proposes that binding and dissociation of BamB drives the conformational changes in BamA. However, Iadanza *et al.* (2016) presented a cryo-EM structure of the BamABCDE complex in a lateral open conformation (fig. 1.10), which suggests that the lateral gating motion of BamA is independent of BamB binding.

Several models of OMP insertion into the OM have been proposed (Gu *et al.,* 2016; Noinaj *et al.,* 2015; Noinaj *et al.,* 2014). Currently favoured models include the 'BamA-assisted model' and the 'BamA-budding model.' OMPs are inserted more efficiently into thin membranes rather than thick membranes (Burgess *et al.,* 2008). In addition, in the absence of BAM, some OMPs can fold spontaneously into the membrane. In the 'BamA-assisted model', BamA primes the localised OM by thinning the membrane and destabilizing the local lipids (Noinaj *et al.,* 2013). Once the OMPs are delivered to the BAM complex and are in close proximity to the locally primed OM, they spontaneously insert into the OM. In the 'BamA-budding model', the BAM complex threads the OMP through the barrel domain of BamA, stimulating the lateral opening of BamA. The β-strands of the OMP integrate with BamA, forming a BamA:OMP

intermediate. Extracellular loops are formed by the substrate exit pore and the β-strands continue to grow off the initial strands of the BamA:OMP intermediate, leading to the formation of a 'super-barrel' (Rollauer *et al.*, 2015). Lastly, the newly formed OMP 'buds' away from BamA towards the OM. The first stand of the OMP has higher affinity for its own last strand. Thus, once the first stand of the OMP enters the membrane, stand exchange occurs, which closes the barrel domain of the OMP and the OMP diffuses away from BamA into the OM. Similarly, Noinaj *et al.* (2014) proposed a 'hybrid model,' which combines both the 'BamA-assisted model' and the 'BamA-budding model.'

In summary, there are numerous models of OMP insertion into the OM. However, these molecular mechanisms might also depend on a number of factors including: the substrate utilised; membrane environment; and spatial clustering of BAM (Knowles *et al.*, 2011; Mahoney et al., 2016; Rassam *et al.*, 2015). A single mechanism might not be sufficient to explain all aspects of BAM activity.

1.11. Conservation of the BAM complex

The BAM complex can vary between organisms, especially in terms of the number of components present/absent. This study mainly focuses on the BAM pathway in *E. coli.* However, this section highlights the differences in the BAM pathway between organisms, using *E. coli* as a reference point. The components that mediate β-barrel protein assembly are conserved in numerous organisms including Gram-negative bacteria and mitochondria (Doerrler *et al.*, 2004; Tashiro *et al.*, 2008; Wu *et al.*, 2005). For example, Tob55/Sam50 is a homologue of BamA found in mitochondria, which also mediates the assembly of β-barrel proteins into the mitochondrial OM (Gentle *et al.*, 2004; Kozjak *et al.*, 2003; Onufryk *et al.*, 2005). Conversely, some components involved in β-barrel protein assembly, for example, Skp,

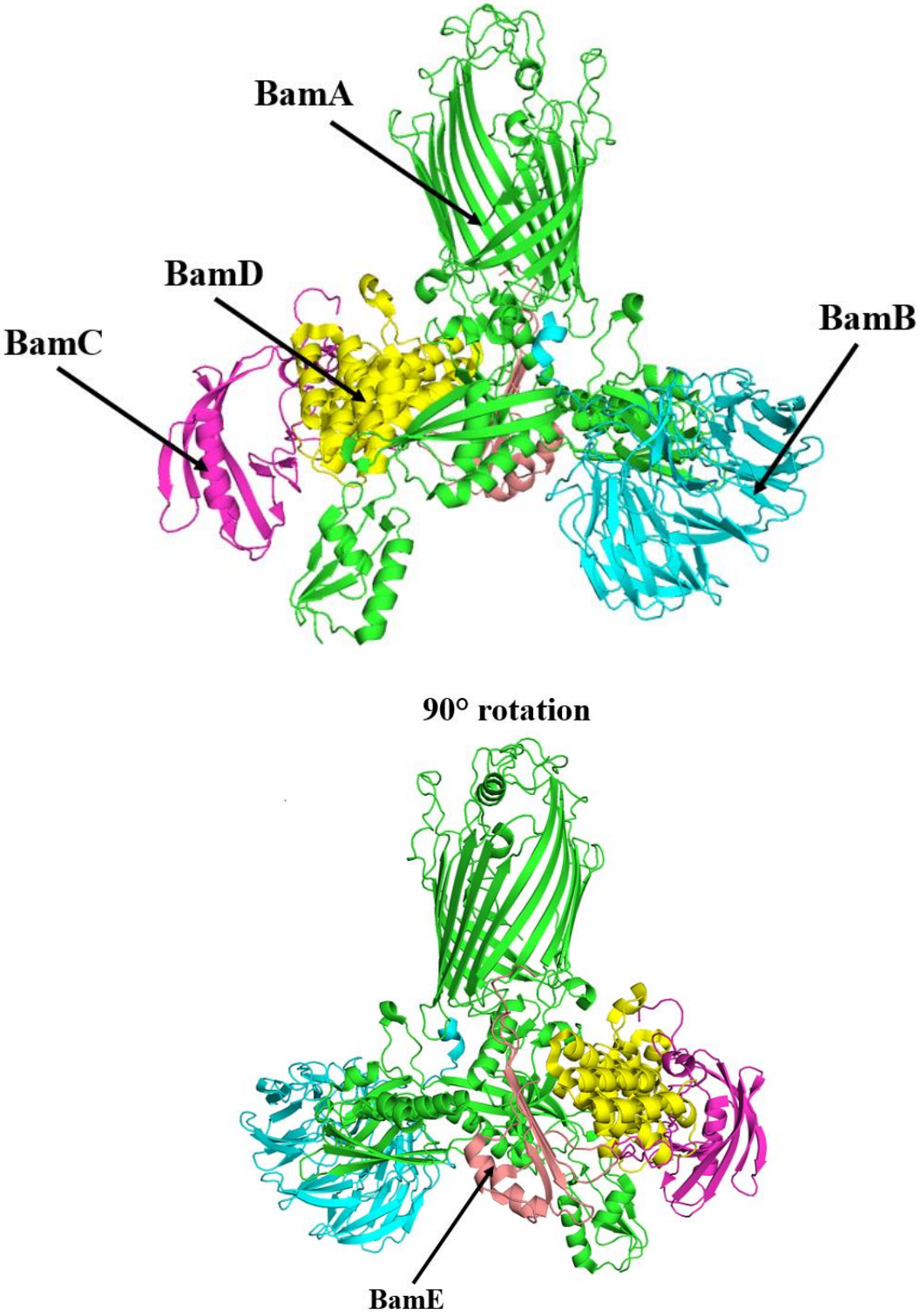


Fig. 1.10 A schematic of the BAM complex. The *E.coli* BAM complex (BamABCDE) as visualised by CryoEM (5LJO; Iadanza *et al.*, 2016), where BamA is shown in Green, BamB is shown in blue, BamC is depicted in dark pink and BamD in yellow. A 90° rotation of the complex was required to visualise BamE (light pink).

BamB or BamC appear to be missing from whole groups of bacteria. Other proteins are conserved but are missing characteristic domains, for example SurA (Gatsos *et al*., 2008).

In *Neisseria meningitidis,* homologs of BamA (Omp85), BamD (ComL), BamC and BamE occur and associate to form an Omp85 complex. However, this organism lacks a BamB homolog but instead contains an additional homolog of BamE (Volokhina *et al.,* 2009). The Omp85 complex also contains an additional component RmpM, which is not required for OMP folding. However, it stabilizes OMP complexes and binds to peptidoglycan (Volokhina *et al.,* 2009). The BamD homolog ComL is also essential for viability and OMP assembly. However, ComL and BamD are not interchangeable. Similarities also exist between BamE and the BamE homologue. Both are non-essential and both contribute to efficient OMP assembly (Volokhina *et al.,* 2009). A null mutation in *bamC* produces only marginal defects in OMP assembly, while the Δ*bamC*Δ*bamE* double mutant was inviable, indicating the importance of BamC in OMP assembly in this organism.

In addition, *Caulobacter crescentus* contains homologs of BamA, BamB, BamD and BamE but not BamC (Gatsos e*t al*., 2008). However, the sequence of BamE is significantly longer, which suggests that it might elicit enhanced activity. Another possibility is that an uncharacterised protein might fulfil the role of BamC in this organism. In summary, although the BAM complex may vary between species, it is a highly conserved essential machinery, making it a great potential target for drug development.

1.12. Using Transposon-directed insertion site sequencing as a genetic screen

In 1965, the first sequencing technologies were developed and in 1972 the first complete protein coding sequence of a bacteriophage MS2 coat protein was obtained (Holley *et al*., 1965; Min Jou *et al*., 1972; Sanger *et al*., 1965). In 1977, the first complete DNA genome was sequenced, a bacteriophage genome referred to as PhiX (Sanger *et al*., 1977). Nowadays,

modern-day high-throughput screens allow the monitoring of numerous mutants and/or conditions simultaneously, aiding the characterisation of genes of unknown function. These screens can involve the production of mutant libraries, which are constructed in two ways, random mutagenesis or targeted mutagenesis.

Targeted mutagenesis in this case refers to construction of gene-deletion mutants, for example the Keio collection (Baba *et al.,* 2006). The Keio collection identified the majority of genes that are not required for the survival of *E. coli* K-12. This library of single mutants is utilised to evaluate fitness under conditions of interest including cell permeability assays, antibiotic assays and biological screens. Random mutagenesis in this case refers to a library of pooled mutants, for example, transposon mutagenesis or transposon-directed insertion-site sequencing (TraDIS). TraDIS is a high throughput technique that combines random transposon mutagenesis with next generation sequencing of transposon mutation sites. This technique profiles a higher number of mutants simultaneously, allowing an entire genome to be assayed at once and provides resolution to the base-pair level (Goodall *et al.,* 2018).

1.12.1. The development of the TraDIS technique

Transposons are linear DNA sequences that can move within a genome, insert itself into different positions and inactivate target genes (McClintock, 1950). In the 1970s, the Tn*5* and Tn*10* transposons were identified, which have the ability to transpose antibiotic-resistant genes. For example, Tn*5* confers resistance to kanamycin and other aminoglycoside antibiotics (Berg *et al.,* 1975). In *E. coli*, transposable elements fall into three general groups that are classified based on their mechanism of transposition. Transposons such as Tn*10* transpose through a conservative cut-and-paste mechanism driven by the enzyme transposase. Transposases acts like 'DNA scissors,' cutting double-stranded DNA to release the transposon from its original position, which is later inserted into a new positon. Other transposons like the Tn*3* transpose through a two-step replicative mechanism, where a fused replicon structure acts as an

intermediate. The third class of transposable elements contain bacteriophage Mu and other related viruses. In this class, transposition occurs through either of the above two mechanisms (Reznikoff, 1993). In bacterial genetics, transposons are manipulated to deactivate target genes and in the absence of transposase, the mutation is stable. The Tn*5* transposon is widely used in numerous species due to its ability to transpose at a high frequency into a wide range of Gram-negative bacteria, with a low insertion specificity and a low excision frequency (Berg and Berg, 1983; de Bruijn and Lupski, 1984).

In early studies of transposon mutagenesis, transposon insertion sites were identified by using position-specific PCR primers (Hare *et al*., 2001). Recently, the technique TraDIS combined random transposon mutagenesis with next-generation sequencing of transposon junctions to screen the entire genome simultaneously on conditions of interest (Langridge *et al*., 2009). TraDIS is mainly utilised to determine gene essentiality and fitness in specific conditions. Similar sequencing methods include HITS, INSeq and Tn-seq (Gawronski *et al*., 2009; Goodman *et al*., 2009; Van Opijnen *et al*., 2009). These protocols vary from TraDIS in precise method, transposon used and the research question asked. For example, INSeq and HITS are utilised to test human infection models. The optimal method to use is dependent upon the strain, resources available and the research question. This Thesis utilises the method TraDIS.

1.12.2. An overview of the TraDIS method

Construction of a TraDIS library consists of transformation of a mini-Tn*5* transposon into electrocompetent *E. coli* cells, where each bacterial cell contains a single random transposon insertion within its genome. The mini-Tn*5* transposon was utilised in this study due to its low/no insertion bias and its relatively small size, which increases its transformation efficiency. In addition, the Tn*5* transposon contains a kanamycin antibiotic cassette, which allows the easy selection of transformants. The successful transformants are pooled, the genomic DNA is extracted, sonicated and numerous PCRs are utilised to amplify the transposon junctions. Next-

generation sequencing technology identifies the amplified transposon junctions, which are then mapped to a reference genome. The presence or absence of insertion sites allows the identification of essential genes, genes with essential regions and non-essential genes of the entire genome simultaneously.

An essential gene can be defined as a gene that when deleted the mutant cannot be isolated following growth (Goodall *et al*., 2018). In terms of TraDIS, an essential gene cannot be disrupted by a transposon and does not result in a viable bacterium. Thus, none or very little mutants will be recovered with transposon insertions within essential genes. Conversely, numerous mutants are recovered with transposon insertions along the length of non-essential genes. In addition, another purpose of TraDIS is to identify conditionally essential genes, such as those required for survival under conditions of stress, for example, upon exposure to an antibiotic. To identify conditionally essential genes, the transposon library is grown under the condition of interest and the input and output pools of mutants are compared. For example, Langridge *et al.* (2009) identified genes in *Salmonella enterica* Typhi that provide tolerance to the substance bile and genes that provide an advantageous or disadvantageous growth effect in rich media. Alternatively, Goodman *et al.* (2009) identified specific genes that are necessary for survival of *Bacteroides thetaiotaomicron* in the colon.

TraDIS can also be utilised to identify conditionally essential genes in a specific gene-deletion mutant, where a parent TraDIS library is compared to a TraDIS library constructed in a defined mutant (fig. 1.11). Such conditionally essential genes are considered ‘synthetic lethal partners’, where each individual mutant is viable, but the two mutations combined together result in demise of the cell. Gene-deletion TraDIS libraries also identify conditionally non-essential genes, which are no longer essential in the mutant of interest but are required for the survival

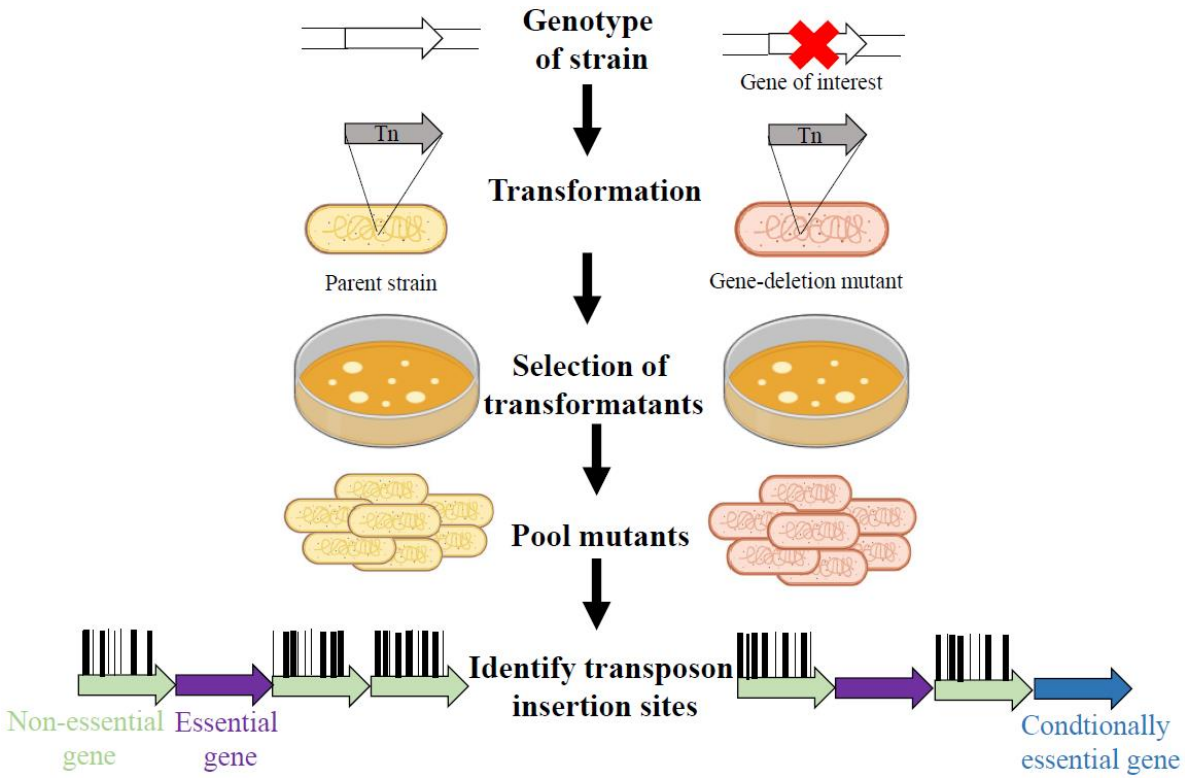


Fig. 1.11 Using TraDIS to identify essential and conditionally essential genes. In a mutant TraDIS library, a gene of interest is disrupted (pink), while in the parent strain the genome is not manipulated (yellow). The mini Tn*5* transposon is transformed into both strains and transformants are isolated on kanamycin. The respective mutants are pooled and transposon insertion sites are identified by sequencing technology. Genes classified as essential (blue) in both the mutant and the parent TraDIS library are genes that contain a relatively low number or no transposon insertions. Genes that are non-essential in both TraDIS libraries (green) contain numerous transposon insertions. Conditionally essential genes (purple) are required for the survival of the mutant but are dispensable in the parent. Conversely, a gene that is essential in the parent that can be viably disrupted in the mutant is referred to as conditionally non-essential.

of the parent strain. Gene-deletion TraDIS libraries help determine the function of unknown genes and increase the understanding of biosynthetic pathways.

TraDIS can also determine the relative frequency of mutants of the same genotype within the final population. For example, in a mutant TraDIS library, a higher number of transposon mutants are recovered with Tn*5* insertions in specific genes, compared to the parent TraDIS library. These mutations are 'enriched for,' which refer to gene deletions that provide an advantage for growth under the condition tested. Conversely, some gene-deletion mutants 'drop out,' which refer to gene deletions that are disadvantageous for growth under the given condition.

Lastly, TraDIS can also be utilised to identify suppressor mutations. Suppressor mutations are secondary mutations that can restore the phenotype of a specific mutant to that of the original parent strain. To identify suppressor mutations the mutant TraDIS library of interest is exposed to conditions that is lethal to the mutant but not to the parent, for example, inhibitory concentrations of vancomycin. Mutants are collected, pooled and sequenced. These mutants contain a secondary gene deletion that either restores the phenotype to the background levels or a mutation that makes the cell more resistant to the condition tested. A parent TraDIS library can be screened alongside the mutant TraDIS library to distinguish between the two categories of suppressors.

1.13. Aims of this thesis

In *Escherichia coli*, the BAM complex consists of two essential components, BamA and BamD and three non-essential components: BamB, BamC and BamE. Despite numerous studies, the roles of the non-essential BAM components remains poorly understood. In addition, critical to understanding OMP biogenesis is understanding the transportation of OMPs to the BAM complex. SurA, Skp and DegP are three known chaperones that transport OMPs across the

periplasm to the BAM complex. Sklar *et al.* (2007) proposed that functional redundancy exists between SurA and Skp and between SurA and DegP. However, there is no molecular evidence to suggest that the functions of SurA and Skp are redundant. Thus, the functional redundancy between the BAM chaperones remains largely unresolved.

In addition, bacteria must synchronize the synthesis, growth and division of the cell envelope components both spatially and temporally. An imbalance in these processes can compromise the permeability barrier and the structural integrity of the cell. Thus, OMP biogenesis must coordinates with other cell envelope pathways including LPS and PG synthesis. However, these mechanisms of coordination are unknown.

The aim of this study was fourfold: to help determine the roles of BamB, BamC and BamE; to increase understanding of the synthetic interactions between the BAM chaperones; to identify potential mechanisms of coordination between OMP biogenesis and other cell envelope pathways; and to identify synthetic lethal partners that could be exploited as drug targets in the development of antimicrobial therapies. The high-throughput technique Transposon-Directed Insertion Site Sequencing (TraDIS) was utilised in an attempt to address these aims. TraDIS libraries were constructed in mutants defective in *bamB, bamC, bamE, surA, skp* and *degP* and were compared to a parent *E. coli* K-12 TraDIS library to identify genes that are essential in the mutant but not in the parent strain. This will identify the cellular factors required upon impairment of OMP biogenesis. In an attempt to identify how OMP biogenesis coordinates with other cell envelope pathways, this study also compared the BAM mutant TraDIS libraries. TraDIS was also utilised in an attempt to identify potential suppressors of *bamB*, *bamC* and *bamE*. The data presented in this Thesis provides a richer understanding of OMP biogenesis that could be harnessed for the development of novel antimicrobials.

CHAPTER 2

Materials and methods

2.1. Bacterial growth media

2.1.1. Culture media

All media were made using distilled water and sterilised by autoclaving at 121°C for 15 min. For standard growth experiments, strains were inoculated into lysogeny broth (LB) comprised of 10 g/l tryptone, 10 g/l NaCl and 5 g/l yeast extract. Low salt LB was used when appropriate, which was comprised of 5 g/l NaCl instead of 10 g/l NaCl. For growth on solid phase media, 1.5% (w/v) of agar was added to the LB. During the construction of the transposon libraries, cells were grown in 2xTY broth, which consisted of 5 g/l NaCl, 16 g/l tryptone and 10g/l yeast extract. Super optimal broth with catabolite repression (SOC) medium was used in the recovery step of competent cell transformations to maximise transformation efficiency. Ready-made SOC solution was purchased from Sigma Aldrich.

2.1.2. Antibiotic supplements

When appropriate, media was supplemented with 100 µg/ml carbencillin disodium salt, 100 µg/ml vancomycin hydrochloride or 50 µg/ml kanamycin sulfate unless otherwise stated. All antibiotic stock solutions were filter sterilised through 0.22 µm syringe filters and stored at -20°C, unless otherwise stated by the manufacturer. A full list of antibiotic stock solutions and their respective solvents are shown in table 2.1.

2.2. Bacterial strains, growth conditions and plasmids

2.2.1. Bacterial strains

Bacterial strains used in this study are outlined in table 2.2. For long term storage, strains were stored as glycerol stocks at -80°C. Glycerol stocks consisted of LB media supplemented with a final concentration of 25% (v/v) glycerol. When required, strains were streaked to single colonies on the appropriate agar plates and incubated overnight at 37°C.

Table 2.1 Stocks of antibiotics used in this study

Antibiotic	Stock concentration	Solvent	Storage	Supplier
Kanamycin sulfate	50 mg/ml	Water	-20°C	Sigma Aldrich
Carbenicillin disodium salt	100 mg/ml	Water	-20°C	Sigma Aldrich
Vancomycin hydrochloride	100 mg/ml	Water	-20°C	Cayman Chemical Company

Table 2.2 Strains used in this study

Name of strain	Description	Source
BW25113	Parent strain of the Keio collection.	Datsenko and Wanner (2000).
BW25113 Δ*bamB*	*bamB* gene deleted by recombination of the kanamycin cassette, which was later removed, leaving a scar.	Created in this study.
BW25113 Δ*bamC*	*bamC* gene deleted by recombination of the kan^R cassette, which was removed, leaving a scar.	Created in this study.
BW25113 Δ*bamE*	*bamE* gene deleted by recombination of the kan^R cassette, which was removed, leaving a scar.	Created in this study.
BW25113 Δ*surA*	*surA* gene deleted by recombination of the kan^R cassette, which was removed, leaving a scar.	Created in this study.
BW25113 Δ*degP*	*degP* gene deleted by recombination of the kan^R cassette, which was removed, leaving a scar.	Created in this study.
BW25113 Δ*skp*	*skp* gene deleted by recombination of the kan^R cassette, which was removed, leaving a scar.	Created in this study.
BW25113 Δ*waaC*::*aph*	*waaC* gene replaced by a kan^R cassette.	Created in this study.
BW25113 Δ*waaF*::*aph*	*waaF* gene replaced by a kan^R cassette.	Created in this study.
BW25113 Δ*waaP*::*aph*	*waaP* gene replaced by a kan^R cassette.	Created in this study.
BW25113 Δ*waaG*::*aph*	*waaG* gene replaced by a kan^R cassette.	Created in this study.
BW25113 Δ*waaY*::*aph*	*waaY* gene replaced by a kan^R cassette.	Created in this study.
BW25113 Δ*ompT*::*aph*	*ompT* gene replaced by a kan^R cassette.	Created in this study.
BW25113 Δ*dapF*::*aph*	*dapF* gene replaced by a kan^R cassette.	Created in this study.
BW25113 Δ*bamB*Δ*dapF*::*aph*	*bamB* gene deleted by recombination of the kan^R cassette, which was later removed. *dapF* gene replaced by a kan^R cassette.	Created in this study.
BW25113 Δ*bamC*Δ*dapF*::*aph*	*bamC* gene deleted by recombination of the kan^R cassette, which was later removed. *dapF* gene replaced by a kan^R cassette.	Created in this study.
BW25113 Δ*bamE*Δ*dapF*::*aph*	*bamE* gene deleted by recombination of the kan^R cassette, which was removed, leaving	Created in this study.

	a scar. *dapF* gene replaced by a *kan*^{R} cassette.	
BW25113 Δ*surA*Δ*dapF*::*aph*	*surA* gene deleted by recombination of the *kan*^{R} cassette, which was removed, leaving a scar. *dapF* gene replaced by a *kan*^{R} cassette.	Created in this study.
BW25113 Δ*degP*Δ*dapF*::*aph*	*degP* gene deleted by recombination of the *kan*^{R} cassette, which was removed, leaving a scar. *dapF* gene replaced by a *kan*^{R} cassette.	Created in this study.
BW25113 Δ*skp*Δ*dapF*::*aph*	*skp* gene deleted by recombination of the *kan*^{R} cassette, which was removed, leaving a scar. *dapF* gene replaced by a *kan*^{R} cassette.	Created in this study.
BW25113 Δ*wecA*::*aph*	*wecA* gene replaced by a *kan*^{R} cassette.	Created in this study.
BW25113 Δ*wecD*::*aph*	*wecD* gene replaced by a *kan*^{R} cassette.	Created in this study.
BW25113 Δ*wecF*::*aph*	*wecF* gene replaced by a *kan*^{R} cassette.	Created in this study.
BW25113 Δ*wzzE*::*aph*	*wzzE* gene replaced by a *kan*^{R} cassette.	Created in this study.
BW25113 Δ*wzxE*::*aph*	*wzxE* gene replaced by a *kan*^{R} cassette.	Created in this study.
BW25113 Δ*rcsF*Δ*lpp*Δ*pgsA* Δ*surA::aph*	*rcsF, lpp* and *pgsA* genes were sequentially replaced by a *kan*^{R} cassette, which were later removed. *surA* gene replaced by a *kan*^{R} cassette.	Created in this study.
BW25113 Δ*rcsF*Δ*lpp*Δ*pgsA* Δ*surA*Δ*waaL::aph*	*rcsF, lpp, pgsA* and *surA* genes were sequentially replaced by a *kan*^{R} cassette, which were later removed. *waaL* gene replaced by a *kan*^{R} cassette.	Created in this study.
BW25113 Δ*surA*Δ*waaL::aph*	*surA* gene deleted by recombination of the *kan*^{R} cassette, which was removed, leaving a scar. *waaL* gene replaced by a *kan*^{R} cassette.	Created in this study.

E. coli K-12 BW25113 (the parent strain) was used for all subsequent experiments and all mutants were constructed in this strain.

2.2.2. Growth conditions of bacteria

Overnight cultures consisted of 5 ml of LB broth inoculated with a single colony in a 25 ml universal bottle. All other liquid cultures were grown in Erlenmeyer flasks. Liquid cultures were incubated in a shaking incubator at 180 rpm at 37°C, unless otherwise stated. Using a spectrometer, growth rates were monitored by measuring optical density at OD_{600}.

2.2.3. Plasmids

Plasmids used in this study are listed in table 2.3. When growing strains that carried a plasmid, growth media was supplemented with the appropriate antibiotic. Some plasmids used in this study are heat sensitive (e.g. pCP20). As a result, strains harbouring these plasmids were incubated at 30°C.

2.3. Gene knockout production

2.3.1. P1 phage transduction

In this study, P1 transduction was one method used to produce a mutant where the gene of interest is replaced by a kanamycin (kan^R) cassette. For example Δ*wecA*::*aph*, Δ*wzzE*::*aph* and Δ*ompT*::*aph* BW25113 strains were produced by P1 transduction. This protocol consisted of transferring a kan^R cassette from the Keio collection into the respective gene of interest in the BW25113 strain. Bacterial cultures of the donor (usually *E. coli* BW25113) and the recipient strain were grown overnight at 37°C. Following which, the donor (50 µl) was inoculated into 5 ml of fresh LB, which contained 25 µl 40% (w/v) glucose (final concentration 0.2%) and 25 µl of 1 M $CaCl_2$ (final concentration 5 mM).

Table 2.3 Plasmids used in this study

Plasmid	Description	Source
pCP20	Temperature sensitive plasmid that expresses FLP recombinase. Amp^R	Datsenko and Wanner (2000)
pKD4	Template for kanamycin resistance cassette flanked by FLP sites. Kan^R	Datsenko and Wanner (2000)
pKD46	Temperature sensitive plasmid with an arabinose inducible promoter. Expresses l Red recombination proteins. Amp^R	Datsenko and Wanner (2000)

Cultures were incubated at 37°C for 45 min with shaking. 100 µl of P1*vir* stock (10^9-10^{10} pfu/ml) was added to the donor culture and incubated for ~3 h or until the culture lysed. To ensure complete cell lysis occurred 100 µl of chloroform was added and the culture was incubated for ~10 min. The supernatant was harvested by centrifugation at 9,200 x *g* for 10 min and transferred to a 5 ml glass bottle for long-term storage at 4°C.

The overnight culture of the recipient cells were centrifuged at 4,000 x *g* for 4 min and the cell pellets were suspended in 750 µl of P1 salts (10 mM $CaCl_2$ and 5 mM $MgSO_4$). 100 µl of cells/P1 salts were mixed with 100 µl of the donor P1 lysate. For the no lysate control, the cells/P1 salts mixture was not mixed with the donor P1 lysate. Following a static 30 min incubation step at 37°C, 1 ml of LB and 200 µl of 1M sodium citrate was added to each sample and the culture was incubated for 1 h at 37°C with aeration. The cells were harvested by centrifugation at 4,000 x *g* for 2 min, the supernatant was discarded and the cells were re-suspended in 100 µl of LB. The cells were spread on agar plates containing 50 µg/ml of kanamycin and 5 mM sodium citrate and were incubated overnight at 37°C. Following mutant confirmation by polymerase chain reaction (PCR), the mutants were re-streaked on plates supplemented with 50 µg/ml kanamycin and 5 mM sodium citrate. This step was repeated three times to ensure there was no P1 phage within the strain.

2.3.2. One-step inactivation of chromosomal genes using PCR products

This study also replaced genes with an antibiotic cassette using the method discussed in Datsenko and Wanner (2000). The kanamycin cassette and the FRT sites were amplified from pKD4 using 56-70 nucleotide long primers, which included 36-50 nt homology extensions and 20 nt priming sequences for pKD4. The 36-50 nt extension was homologous to the chromosomal insertion site. Following amplification, the fragment was separated by gel electrophoresis and extracted using a gel extraction kit (Qiagen).

The target strain was first transformed with the temperature sensitive plasmid pKD46, which codes for an arabinose inducible lambda red recombination system. This strain was then grown at 30°C overnight in LB supplemented with carbenicillin. The overnight (500 µl) was inoculated into 50 ml of fresh LB, which also contained 0.2% (w/v) arabinose and 100 µg/ml carbenicillin and was grown to an OD_{600} of ~0.6 at 30°C. The cells were pellet and made electrocompetent using the steps outlined in 2.5.2.

The competent cells (50 µl) were mixed with insert DNA in a 1.5 ml microcentrifuge tube. Following a 30 min incubation step on ice, the samples were transferred to a 2 mm electroporation cuvette (Cell Projects) and electroporated at 2,200 V using an Eppendorf Eporator. SOC media (1 ml) was added to the cells and the cells were recovered for 2 h at 37°C with aeration. The cells were harvested by centrifugation at 4,000 x *g*, the supernatant was removed and the cell pellet was resuspended in 100 µl of LB. Transformants were isolated on LB agar plates supplemented with the appropriate antibiotic and the plates were incubated overnight at 37°C. Mutants were confirmed via PCR using flanking primers.

2.3.3. Removal of the kanamycin cassette

In a number of mutants utilised in this study, the resistance cassette that replaced a gene of interest was removed. The kanamycin cassette contains two FRT sites either side of the resistance gene, which can be utilised to flip the resistance gene out, producing a scar region of ~ 100 bp. Firstly, the strain of interest, was transformed with the temperature sensitive pCP20 plasmid. The pCP20 plasmid codes for the yeast recombinase Flp, which recombines with the FRT sites, either side of the kanamycin cassette (Cherepanov and Wackernagel, 1995). The transformants were isolated on agar pates supplemented with 100 µg/ml carbenicillin.

In an attempt to remove the pCP20 plasmid, the transformants were re-streaked onto LB agar plates and incubated overnight at 37°C. Individual colonies were then isolated and re-streaked onto LB agar, LB agar supplemented with kanamycin (deletion confirmation) and LB agar supplemented with carbenicillin (plasmid loss conformation). The colonies that did not grow on the LB agar supplemented with kanamycin or carbenicillin but grew on the LB only agar were screened by colony PCR to confirm the deletion of interest. This method was utilised in the construction of Δ*bamB*; Δ*bamC*; Δ*bamE*; Δ*surA*; Δ*skp* and Δ*degP* mutants.

2.4. Molecular genetics techniques

Kits were used following the manufacturer's instructions, unless otherwise stated.

2.4.1. Preparation of genomic DNA and plasmid DNA

Genomic DNA was isolated using the STRATEC RTP Bacteria DNA Mini kit, following the protocol for Gram-negative bacteria. Plasmid DNA was isolated from an overnight culture using the Qiagen QIAprep Spin Miniprep kit.

2.4.2. Qubit quantification of DNA

DNA was routinely quantified using Qubit™ dsDNA HS Assay kit (ThermoFisher), using a 1:1 ratio of DNA to Dye.

2.4.3 Polymerase chain reaction (PCR)

The primers used in this study are listed in table 2.4. DNA fragments (10 ng/µl plasmid) were amplified using Velocity High-Fidelity DNA polymerase (Bioline). The PCR reactions also contained 5X Hi-Fi reaction buffer and 5% (v/v) DMSO. For colony PCR, a single colony was resuspended in 100 µl of water, boiled for 10 min at 100°C and pelleted at 11,000 x *g* for 30 s. 2 µl of supernatant was utilised as the PCR template with 12.5 µl of MyTaq

Red Mix (Bioline), 8.5 µl water and 1 µl 10 mM forward and reverse primer. Routine PCR thermal profiles are listed in table 2.5.

2.4.4. PCR DNA purification

Standard PCR products amplified by Velocity were purified using the Qiagen QIAquick PCR purification kit, following kit instructions with minor modifications. The samples were eluted in 50 µl nuclease-free water (Ambion) and the elution incubation time was increased to 4 min. AMPure XP beads were utilised for PCR clean-up during the TraDIS library preparation protocol.

2.4.5. Agarose gel electrophoresis

DNA was separated on a 1% (w/v) agarose gel in 1x TAE buffer (50x TAE buffer, 2 M Tris base, 1 M acetic acid and 0.05 M EDTA in water). To every 100 ml of agarose, 5 µl Midori Green DNA dye (Nippon Genetics) was added. Prior to loading DNA into the well, isolated DNA was mixed with a DNA loading dye in a 1:1 ratio (0.025% (w/v) bromophenol blue, 20% (v/v) glycerol, 0.025% (v/v) xylene cyanol F, 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA). A 1 kb DNA ladder (Bioline) was utilised as a DNA marker unless otherwise stated. Samples were separated by electrophoresis at 3-5 V/cm for 30-60 min in 1x TAE buffer and imaged using a Gel Doc light box (Bio-Rad).

2.4.6. Extraction of DNA fragments from agarose gels

Using a razor blade, DNA bands were cut out from the gel and transferred to a 1.5 ml eppendorf. DNA was extracted using the QIAquick Gel Extraction kit (Qiagen) and samples were eluted in 50 µl of nuclease-free water (Ambion).

Table 2.4 Primers used in this study

Name	Primer sequence	Description
*bamB*_check forward	GCGCGTAGTGCATGGGAAGC	To verify if the *bamB* gene is absent/present.
*bamB*_check reverse	CAACGCACGCTATATTCGCG	To verify if the *bamB* gene is absent/present.
*bamC*_check forward	GGTCTTGTGGCGACCGATAC	To verify if the *bamC* gene is absent/present.
*bamC*_check reverse	CCTTATCCGAACTACGTCCG	To verify if the *bamC* gene is absent/present.
*bamE*_check forward	GCTTCACGGTCAGAGTAAAC	To verify if the *bamE* gene is absent/present.
*bamE*_check reverse	GAGCTTCGCAGGCAACGAGC	To verify if the *bamE* gene is absent/present.
*degP*_check forward	GGAACTTCAGGCTATAAAAC	To verify if the *degP* gene is absent/present.
*degP*_check reverse	GAAGATGTATGGAGTTGTGG	To verify if the *degP* gene is absent/present.
*surA*_check forward	GCGGTATATGACAACGCAATC	To verify if the *surA* gene is absent/present.
*surA*_check reverse	CGGAGGGTGAGCGGCAAAC	To verify if the *surA* gene is absent/present.
*skp*_check forward	GGAATGTAGTGGTAGTGTAG	To verify if the *skp* gene is absent/present.
*skp*_check reverse	CCGGTGATGACGATATCGCC	To verify if the *skp* gene is absent/present.
*gmhA*_Kan^R forward	TACTTGTAGGAGGTCTGACC	Used in the construction of the Δ*gmhA*::*aph* strain and to verify if the *gmhA* gene is absent/present.
*gmhA*_Kan^R reverse	CAATTCGCACATGGGTAACC	Used in the construction of the Δ*gmhA*::*aph* strain and to verify if the *gmhA* gene is absent/present.
*gmhB*_Kan^R forward	ATAAGCGTACTCTTACCCGC	Used in the construction of the Δ*gmhB*::*aph* strain and to verify if the *gmhB* gene is absent/present.
*gmhB*_Kan^R reverse	GTTAAAGAGCAGTTGCGACG	Used in the construction of the Δ*gmhB*::*aph* strain and to verify if the *gmhB* gene is absent/present.
*hldE*_Kan^R forward	GCAATTAGCATCCTTGCACC	Used in the construction of the Δ*hldE*::*aph* strain and to verify if the *hldE* gene is absent/present.
*hldE*_Kan^R reverse	GAGGCATAGGAATAGCTTCG	Used in the construction of the Δ*hldE*::*aph* strain and to verify if the *hldE* gene is absent/present.

*waaD*_Kan^R forward	GCAATTAGCATCCTTGCAC C	Used in the construction of the Δ*waaD*::*aph* strain and to verify if the *waaD* gene is absent/present.
*waaD*_Kan^R reverse	GAGGCATAGGAATAGCTTC G	Used in the construction of the Δ*waaD*::*aph* strain and to verify if the *waaD* gene is absent/present.
*waaC*_Kan^R forward	GTGATCCGTTTGATTACCG G	Used in the construction of the Δ*waaC*::*aph* strain and to verify if the *waaC* gene is absent/present.
*waaC*_Kan^R reverse	GTAAGTAGCACGAAATGGC G	Used in the construction of the Δ*waaC*::*aph* strain and to verify if the *waaC* gene is absent/present.
*waaF*_Kan^R forward	GGCTTATCACAAGAAAGGC C	Used in the construction of the Δ*waaF*::*aph* strain and to verify if the *waaF* gene is absent/present.
*waaF*_Kan^R reverse	TTGCCACAGGAATAACTCG C	Used in the construction of the Δ*waaF*::*aph* strain and to verify if the *waaF* gene is absent/present.
*waaP*_Kan^R forward	GGGTACGCGCATTATATTG C	Used in the construction of the Δ*waaP*::*aph* strain and to verify if the *waaP* gene is absent/present.
*waaP*_Kan^R reverse	CAGCCATGCATTATCCATC	Used in the construction of the Δ*waaP*::*aph* strain and to verify if the *waaP* gene is absent/present.
*waaG*_Kan^R forward	ATTTGGTGCAACGGATCAC G	Used in the construction of the Δ*waaG*::*aph* strain and to verify if the *waaG* gene is absent/present.
*waaG*_Kan^R reverse	TCACTCAGGCGATGAATAG C	Used in the construction of the Δ*waaG*::*aph* strain and to verify if the *waaG* gene is absent/present.
*waaY*_Kan^R forward	CTAAACCGTGGCACAAATG G	Used in the construction of the Δ*waaY*::*aph* strain and to verify if the *waaY* gene is absent/present.
*waaY*_Kan^R reverse	GGTATAACGACTTCTCTGG C	Used in the construction of the Δ*waaY*::*aph* strain and to verify if the *waaY* gene is absent/present.
*wecA*_check forward	CTATCGACTACAACCGTTC TG	To verify if the *wecA* gene is absent/present.
*wecA*_check reverse	GATCAAATGGTGAATATGC TG	To verify if the *wecA* gene is absent/present.
*wecD*_check forward	CTCCATCAACCAGATGTAC C	To verify if the *wecD* gene is absent/present.
*wecD*_check reverse	CTCCATCAACCAGATGTAC C	To verify if the *wecD* gene is absent/present.
*wecF*_check forward	GGTGAGCAGTATTTCCGAT G	To verify if the *wecF* gene is absent/present.

*wecF*_check reverse	CACGCCGGAGACTTCACTG	To verify if the *wecF* gene is absent/present.
*wzzE*_check forward	TTGTGCTGATTACCCTTGCC	To verify if the *wzzE* gene is absent/present.
*wzzE*_check reverse	GCAGTGACGCAAACTTTAGC	To verify if the *wzzE* gene is absent/present.
*wzxE*_check forward	CTCCAGCTATTTGATGTCCGATC	To verify if the *wzxE* gene is absent/present.
*wzxE*_check reverse	CACTGGAAAGCTCATACAGGTC	To verify if the *wzxE* gene is absent/present.
*ompT*_check forward	ACATAGTAATTGAGGATAAACCTCATGC	To verify if the *ompT* gene is absent/present.
*ompT*_check reverse	AGCCCAGAAATGTGGCTATAACAG	To verify if the *ompT* gene is absent/present.
*dapF*_check forward	GAAAGGTCCGCTCTATTTCC	To verify if the *dapF* gene is absent/present.
*dapF*_check reverse	GAACATGAATATGATTACGTGC	To verify if the *dapF* gene is absent/present.
TKK_Forward	ACCTGCAGGCATGCAAGCTTCAGG	Forward primer for amplification of the kanamycin transposon junction.
TKK_Reverse	GACTGGAGTTCAGACGTGTGCTCTTCCGATC	Reverse primer for amplification of the kanamycin transposon junction.
TKK_inline index_6.1.	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTACGAGCTTCAGGGTTGA GATGTGTA	Inline barcode.
TKK_inline index_6.3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTACGTAAGCTTCAGGGTTGA GATGTGTA	Inline barcode.
TKK_inline index_7.2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTGCTAGCTAGCTTCAGGGTTG AGATGTGTA	Inline barcode.
TKK_inline index_7.4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTAGCTAGAGCTTCAGGGTTG AGATGTGTA	Inline barcode.
TKK_inline index_8.2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC	Inline barcode.

	TGCATGCATAGCTTCAGGG TT GAGATGTGTA	
TKK_inline index_8.3	AATGATACGGCGACCACCG AGATCTACACTCTTTCCCT ACACGACGCTCTTCCGATC TCATGCATGAGCTTCAGGG TT GAGATGTGTA	Inline barcode.
TKK_inline index_8.4	AATGATACGGCGACCACCG AGATCTACACTCTTTCCCT ACACGACGCTCTTCCGATC TATGCATGCAGCTTCAGGG TTGAGATGTGTA	Inline barcode.
TKK_inline index_9.2	AATGATACGGCGACCACCG AGATCTACACTCTTTCCCT ACACGACGCTCTTCCGATC TATCGATCGAAGCTTCAGG GT TGAGATGTGTA	Inline barcode.
TKK_inline index_9.3	AATGATACGGCGACCACCG AGATCTACACTCTTTCCCT ACACGACGCTCTTCCGATC TTCGATCGATAGCTTCAGG GT TGAGATGTGTA	Inline barcode.
TKK_inline index_9.4	AATGATACGGCGACCACCG AGATCTACACTCTTTCCCT ACACGACGCTCTTCCGATC TCGATCGATCAGCTTCAGG GT TGAGATGTGTA	Inline barcode.
TKK_Illumnia index_1	CAAGCAGAAGACGGCATA CGAGATCGTGATGTGACTG GAGTTCAGACGTGTGCTCT TCCGATC	Illumnia barcode.
TKK_Illumnia index_2	CAAGCAGAAGACGGCATA CGAGATACATCGGTGACTG GAGTTCAGACGTGTGCTCT TCCGATC	Illumnia barcode.
TKK_Illumnia index_3	CAAGCAGAAGACGGCATA CGAGATGCCTAAGTGACTG GAGTTCAGACGTGTGCTCT TCCGATC	Illumnia barcode.
TKK_Illumnia index_4	CAAGCAGAAGACGGCATA CGAGATTGGTCAGTGACTG GAGTTCAGACGTGTGCTCT TCCGATC	Illumnia barcode.
TKK_Illumnia index_5	CAAGCAGAAGACGGCATA CGAGATCACTGTGTGACTG GAGTTCAGACGTGTGCTCT TCCGATC	Illumnia barcode.
TKK_Illumnia index_6	CAAGCAGAAGACGGCATA CGAGATATTGGCGTGACTG	Illumnia barcode.

	GAGTTCAGACGTGTGCTCT TCCGATC	
TKK_Illumnia index_7	CAAGCAGAAGACGGCATA CGAGATGATCTGGTGACTG GAGTTCAGACGTGTGCTCT TCCGATC	Illumnia barcode.
TKK_Illumnia index_8	CAAGCAGAAGACGGCATA CGAGATTCAAGTGTGACTG GAGTTCAGACGTGTGCTCT TCCGATC	Illumnia barcode.
TKK_Illumnia index_9	CAAGCAGAAGACGGCATA CGAGATCTGATCGTGACTG GAGTTCAGACGTGTGCTCT TCCGATC	Illumnia barcode.
TKK_Illumnia index_10	CAAGCAGAAGACGGCATA CGAGATAAGCTAGTGACTG GAGTTCAGACGTGTGCTCT TCCGATC	Illumnia barcode.
TKK_Illumnia index_11	CAAGCAGAAGACGGCATA CGAGATGTAGCCGTGACTG GAGTTCAGACGTGTGCTCT TCCGATC	Illumnia barcode.
TKK_Illumnia index_12	CAAGCAGAAGACGGCATA CGAGATTACAAGGTGACTG GAGTTCAGACGTGTGCTCT TCCGATC	Illumnia barcode.

Table 2.5 PCR thermal profiles

MyTaq red polymerase			
Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	95°C	1 min	1
Denaturation	95°C	15 s	25-35
Annealing	Determined by user, dependent on Tm of primers	15 s	
Extension	72°C	10 s/kb	
Final extension	72°C	4-10 min	1
Hold	4°C	Infinity	-

Velocity polymerase			
Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	98°C	2 min	1
Denaturation	98°C	30 s	25-35
Annealing	56°C	30 s	
Extension	72°C	30 s/kb	
Final extension	72°C	4-10 min	1
Hold	4°C	Infinity	-

2.4.7. Restriction digestion of DNA

Prior to digestion, purified PCR products and plasmids were quantified using Qubit™ dsDNA high sensitivity assay kit (Invitrogen). Both products were digested independently, using either one restriction enzyme or two restriction enzymes (table 2.6). Digested plasmids used for cloning were incubated with 1 µl (10 units) calf alkaline phosphatase (CIP, New England Biolabs) per 50 µl reaction at 37°C for 1 h.

2.4.8. DNA ligation

Following quantification of the vector and insert DNA using Qubit™ reagents, 50 ng of the vector was mixed with the insert DNA at a ratio of 1:3. 2 µl of T4 DNA ligase buffer (10x), 1 µl T4 DNA ligase (New England BioLabs) and water was added to the reaction to produce a final volume of 20 µl. The samples were incubated at room temperature (RT) for 10-15 min and a ligation control consisted of all of the reagents except for the insert.

2.5. Preparation of competent cells

2.5.1. Preparation of calcium competent cells

A single colony was inoculated into 5 ml of LB and was grown overnight at 37°C. The overnight culture (500 µl) was inoculated into 49.5 ml of fresh LB, which was grown at 37°C to an OD_{600} of 0.4-0.6. Following a 10 min incubation step on ice, the cells were harvested by centrifugation at 5,000 x *g* at 4°C for 10 min, the supernatant was discarded and the cell pellet was resuspended in 25 ml ice-cold 0.1 M $CaCl_2$. The sample was incubated on ice for 30 min and the cells were centrifuged again at 5,000 x *g*, 4°C for 10 min. The supernatant was discarded and the cell pellet was resuspended in 0.1 M $CaCl_2$ and 15% (v/v) glycerol. Following an additional incubation step, the component cells were separated into aliquots and stored at -80°C. These cells were utilised in the transformation of plasmids or PCR products as per 2.5.3.

Table 2.6 Restriction digestion

Restriction digest using one restriction enzyme	
DNA	250 µg
Enzyme	0.5 µl
10X Cutsmart Buffer	1 µl
H_2O	8.5 µl - x
Total	**10 µl**
Restriction digest using two restriction enzymes	
DNA	250 µg
Enzyme 1	0.25 µl
Enzyme 2	0.25 µl
10X Cutsmart Buffer	1 µl
H_2O	8.5 µl - x
Total	**10 µl**

2.5.2. Preparation of electrocompetent cells

To make 500 µl of competent cells, 500 µl of the overnight culture was inoculated into 50 ml of fresh LB media and grown to an OD_{600} of 0.4-0.6. The culture was then incubated on ice for 30 min and centrifuged at 4,000 x *g* for 10 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 50 ml of ice cold 10% (v/v) glycerol. The centrifuge and resuspension steps were repeated four times with decreasing volumes of ice cold 10% (v/v) glycerol (50 ml, 25 ml, 5 ml and 500 µl). Cells were separated into aliquots in pre-chilled microcentrifuge tubes. These cells were utilised in the transformation of plasmids or PCR products as per 2.5.3.

2.5.3. Transformation of competent cells

Electrocompetent or chemical competent cells (50 µl) were incubated with 50 ng of DNA in a pre-chilled microcentrifuge tube for 30 min on ice. DNA was transformed into chemical competent cells by heat shock, where cells were incubated in a 42°C water bath for 90 s, followed by a 5 min incubation on ice. In the case of electrocompetent cells, the sample was transferred to a pre-chilled 2 mm electroporation cuvette (Cell Projects) and electroporated at 2,200 V using an Eporator (Eppendorf). In both cases, 1 ml of SOC media was added to the samples and the cells were recovered in 15 ml centrifuge tubes at 37°C or where appropriate 30°C for 1 h with aeration. Following the recovery step, the cells were centrifuged at 4,000 x *g*, the supernatant was removed and the cell pellet was resuspended in 100 µl of LB. Transformants were isolated on LB agar plates supplemented with the appropriate antibiotic and the plates were incubated overnight at 37°C.

2.6. Transposon directed insertion-site sequencing (TraDIS) protocol

2.6.1. Construction of the library

A single colony of the mutant of interest was grown overnight at 37°C in 5 ml of 2xTY broth (16 *g* tryptone, 10 *g* yeast extract, 5 *g* NaCl in 1 litre of distilled water). In a 2 litre conical flask, the overnight was inoculated into 800 ml of fresh 2xTY broth and was grown at 37°C to an OD_{600} of 0.9 with aeration. The 800 ml of bacterial culture was separated into 50 ml falcons and incubated on ice for 30 min. Following the incubation period, the cells were centrifuged at 5,000 x *g* for 10 min at 4°C, the supernatant was removed and the cell pellet was gently resuspended in 50 ml ice-cold 10% (v/v) glycerol. The centrifugation step was repeated. However, the cell pellets were only resuspended in 25 ml of 10% (v/v) glycerol. Two of the 25 ml cultures were combined and the process of centrifugation and resuspension in 50 ml of 10% (v/v) glycerol, followed by 25 ml of 10% (v/v) glycerol was repeated until all of the samples were combined to produce one 50 ml culture resuspended with 10% (v/v) glycerol.

The centrifugation step was repeated one more time (5,000 x *g* for 10 min at 4°C), the supernatant was discarded and the bacterial pellet was resuspended in 1 ml 10% (v/v) glycerol. Aliquots of 200 µl cells were separated into 1.5 ml microcentrifuge tubes and 0.2 µl EZ-Tn*5*™ transposome (Epibio) was mixed with each cell aliquot. Following a 30 min incubation step on ice, samples were transferred to 2 mm gap electroporation cuvettes (Cell Projects Ltd.) and electroporated at 2.2 kv/2,200 V. Following electroporation, warm SOC media was added immediately and each sample was transferred to a 15 ml falcon. Samples were recovered for 2-4 h with aeration. Each aliquot was diluted with 5 ml of LB and 4-5 drops were spread onto LB agar plates supplemented with 50 µg/ml kanamycin, which allowed successful isolation of transformants. The plates were incubated overnight at 37°C.

The next day, the number of colonies were counted on three random plates and an average colony count was calculated. The cells were resuspended in LB and collected. The LB containing the resuspended colonies was mixed with 10% (v/v) glycerol in a 1:10 ratio. When ~1 million colonies were collected all of the transposon mutants were pooled and mixed thoroughly. The pooled library was separated into aliquots and stored at -80°C.

2.6.2. Preparation of TraDIS libraries for sequencing

Harvested cells were prepared for sequencing in a number of steps as outlined in figure 2.1. Using a STRATEC RTP bacteria DNA mini kit, genomic DNA was extracted according to the manufacturer's instructions. Using the Quibet method, DNA was quantified as outlined in section 2.4.2 and diluted in nuclease-free water (1 µg per 500 µl). Using a bioruptor (Diagenode), DNA samples were mechanically fragmented via ultrasonication. This program fragmented DNA to an average length of ~300 bp and consisted of 30 s of shearing followed by a 90 s break at low intensity. Following shearing, samples were condensed using a vacuum concentrator (Eppendorf, concentrator 5301) to a final volume of 55.5 µl.

The NEBNext Ultra 1 kit (New England Biolabs) was utilised to repair ends of fragmented DNA. The gDNA was then size selected (250 bp) using AMPure XP SPRI beads (Beckman Coulter), as per manufacturer's instructions. A PCR amplification step was introduced to enrich for DNA fragments that contained a transposon junction. The PCR reaction contained 25 µl of NEB Next Q5 Hot Start Hifi PCR master mix, 2.5 µl TKK_Forward primer (10 µM), 2.5 µl TKK_Reverse primer (10 µM), 15 µl sample and 5 µl nuclease-free water. The PCR cycling conditions were as shown in table 2.7.

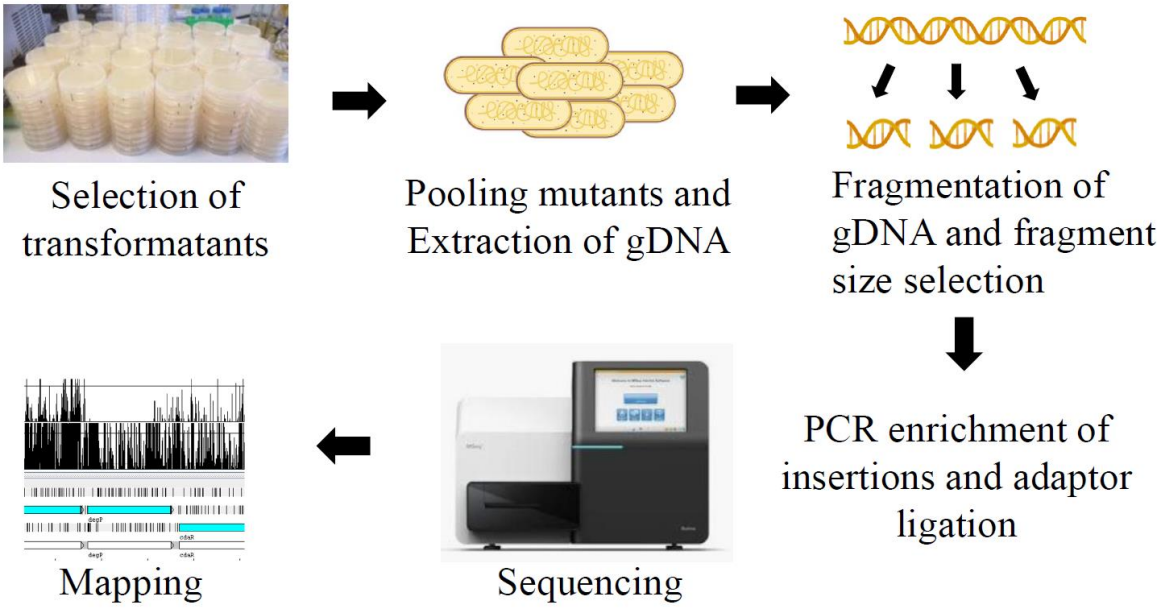


Fig. 2.1 A schematic of the TraDIS protocol. Transformants containing the min Tn*5* transposon were isolated on LB agar supplemented with kanamycin. Genomic DNA was extracted, pooled and fragmented. Fragments were size selection. Multiple PCRs enriched for DNA fragments that contained a transposon junction and added adaptors, Illumnia and inline index barcodes. Lastly, the samples were sequenced using Illumnia technology. The insertion site sequences were mapped to the BW25113 reference genome and were visualised by the Artemis genome browser.

Table 2.7 PCR cycling conditions for enrichment of transposon junctions

PCR cycling conditions			
Cycle step	**Temperature**	**Time**	**Cycles**
Initial Denaturation	98°C	48 s	1
Denaturation	98°C	15 s	10
Annealing	65°C	30 s	10
Extension	72°C	30 s	10
Final Extension	72°C	1 min	1
Hold	4°C	Infinity	--

The PCR product was purified using AMPure XP SPRI beads (Beckman Coulter) at a ratio of 0.9:1 beads to sample. Following purification, an additional PCR step prepared the sample for sequencing through the addition of sequencing specific p5 and p7 adapters and the addition of Illumnia and inline index barcodes (fig. 2.2B). The p5 and p7 adapter sequences are required for binding to the Illumina flow cell. The Illumina barcode is a distinct nucleotide sequence that identifies the dataset of interest from other TraDIS datasets. The inline index barcodes are custom-made barcodes of variable length that increase indexing capacity while staggering the introduction of the transposon sequence to increase base diversity during sequencing.

This PCR step consisted of 15 µl of adapted ligated DNA fragments, 25 µl of NEBNext Q5 Hot Start Hifi PCR master mix, 2.5 µl of a custom inline forward primer (TKK_X.Y, 10 µM), 2.5 µl of a NEB Next Illumnia index reverse primer (10 µM), made up to a total reaction volume of 50 µl with nuclease-free water. The PCR cycling conditions followed are outlined in table 2.7. Following which, the PCR product was purified using AMPure XP SPRI beads (Beckman Coulter) at a ratio of 0.9:1 beads to sample. Genomic DNA was eluted in 33 µl nuclease-free water and prepped libraries were stored at -20°C.

2.6.3. Quantification of prepped library samples prior to sequencing

Prior to sequencing, the concentration of prepped genomic libraries were determined using quantitative polymerase chain reaction (qPCR). The KAPA Library Quant Kit (Illumina) Universal qPCR Mix (Kapa Biosystems) was used as per kit instructions at half volume (10 µl) reactions. To obtain the most accurate readings, each library was quantified using three independent replicates, at two different dilutions: 1/50,000 and 1/500,000, alongside the six standards provided in the kit. Samples were quantified using an Mx3005P qPCR system (Agilent Technologies) and the thermal profile was followed as per table 2.8.

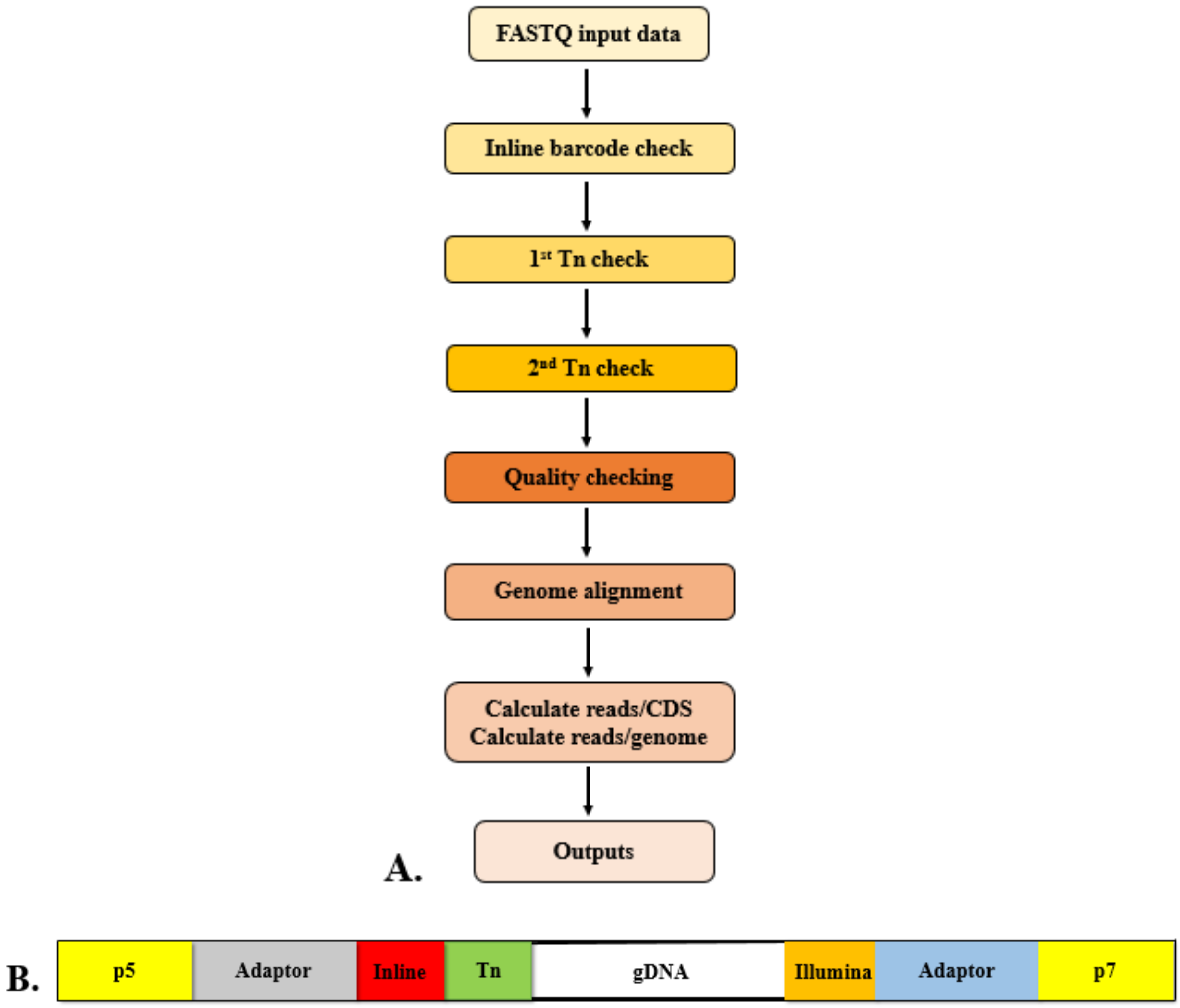


Fig. 2.2 Preparing and processing TraDIS data. (A) The TraDIS data processing pipeline. The input FASTQ files were processed using custom scripts. Data were separated based on inline and Illumnia barcodes and the presence of a transposon was checked in two steps. The reads were quality checked and then aligned to a reference genome. The number of unique insertion points were calculated per CDS/reads. Data that survives each process is passed onto the next stage, which leads to the production of output files: insertion_indices.csv; position_depth_count.txt; CDS insertion counts; and genome insertion summary. (B) Genomic DNA contains a transposon junction that is amplified (Tn shown in green). Ligated to the product is an inline index barcode (red) and an Illumnia index barcode (orange). Adaptors are also ligated (grey and blue), which act as sequencing priming sites. The p5 and p7 sequences (yellow) allow binding of the fragments to the flow cell.

Table 2.8 Thermal profile for quantifying library samples

Thermal profile for qPCR		
Temperature	**Time**	**Cycles**
95°C	5 min	1
95°C	30 s	35
60°C	30 s	35
72°C	30 s	35
95°C	30 s	1
65°C	30 s	1
95°C	30 s	1

2.6.4. MiSeq sequencing protocol

As previously discussed, qPCR was used to quantify the concentration of prepped libraries. The prepped libraries were then diluted to 8 nM stock concentrations and pooled in a nuclease free 1.5 ml microcentrifuge tube. The pooled amplified library was denatured and diluted with ultra-pure 10 mM Tris-Cl, pH 8.0. The sample was spiked with 5% denatured PhiX (20 pM), which acts as a positive sequencing control (Min Jou *et al.,* 1972; Sanger *et al.*, 1977). The sample was then loaded onto the MiSeq (Illumina) machine using a 150 cycle v3 cartridge. The optimal cluster density aimed for was 1,000 clusters per mm^2.

2.6.5. Data analysis

The output FASTQ files were processed in a number of steps as discussed in Goodall *et al.* (2018) (fig. 2.2A). Using FASTX barcode splitter and trimmer tools, the sequenced reads were trimmed and separated based on inline index barcodes. The accuracy of the transposon sequences were checked in two steps. The first ~25 bases were screened, allowing up to three nucleotide base mismatches and the last 10 bases of the transposon were checked, allowing up to one mismatch. Using Trimmomatic, transposon-containing sequences that were less than 20 bases in length were removed (Bolger *et al.,* 2014). Using the aligner bwa with the mem algorithm (0.7.8-r455 (Li and Durbin, 2009)), the surviving sequences were aligned to the *E. coli* BW25113 reference genome CP009273.1, available from NCBI (Tatusova *et al.,* 2014). SAMtools converted SAM (sequence alignment/map) files to BAM files (binary version of SAM), which sorted and indexed the data, making it easily accessible (Li *et al.,* 2009). The BEDtools suite was used to create BED (browser extensible data) files. These were intersected against the coding sequence boundaries defined in general feature format (.gff) files obtained from NCBI. Custom python scripts were used to quantify the location and frequency of transposon insertion sites and the data were manually inspected using

Artemis (Rutherford *et al.,* 2000). The output files included insertion_indices.csv, position_depth_count.txt, CDS insertion counts and genome insertion summary.

2.6.6. Essential gene prediction

The frequency of the insertion index scores (IIS) were plotted on a histogram using the Freedman-Diaconls rule for choice of bin widths. Using the R MASS library (http://www.r-project.org), an exponential distribution was fitted to the left i.e. the essential mode. A gamma distribution was fitted to the right, the non-essential mode. For a given IIS, the likelihood of a gene falling into one mode rather than another (essential or non-essential) was calculated. The ratio of these values was termed the log-likelihood score. A 12-fold likelihood threshold was used and based on the log-likelihood scores, genes were defined as essential if they were 12 more times likely to belong to the left mode than the right mode. A gene was deemed non-essential if it was 12 times more likely to belong to the right mode than the left mode. Genes were deemed as 'unclear' if the log likelihood score was between the upper and lower log2(12) threshold values of 3.6 and -3.6, respectively. This cut-off is consistent with analysis used by Goodall *et al.* (2018).

2.7. Condition screening of TraDIS libraries

The Δ*bamB*, Δ*bamE* and the parent TraDIS libraries were screened on inhibitory concentrations of vancomycin in an attempt to identify potential gene-deletion suppressors. Firstly, the parent and the Δ*bamB* and Δ*bamE* mutants were spot plated onto various concentrations of vancomycin as described in 2.8.1. to identify approximate inhibitory concentrations of vancomycin (MIC). The respective TraDIS libraries were diluted to an OD_{600} of 1.0 with LB and 50 µl of the diluted library was plated onto five square LB plates supplemented with a suitable concentration of vancomycin (2 x MIC or 1.5 x MIC). The plates were incubated overnight at 37°C. The following day, the suppressor colonies were

collected, pooled and supplemented with 10% (v/v) glycerol. Genomic DNA was extracted and the sample was processed as discussed in sections 2.6.2-2.6.6

2.8. Phenotypic Assay

2.8.1. 96-well plate growth kinetics

Growth kinetics were assessed in 96-well U-bottom plates (greiner) in a CLARIOstar 96-well plate reader (BMG Labtech). LB media (200 µl) was inoculated with overnight bacterial cultures to produce a starting OD_{600} of 0.02. Plates were incubated at 37°C with shaking and optical density readings were taken every 5 min for up to 24 h.

2.8.2. Microdilution spot plate

The overnight cultures were normalised to an OD_{600} of 0.1 using LB. In a 96-well microtiter plate, 200 µl of each culture was transferred to row A. Following which, 20 µl of row A and 180 µl of LB was transferred to row B. This was repeated at far as row H to produce a 10-fold serial dilution for each culture. Using a multi-channel pipette, 2 µl from each well was inoculated onto the LB agar plates supplemented with the appropriate antibiotic. The plates were incubated overnight at 37°C.

2.8.3. Membrane fluidity assay

Membrane fluidity was measured using the membrane fluidity kit (Abcam: ab189819) as previously described with minor modifications (Storek *et al.,* 2019). Bacterial strains were grown to log phase (~2 h) in LB medium. Following which, cells were harvested by centrifugation, washed with PBS and incubated with labelling mix (10 µM pyrenedecanoic acid (PDA), 0.08% pluronic F-127, in PBS) in the dark for 20 min at 25°C with rocking. The cells were washed twice in PBS and re-suspended in PBS. Fluorescence was recorded on a plate reader, with excitation at 350 nm and emission at 400 nm and 470 nm. By measuring the ratio of excimer (470 nm) to monomer (400 nm) fluorescence, membrane fluidity was

quantitatively measured. The emission spectra were compared to unlabelled cells to confirm membrane incorporation. Each triplicate experiment was repeated three times. Membrane fluidity of the mutants of interest were expressed relative to the parent *E. coli* strain.

2.8.4. OmpT in vivo folding assay

The OmpT in vivo assay for monitoring BAM activity was performed as described previously with minor modifications (Hagan *et al.,* 2010; Storek *et al.,* 2019; Iadanza *et al.,* 2016). Bacterial strains were grown to log phase (~2 h) in LB medium and were normalised to an OD_{600} of 0.2 in growth media. Bacterial cells (5 µl) were added to 95 µl of 25 µM fluorogenic peptide Abz-ARRAY(NO_2)-NH_2 diluted in PBS. Fluorescence emission was immediately measured at 430 nm following excitation at 325 nm over a period of 5 h with readings every 20 s.

2.9. Time lapse microscopy

Bacterial strains were grown to early log phase (~1 h). Microscope slides were prepared as per Jong *et al*. (2011) with some minor modifications. Agarose (1.5%) was dissolved in 30 ml of LB and 500 µl of LB/agarose was transferred to the centre of a gene frame (ABgene; 1.7 x 2.8 cm), placed on a glass slide (1 mm). A cover slide was placed on top of the gene frame (Abcam) and the slides were then incubated at RT for a minimum of 30 min. Following solidification of the agarose-LB (30-45 min), the top slide was removed and 2.5 µl of bacterial cells were spread onto the solid medium. A clean cover slip was added and using the 100 x objective, a Zeiss Axio Observer Z1 microscope with a pre-warmed incubator was used to capture the growth of cells for 2-4 h.

2.10. Lipopolysaccharide analysis

2.10.1. Aqueous phenol, chloroform and light petroleum extraction (PCP)

Lipopolysaccharide was extracted according to the PCP procedure (Galanos *et al.*, 1969). The bacterial strains were grown until stationary phase and the bacterial cell pellets were harvested by centrifugation. The pellets were re-suspended in PCP solution (90% aqueous phenol, chloroform and light petroleum in the proportion 2:5:8 (v/v/v), ~2.5%, w/v) and stirred at RT for 30 min. The suspension was homogenised with the Ultra-Turrax homogenisator for 2 min. Following which, the suspension was centrifuged and the supernatant collected.

The remainder of the previous bacterial pellet was mixed with the PCP solution (~2.5%, w/v) for 30 min. The centrifugation step was repeated and the supernatant was added to the supernatant previously extracted. Petroleum ether and chloroform was removed via rotary evaporation and the LPS was precipitated using water and left overnight. Both phases were separated by centrifugation, dialysed (until no phenol smell, approximately 48 h) and freeze-dried. The phenol supernatant was diluted ten times in water prior to dialysis.

2.10.2. SDS-PAGE analysis

Proteins samples were analysed on polyacrylamide gels (15% resolving gel with a 5% stacking gel with 1.5 mm thickness). The 15% resolving gel consisted of 3.6 ml water; 3.75 ml 1 M Tris-HCl, pH 8.8; 0.075 ml 20% (w/v) SDS; 7.5 ml Acrylamide/Bisacrylamide (30%/0.8% w/v, Protogel); 0.075 ml 10% (w/v) ammonium persulfate (APS); and 0.01 ml TEMED. The APS and TEMED was added last. The lipopolysaccharides were dissolved in sample buffer (62 mM Tris-HC1 of pH 6.9, 10% SDS, 1% mercaptoethanol, 20% glycerol) at 100°C for 5 min. About 8 μg of each sample was loaded onto the gel. The gels were run for 45 min at 200 V and then silver stained.

2.10.3. Silver staining

Firstly, the LPS samples were screened by SDS-PAGE (15%) as discussed in section 2.10.2 Following which, the LPS was visualised by silver staining (Kittelberg *et al*., 1993). All steps shown in table 2.9 were performed at RT and in 100 ml volumes per gel.

2.10.4. Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) was utilised for the analysis of monosaccharides and lipids. The fatty acids were recovered as ester derivatives after methanolysis by extracting the crude reaction with hexane (De Castro *et al.*, 2010). LPS samples extracted from PCP were dried over P_2O_5 overnight and treated with 1 M HCl/ CH_3OH (1 ml) at 80°C for 30 min. The crude reactions were extracted twice with hexane and the two extracts were pooled, dried under a stream of air and analysed by GC-MS. The remaining methanol phase after hexane extraction was dried and acetylated.

Monosaccharide composition was evaluated by analysing both the acetylated and methylglycoside derivates by GC-MS. GC-MS was analysed on Agilent Technologies 7820A (Santa Clara, CA, USA) equipped with a mass selective detector 5973N and a Zebron ZB-5 capillary column (Phenomenex, 30 m x 0.25 mm i.d., 0.25 μm as film thickness, flow rate 1 mL/min, He as carrier gas). Electron impact mass spectra were recorded with ionization energy of 70 eV and an ionizing current of 0.2 mA. The temperature program used consists of 150°C for 5 min, 150°C up to 300°C at 10°C/min, 300°C for 12 min.

2.10.5. Proton nuclear magnetic resonance (^{1}H NMR)

The saccharide portion of the LPS was extracted and profiled by proton nuclear magnetic resonance. The LPS (10 mg/ml) was treated with a mild acid treatment (1% Acetic acid (100°C for 2 h)) to cleave the core region of each LPS from the lipid A moiety. The oligosaccharides were separated from lipid A by centrifugation (10,000 x *g*). For structural assignment, one-dimensional ^{1}H NMR spectra were recorded using a solution consisting of

Table 2.9 Silver staining protocol

Fixation/oxidation		
fixation	fixative: 30% ethanol, 10% acetic acid	overnight
oxidation	0.7% periodic acid in fixing solution	10 min
wash 3x	ddH_2O	30 min
Silver staining		
silver	0.1% silver nitrate	30 min
wash 1x	ddH_2O	10 s
developer	3% sodium carbonate, 0.02% formaldehyde	Until sample develops (usually 5 min)
stop	1% acetic acid	5 min
wash 3x	ddH_2O	10 min
reduction	farmers reducer (0.3% sodium thiosulfate, 0.15% potassium ferricyanide, 0.05% sodium carbonate)	10-30 s. This step is only used if the developer is left too long to remove some of the colour.
wash 3x	ddH_2O	10 min
Recycling		
silver	0.1% silver nitrate	30 min
wash 1x	ddH_2O	10 s
developer	3% sodium carbonate, 0.02% formaldehyde	10 min
stop	1% acetic acid	5 min
wash 3x	ddH_2O	10 min
reduction	farmers reducer (0.3% sodium thiosulfate, 0.15% potassium ferricyanide, 0.05% sodium carbonate)	10-30 s
wash 2x	ddH_2O	10 min
store	7% acetic acid	

0.3 mg in 0.5 ml of D2O at 298 K and pD 7 (uncorrected value) with a Bruker 600 DRX spectrometer equipped with a cryoprobe. Spectra were calibrated with internal acetone (H 2.225, C 31.45).

2.11. Phospholipid techniques

2.11.1. Phospholipid extraction

Phospholipids were extracted using an adapted Bligh-Dyer method (Bligh and Dyer, 1959). Bacterial cultures were grown overnight and cells were harvested from centrifugation at 4,000 x *g* for 10 min. The bacterial cell pellets were normalised to OD_{600} of 3 in PBS. In glass tubes, the pellets were mixed with methanol and chloroform with a ratio of 2:1. The control consisted of 1.5 ml PBS with no bacteria. The samples were vortexed well and incubated at 50°C for 30 min. Additional chloroform and water was added to the samples to produce a final ratio of 2:2:1.8 of methanol:chloroform:water. The samples were centrifuged for 1000 rpm for 10 min. The bottom phase, which contained the phospholipids was transferred into a new glass tube using a glass pasteur pipette. The clean upper phase from the controls were added to the samples and vortexed well. The samples were centrifuged at 1000 rpm for 10 min. The phospholipid-containing bottom phase was collected and transferred into a new glass tube and stored at -20°C.

2.11.2. Thin layer chromatography

Using a glass capillary tube (Sigma Aldrich), 5-10 µl of a phospholipid sample was spotted onto a TLC silica gel membrane (Merck). Once dry, the membrane was exposed to a solvent system that consisted of chloroform, methanol and acetic acid with a ratio of 65:25:10, respectively. The samples were separated for ~20 min or until the solvent front had migrated up a sufficient level of the membrane. The membrane was removed and air-dried at RT. The

phospholipid membrane was stained with phosphomolybdic acid (PMA) and warmed with a heat gun until the lipid species were visible.

CHAPTER 3

Identification of conditionally essential genes in a Δ*bamB* mutant

3.1. Introduction

Essential genes are desirable targets for antimicrobial chemotherapy as they are highly conserved amongst strains. Therefore, many efforts have been made to define genes that are essential for bacterial growth and survival. Generally, two approaches have been used to identify essential genes: (1) targeted gene deletions, for example, the Keio collection of mutants (Baba *et al.,* 2006); and (2) random mutagenesis (Yamazaki *et al.,* 2008).

An essential gene can be defined as a gene that when deleted generates a mutant that is unable to survive and grow. Certain genes become essential under specific conditions, for example, upon exposure to an antibiotic or when another gene is deleted. These genes are considered to be conditionally essential. For example, the protease DegP is required for growth at high temperatures (Strauch *et al.,* 1989). Conditionally non-essential genes are normally required for growth, but become non-essential under the conditions imposed. For example, genes required for the survival of the parent strain that are dispensable in the mutant tested.

The β-barrel assembly machinery (BAM) consists of two essential subunits, BamA and BamD and three non-essential subunits: BamB, BamC and BamE. The BAM complex folds and inserts outer membrane proteins into the outer membrane. Without the BAM complex, β-barrels are not inserted, leading to membrane disruption and demise of the cell. Thus, an increase in the understanding of the BAM complex might help the identification of new antimicrobial targets. Many studies have highlighted the importance of the essential BAM components. However, there is a lack of understanding regarding the function of the non-essential BAM subunits, including BamB. Noinaj *et al*. (2011) suggested that BamB might act as a scaffold to the BAM complex. Another suggested function was that BamB might act in the early steps of OMP assembly. Recently, Gunasinghe *et al.* (2018) suggested that

regions form within the membrane that contain multiple BAM complexes and in the absence of BamB, these regions disperse, which suggests that BamB mediates the interactions between the BAM complexes.

In this study, transposon-directed insertion-site sequencing (TraDIS) was utilised to identify genes that are essential in an *E. coli* K-12 mutant defective in *bamB*. The data generated were compared to a TraDIS library of an isogenic parental strain to identify essential and non-essential protein coding sequnces specific to the Δ*bamB* mutant. Genes required for the survival of the Δ*bamB* mutant that are not required in the parent were identified and investigated in this chapter to determine the effect upon loss of BamB on outer membrane biogenesis. Synthetic lethal partners can be utilised in the identification of compounds that target non-essential proteins. For example, a compound that targets *bamB* could be identified in a Δ*degP* mutant as *degP* is required for the survival of a Δ*bamB* mutant.

3.2. Results

3.2.1. Construction and data analysis of the respective TraDIS libraries

In order to identify all genes that have a synthetic genetic interaction with *bamB*, TraDIS libraries were constructed in the parent and in a mutant devoid of *bamB*. The TraDIS method utilised in this study was based on *E. coli* studies such as Langridge *et al.* (2009). However, this method was modified by Goodall *et al.* (2018), where more stringent parameters were utilised in the classification of genes into essential, non-essential and unclear modes. This was consistent with analysis used by Phan *et al.* (2013). A mini-Tn*5* kanamycin transposon was transformed into electrocompetent BW25113 mutants lacking *bamB*. The transformants were isolated on LB medium supplemented with kanamycin and the bacterial colonies were collected and pooled. This was repeated until ~1 million mutants had been collected. Genomic DNA was extracted, fragmented and fragments were size selected. Using multiple

PCRs, the fragments that contained the transposon insertions were amplified and sequenced using Illumina technology.

Sequenced reads were filtered based on Illumnia and inline barcodes, allowing the separation of datasets and independent replicates. Only FASTQ data that contained an inline index barcode with no mismatches were included in the next step in the process. The barcode was trimmed using FASTX and all other data were removed. The presence of a transposon was checked in two steps in which the first 25 and the last 10 nucleotides were screened. The quality of the data were checked and poor quality short reads (<20 bp) were removed. Sequenced reads were then mapped to the *E. coli* BW25113 genome (CP009273.1). SAMtools converted SAM (sequence alignment/map) files to BAM files (binary version of SAM), which sorted and indexed the data to make it easily accessible. The BEDtools suite created the BED (browser extensible data) files, which were intersected against the coding sequence boundaries defined in general feature format (.gff) files obtained from the NCBI. Custom python scripts quantified the location and frequency of transposon insertion sites and lastly, using Artemis, the data were manually inspected.

3.2.2. Putative essential genes.

Genes were classified as either essential or non-essential depending on the number of transposon insertions in the protein coding sequence of the gene. However, the length of the gene needs to be taken into account. Consequently, the number of unique insertion points within the CDS was divided by the CDS length in bases. This value is referred to as the insertion index score (IIS) and acts as a measure of essentiality in many studies (Goodall *et al.,* 2018; Langridge *et al.,* 2009; Phan *et al.,* 2013). The frequency of insertion index scores were plotted on a histogram using the Freedman-Diaconis rule for choice of bin widths. This histogram was bimodal for the Δ*bamB* TraDIS dataset (fig. 3.1).

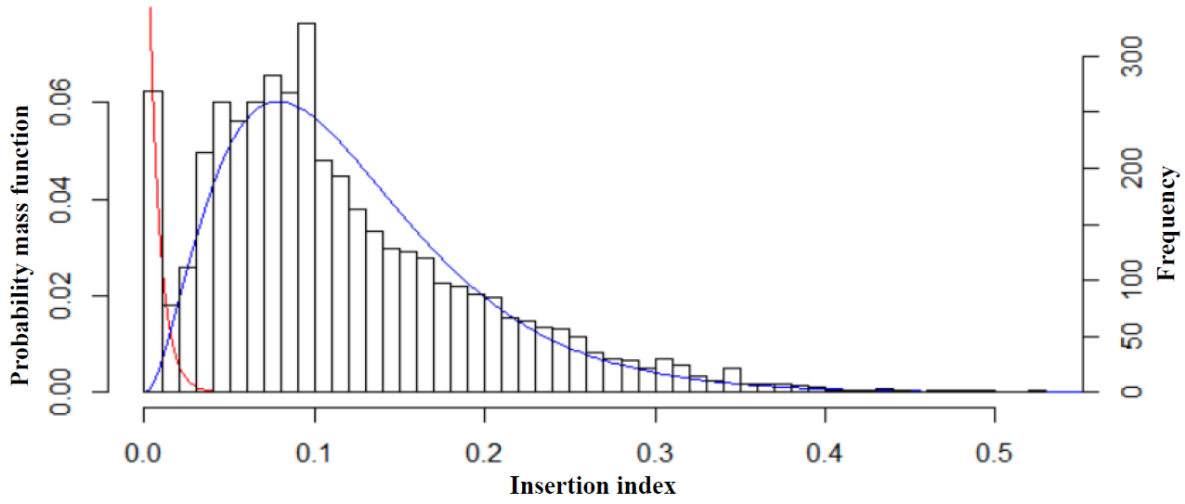


Fig. 3.1 Histogram illustrating the binomial frequency distribution of the genome-wide insertion sites of the Δ*bamB* TraDIS library. An exponential distribution model was fitted to the left (red). This is the essential mode and contains genes with low or no transposon insertions. A gamma distribution model was fitted to the right (blue), which is the non-essential mode. This mode contains genes with numerous transposon insertions.

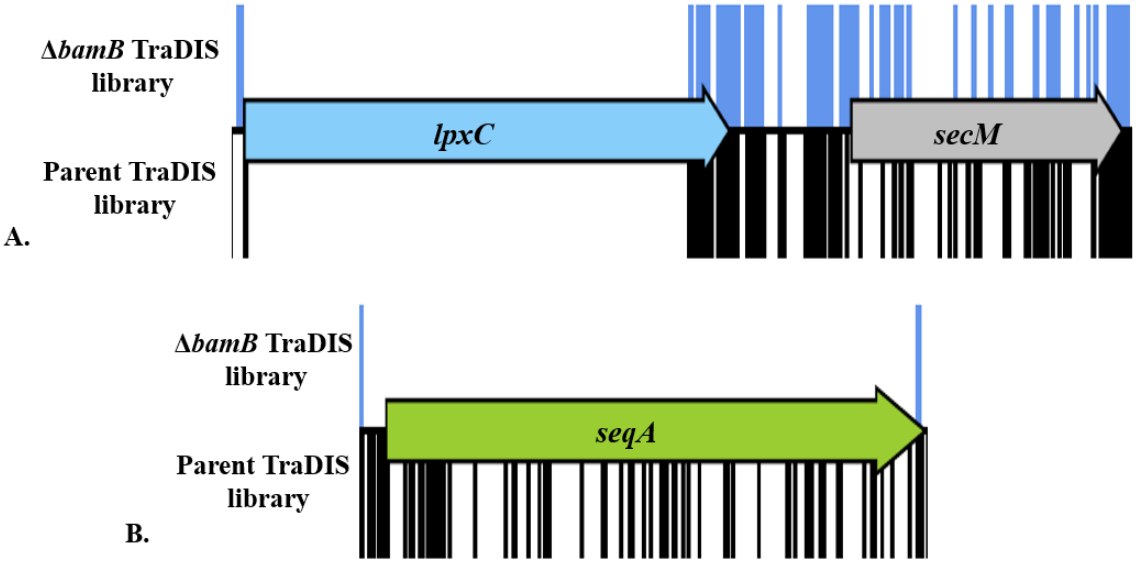


Fig. 3.2 An example of an essential, non-essential and conditionally essential gene. Transposon insertions in the parent and the Δ*bamB* TraDIS libraries are represented by black and blue lines, respectively. (A) No mutants were recovered with transposon insertion in the gene *lpxC*. As a result, *lpxC* was classified as essential in both TraDIS libraries. Conversely, numerous mutants with transposon insertions in the gene *secM* were recovered. Thus, this gene was classified as non-essential in both TraDIS libraries. (B) In the parent TraDIS library, mutants that contained transposon insertions in the gene *seqA* were viable. However, in the Δ*bamB* TraDIS library, mutants that contained transposon insertions in the gene *seqA* were not recovered. Thus, this gene is conditionally essential in a Δ*bamB* mutant.

The R MASS library (http://www.r-project.org) was used to fit an exponential distribution to the left, which contained genes with a low number of transposon insertions. These genes were deemed to be essential. A gamma distribution was fitted to the right, which contained genes with numerous transposon insertions. Genes in this mode are assumed to be non-essential. Figure 3.2 shows examples of the transposon insertion profiles of an essential gene, *lpxC*, a non-essential gene, *secM*, and a conditionally essential gene, *seqA*.

For a given IIS, the likelihood of a gene falling into one mode rather than another (essential or non-essential) was calculated. The ratio of these values was termed the log-likelihood score. A threshold cut off of log2(12) was used, which means, based on the log likelihood scores, genes classified as essential were 12 times more likely to belong to the left mode than the right mode. Similarly, genes classified as non-essential were 12 times more likely to belong to the non-essential mode than the essential mode. Genes with a log likelihood score between the upper and lower log2(12) threshold values, 3.6 and -3.6 respectively, were categorised as 'unclear'. This cut off is consistent with analysis used by Goodall *et al.* (2018).

3.2.3. The parent TraDIS library

The parent BW25113 kanamycin TraDIS library used in this study was constructed and sequenced by a previous student, Georgia Isom. This library is a highly saturated library comprised of ~1 million mutants. Data were checked for the presence of an inline index barcode, followed by the transposon sequence in two steps as previously discussed. The analysis identified over 5.6 million reads and 710,181 unique transposon insertion sites (UIS) (table 3.1), which equates to a transposon insertion site approximately every 6.5 bp throughout the genome. This library identified 341 essential genes and 3,719 non-essential genes. The mutant TraDIS libraries were compared to the parent TraDIS library to identify genes required for the survival of the mutant that were not required for survival of the parent.

Table 3.1 The number of reads at each stage of the data processing pipeline for technical replicates of the parent and Δ*bamB* TraDIS libraries

Library	Sample	Tn1 check	Tn2 check	Mapped reads	UIS
Δ*bamB*	Rep 1	9,551,607	9,400,448	6,486,647	471,758
	Rep 2.	3,108,259	2,882,945	2,180,697	316,563
	Combined	12,659,866	12,283,393	8,667,345	521,215
Parent	Rep 1	3,488,362	3,314,847	2,846,382	328,988
	Rep 2.	3,312,044	3,241,859	2,831,777	588,294
	Combined	6,556,706	6,079,630	5,678,155	710,181

3.2.4. Sequencing and comparison of independent replicates of the Δ*bamB* TraDIS library

For an internal measure of quality control, two replicates of each transposon library were sequenced. The gene insertion index scores were compared between each technical replicate to ensure there was a high correlation within datasets. The R-squared value acts as a measure of quality control. The closer this value is to one, the higher the similarity between the two replicates. The R-squared value for the Δ*bamB* TraDIS library was 0.95, indicating there is a high correlation between replicates (fig. 3.3). As a result, data from replicates were pooled.

3.2.5. Genome-wide transposon insertion sites

To quantify the transposon insertion density around the genome, the transposon insertion sites for the relevant TraDIS libraries were mapped to the reference *E. coli* BW25113 genome and visualised in a closed genome map (fig. 3.4). The transposon insertion sites were evenly mapped throughout the BW25113 genome, with the exception of an increased density occurring around the origin. A bias for transposon insertions around the origin was noted in figure 3.4. To further characterise this bias, the insertion index score of each coding sequence of the Δ*bamB* TraDIS library, was plotted in order of annotation (fig. 3.5). The plotted IISs followed a wave, with a peak at the origin of replication (around CDS 3750) and a dip at the genome terminus (around CDS 1600). Chao *et al.* (2016) and Goodall *et al.* (2018) also reported an increased transposon insertion bias at the origin. Thus, genomic position influences the insertion index scores of genes. These artefacts are due to incomplete rounds of DNA replication, where chromosome replication was initiated before completion of the previous replication event. Consequently, the copy number of genes in close proximity to the origin was higher than genes located near the terminus.

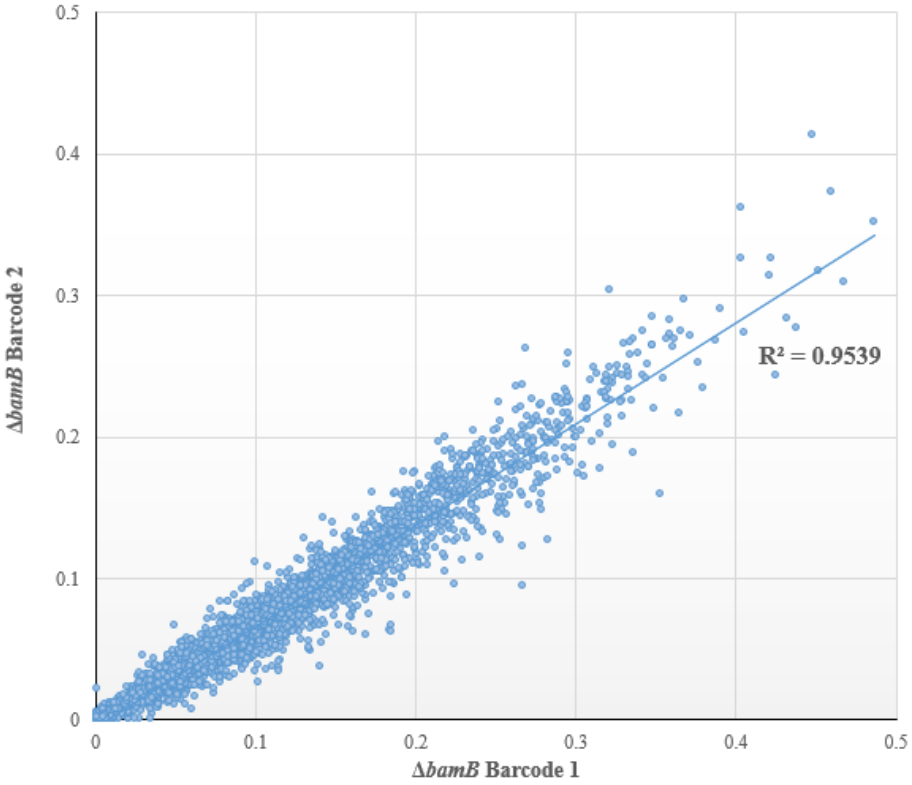


Fig. 3.3 Sequencing of two independent replicates. The correlation between the gene insertion index scores for sequenced replicates of the Δ*bamB* TraDIS library. The R-squared value for the Δ*bamB* TraDIS library was 0.95.

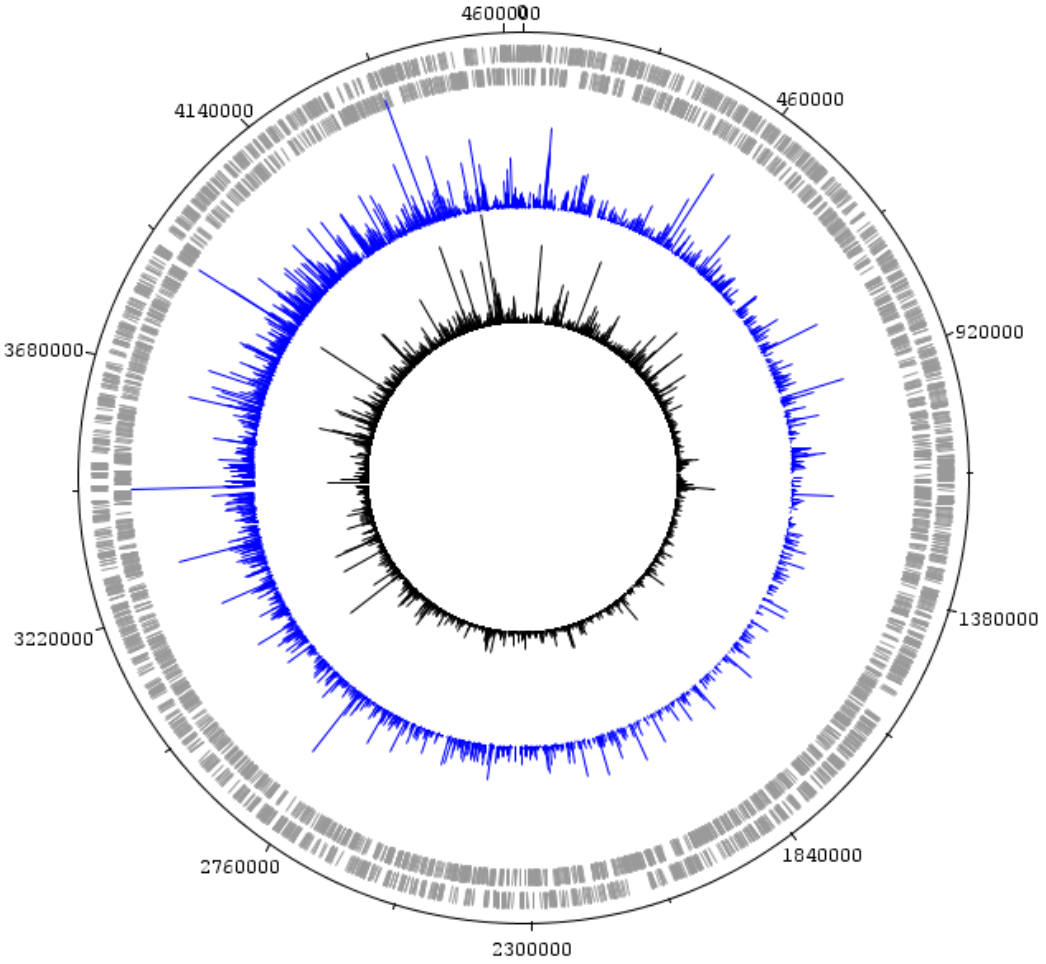


Fig. 3.4 Frequency and location of transposon insertion sites throughout the Δ*bamB* and the parent TraDIS libraries. The outer track marks the BW25113 genome in base pairs, starting at the annotation origin. The next two inner tracks correspond to sense and antisense CDS, respectively (grey). The two inner circles correspond to the frequency and location of transposon insertion sites in the Δ*bamB* TraDIS library (blue) and the parent TraDIS library (black), mapped to the BW25113 genome (CP009273.1). This figure was created using DNAPlotter.

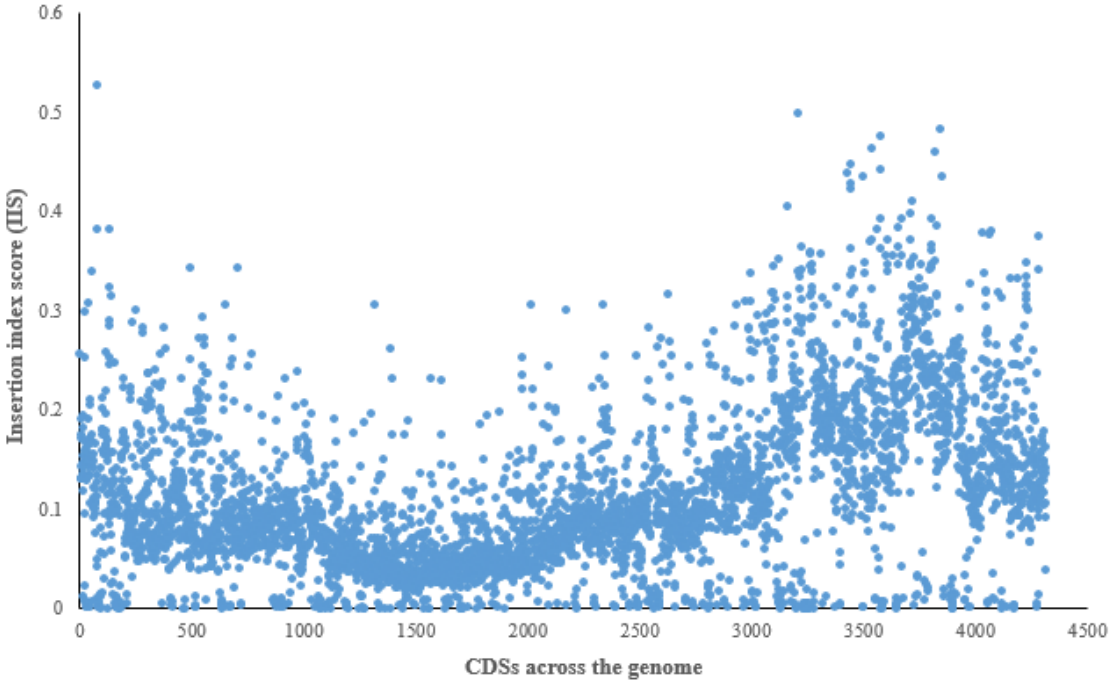


Fig. 3.5 The IISs of the relative CDSs across the genome of the Δ*bamB* TraDIS library.

The insertion index scores of the Δ*bamB* TraDIS library was ordered based on annotation (1-4313). This plot demonstrates that the insertion index scores of genes in the Δ*bamB* TraDIS library was influenced by genomic position.

3.2.6. Comparison of essential genes between the Δ*bamB* and the parent TraDIS library

The Δ*bamB* TraDIS library was a highly saturated library. It comprised of around 800,000 mutants and the analysis identified 521,215 unique transposon insertion sites and over 8 million reads. Data recovered are summarized in table 3.1. In the Δ*bamB* TraDIS library, a transposon insertion site occurred approximately every 8.9 bp throughout the genome. No insertions were found within the *bamB* gene, which indicated that a successful library had been constructed. In the Δ*bamB* TraDIS library, 316 genes were identified as essential. These were compared to the essential genes of the parent TraDIS library. Both datasets shared 287 essential genes, which were not investigated further (fig. 3.6). Using the IIS as a proxy for essentiality, twenty-nine genes were identified to be required for the survival of the Δ*bamB* mutant that were not required for the survival of the parent.

3.2.7. Manual inspection of the Δ*bamB* TraDIS dataset

Numerous experimental approaches are utilised to identify gene essentiality. Discrepancies are often found when lists of essential genes generated by different analytical procedures are compared, even for the same organism grown under similar conditions (Baba *et al.,* 2006; Gerdes *et al.,* 2006; Glass *et al.,* 2006; Winterberg *et al.,* 2005). These discrepancies can arise from various sources that include: small differences in growth conditions; use of different transposons with different insertion sequence bias; and the use of different statistical methods to analyse large datasets.

Overreliance on automated analysis of data can lead to over-estimation of the number of essential genes and/or failure to identify essential genes (Grenov and Gerdes 2008). Over reporting of essential genes can occur when the IIS of a non-essential gene falls below the statistical cut-off threshold. This can occur due to polarity effects, where mutants are not

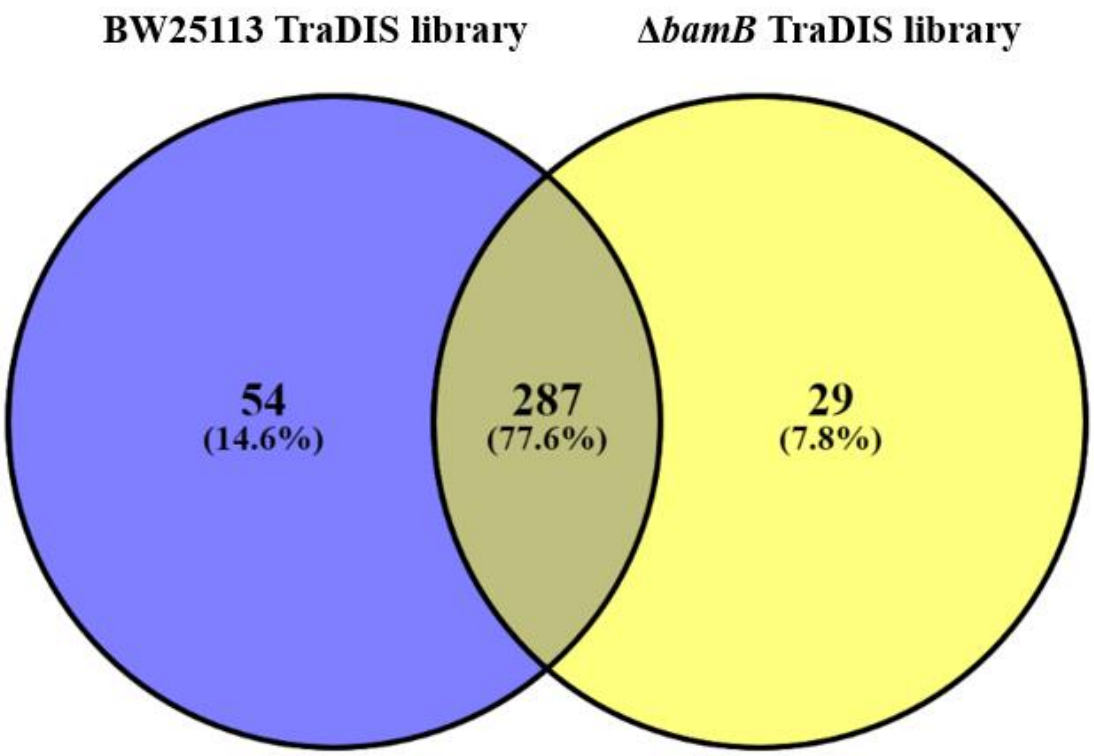


Fig. 3.6 Scaled Venn diagram illustrating the number of essential genes shared by the Δ*bamB* and parent TraDIS datasets. Both datasets shared 77.6% of the essential genes identified. The essential genes specific to the parent and the Δ*bamB* TraDIS library were 54 and 29, respectively.

Table 3.2 Essential genes required for the survival of the Δ*bamB* mutant that were not required for the survival of the parent

Name of gene	Function of gene
dapF	DapF catalyses the last step in the biosynthesis of lysine: the conversion of LL-diaminopimelate (LL-DAP) to *meso*-diaminopimelate (*meso*-DAP).
dnaK	A chaperone and heat shock protein involved in regulation of cell division, protein phosphorylation, modulation of proteolysis, survival of bacteria in different stress conditions and transport of proteins across the IM.
gmhA	The gene product of *gmhA* is sedoheptulose 7-phosphate isomerase, which catalyses the first step in the biosynthesis of heptose.
hda	Hda inhibits the reinitiation of DNA replication (Kato and Katayama, 2001).
holC	DNA polymerase III subunit **X.**
holD	DNA polymerase III subunit **Ψ.**
menI	1,4-dihydroxy-2-naphthoyl-CoA thioesterase enzyme is involved in the synthesis of menaquinone (Chen *et al*., 2013).
obgE	ObgE prevents the assembly of the intact ribosome and is involved in cell cycle progression and chromosome partitioning (Dutkiewicz *et al*., 2002; Feng *et al*., 2014; Foti *et al*., 2007).
pbl	Transglycosylase, remnant of a pathogenicity island (Ren *et al*., 2004).
priA	PriA aids the restart of stalled replication forks (Rangarajan *et al.,* 2002, Gregg *et al.,* 2002).
rlmE	Methyltransferase that elicits a role in the methylation of 23S rRNA (Caldas *et al.*, 2000).
rnpA	A component of ribonuclease P holoenzyme that processes tRNA precursor molecules and the stable 4.5 sRNA precursor (Chang and Carbon, 1975; Bothwell *et al*., 1976).
rpmF	A component of the 50S subunit of the ribosome.
secB	A cytoplasmic chaperone that forms part of the Sec pathway.
seqA	A negative regulator of replication initiation (Lu *et al.,* 1994).
yceD	Unknown function.
yceQ	Unknown function.

yciS (lapA)	Lipopolysaccharide assembly protein (LapA) might be involved in LPS transport (Klein *et al.*, 2014).
yddM	Putative DNA-binding transcriptional regulator (Gao *et al.*, 2018).
ydfB	Function unknown.
ydiE	Belongs to the Fur regulon (McHugh *et al*., 2003).
ygeF	Unknown function, remnant of an ETT2 (type III secretion system) pathogenicity island (Ren *et al*., 2004).
ygeG	Unknown function, remnant of a pathogenicity island of a type III secretion system (Ren *et al*., 2004).
ygeI	Function unknown, remnant of an ETT2 (type III secretion system) pathogenicity island (Ren *et al*., 2004).
ygeN	Function unknown, part of an operon that is a remnant of an ETT2 (type III secretion system) pathogenicity island.
yncJ	Function unknown.
ynhF	Function unknown.
yoaI	Function unknown.
zwf	The gene *zwf* encodes glucose-6-phosphate 1-dehydrogenase, which is involved in the pentose phosphate pathway.

*Where no reference was given, the functions were taken from the Ecocyc website: https://ecocyc.org/

recovered with transposon insertions in a non-essential gene because the gene is upstream of a co-transcribed essential gene. Alternatively, the gene might be located close to the replication terminus or the gene might be inaccessible to transposition because of extreme DNA structure (Goodall *et al.*, 2018). In addition, automated analysis can fail to identify essential regions within genes, such that loss of the gene is lethal but mutants can be recovered with transposon insertions in a region of the gene.

In this study, to minimize the possibility of incorrectly classifying genes as essential or non-essential, the insertion distribution in each gene was visually inspected, this is referred to as manual inspection. Manual inspection of the TraDIS dataset can reveal important information that might otherwise be misinterpreted or go unnoticed by reliance on the high throughput analysis pipeline.

Manual inspection allows the ability to determine, at the bp level, the boundaries of essential regions within a gene. Genes that contain both a transposon-free region and insertions in a non-essential region would be classified as non-essential by the statistical analysis. However, manual inspection would reveal that the gene is essential. An example was reported in Goodall *et al.* (2018), where no mutants with transposon insertions in the N-terminal domain of the gene *ftsK* were recovered. This region is required for localization of the protein to the septum. However, numerous mutants were recovered that contained transposon insertions in the non-essential region of this gene. This is consistent with previously published literature (Dubarry *et al.*, 2010; Wang *et al.*, 1998). In this current study, manual inspection revealed the gene *degP* contained an essential region in the Δ*bamB* TraDIS dataset (section 3.2.9).

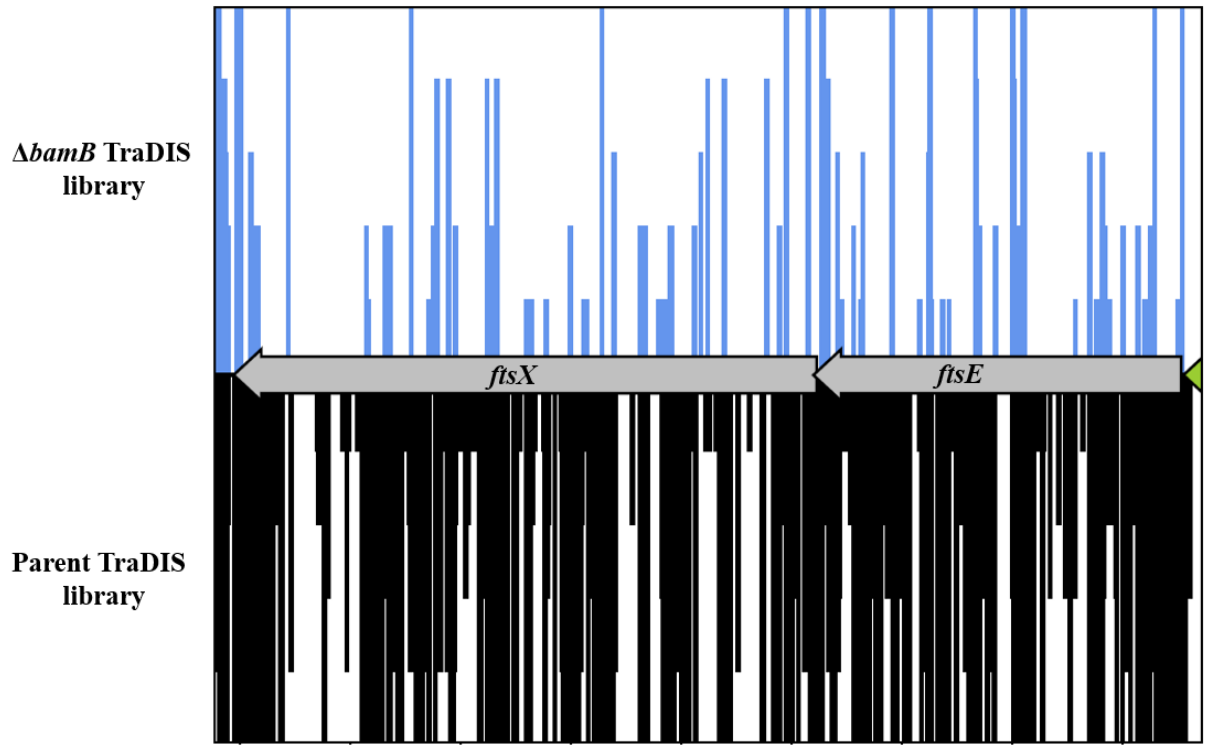


Fig. 3.7 Manual inspection of the data identifies genes that might be functionally important in the condition tested. In the Δ*bamB* TraDIS library, fewer mutants were recovered with transposon insertions in the genes *ftsX* and *ftsE*, compared to the parent counterpart. Blue lines (Δ*bamB* TraDIS library) and black lines (parent TraDIS library) represent transposon insertions. The longer the line, the higher the number of mutants recovered with transposon insertions in that location. A transposon cut-off point of 10 was utilised in the construction of this figure.

Manual inspection also identifies genes that contain sparse transposon insertions. The analysis pipeline fails to classify these genes as functionally important in the condition tested. For example, significant fewer mutants with transposon insertions in the genes *ftsX* and *ftsE* were recovered in the Δ*bamB* TraDIS library than in the parent TraDIS library (fig. 3.7). These mutants were outcompeted due to severe impairments on cell division and OMP biogenesis. FtsX and FtsE localize to the cell division site and are important for assembly and stability of the septal ring (Schmidt *et al.,* 2004). Thus, some non-essential genes involved in cell division are functionally more important in the Δ*bamB* mutant than in the parent strain.

Manual inspection of the TraDIS data prevents loss of information due to the limitations of the statistical analysis tools. In the Δ*bamB* TraDIS dataset, genes identified by manual inspection to have fewer transposon insertions than in the parent TraDIS dataset are listed in table 3.3. Both the 29 genes found to be synthetically lethal with a Δ*bamB* mutant via our automated bioinformatic pipeline and the 17 genes of interest observed by manual inspection were examined further.

3.2.8. Classification of synthetically lethal partners of BamB based on Gene Ontology

To further understand why the identified conditionally essential genes are required for the survival of the Δ*bamB* mutant, the genes were classified based on function and the literature was searched for explanations. The eggNOG database categorises each gene based on its function into one or more Gene Ontology (GO) classifications (Huerta-Cepas *et al.*, 2016). However, this database does not determine whether the GO categories identified are statistically more often in the input list than expected by chance.

Table 3.3 Genes identified by manual inspection of the data that are functionally important in the Δ*bamB* TraDIS library

Name of Gene	Function of gene
cydA	Subunit I of cytochrome *bd*-I terminal oxidase (Newton and Gennis, 1991).
cydB	Subunit II of cytochrome *bd*-I terminal oxidase (Newton and Gennis, 1991).
cydC	Component of a heterodimeric ABC transporter that is required for assembly of periplasmic cytochrome C and cytochrome Bd (Poole *et al.*, 1994).
cydD	Component of a heterodimeric ABC transporter that is required for assembly of periplasmic cytochrome C and cytochrome Bd (Poole *et al.*, 1994).
degP	Periplasmic protease that degrades damaged and aggregated proteins (Strauch *et al.*, 1988).
degS	High temperature requirement protease that senses and reacts to damaged and mislocalised proteins.
dnaT	DnaT is required for chromosomal DNA replication and for the induction of replication during the SOS bacterial stress response (Lark *et al.*, 1978; Masai *et al.*, 1986).
ftsE	Cell division protein important for assembly and stability of the septal ring (Schmidt *et al.*, 2004).
ftsX	Cell division protein important for assembly and stability of the septal ring (Schmidt *et al.*, 2004).
gpsA	Glycerol-3-phosphate dehydrogenase (GpsA) catalyses the reduction of dihydroxyacetone-phosphate to produce glycerol-3-phosphate, a precursor utilised in the biosynthesis of phospholipids (Hsu and Fox, 1970; Edgar and Bell, 1979).
hldE (*gmhC*)	HldE catalyses two reactions in the heptose biosynthesis pathway, a building block of LPS (Kneidinger *et al*., 2001).
lon	A cytoplasmic ATP-dependent protease that degrades misfolded proteins and key regulatory proteins (Laskowska *et al.*, 1996; Schoemaker *et al.*, 1984; Torres-Cabassa and Gottesman, 1987).
recA	RecA is a DNA recombination and repair protein that functions in homologous recombination.
rpmE	50S ribosomal subunit protein L31 that is involved in translation initiation, reading frame maintenance and formation of 100S ribosomes in stationary phase (Ueta *et al.*, 2017).

upp	Uracil phosphoribosyltransferase catalyses the synthesis of uridine 5'-monophosphate (UMP) from uracil and 5-phospho-α-D-ribose 1-diphosphate (PRPP).
waaC	WaaC transfers the first heptose sugar onto the Kdo_2 moiety of LPS (Gronow *et al*., 2000).
waaD (hldD)	WaaD catalyses the last step in the synthesis of heptose (Kneidinger *et al*., 2001).

*Where no reference was given, the functions were taken from the Ecocyc website: https://ecocyc.org/

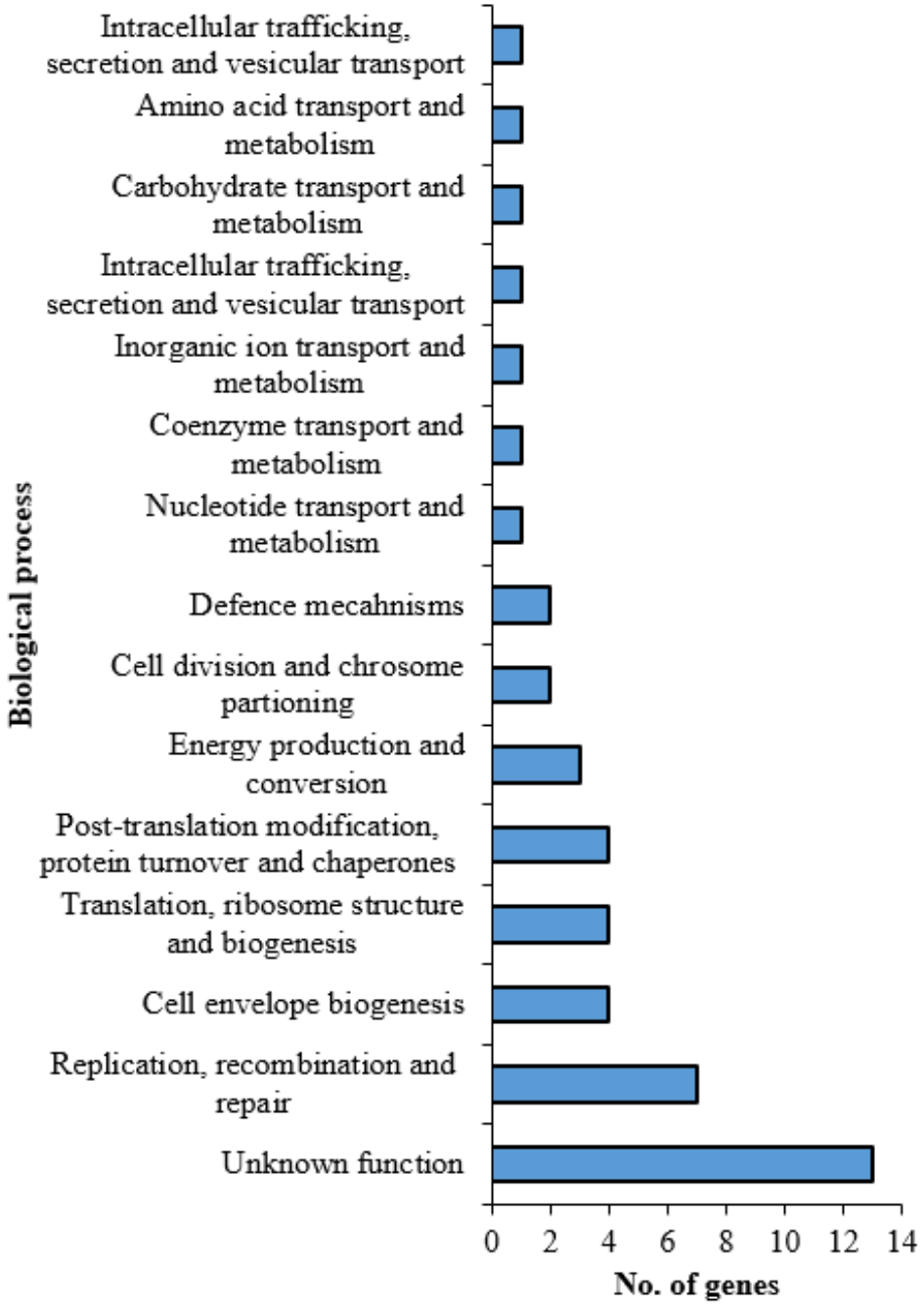


Fig. 3.8 Functional classification of the genes of interest identified in the Δ*bamB* TraDIS dataset. Genes that were deemed to be functionally important in the Δ*bamB* mutant were grouped into GO categories: 13 were genes of unknown function; 7 were involved in replication, recombination and repair; and 4 were involved in cell envelope biogenesis. The eggNOG database did not determine whether the GO categories identified were significantly enriched.

Genes from the Δ*bamB* TraDIS dataset that were classified as functionally important were processed using the eggNOG database, to quantify the proportion of genes that code for products in a specific pathway or cellular process (fig. 3.8). Many of these genes were genes of unknown functions (13 genes), while the second largest GO category was 'replication, recombination and repair,' which contained seven genes. The other GO categories that were functionally more important in the Δ*bamB* mutant than in the parent strain were 'cell envelope biogenesis;' 'translation, ribosome structure and biogenesis;' 'post-translational modification, protein turnover and chaperones;' 'energy production and conversion;' 'cell division and chromosome partitioning;' and 'defence mechanism' (fig. 3.8). The GO categories that contained one gene each were: 'nucleotide transport and metabolism;' 'coenzyme transport and metabolism;' 'inorganic ion transport and metabolism;' 'intracellular trafficking, secretion and vesicular transport;' 'carbohydrate transport and metabolism;' 'amino acid transport and metabolism;' and 'intracellular trafficking, secretion and vesicular transport.'

3.2.9. The gene *degP*, a synthetic lethal partner of *bamB*

SurA, Skp and DegP are three known chaperones that deliver OMPs to the BAM complex. Viable knockouts include single mutants Δ*surA*, Δ*skp*, Δ*degP* and the double mutant Δ*skp*Δ*degP*. The double mutants Δ*surA*Δ*skp* and Δ*surA*Δ*degP* are synthetically lethal. Consequently, Sklar *et al.* (2007) proposed that these three chaperones make up two parallel pathways of OMP transport, where SurA functions in one pathway and the combination of Skp and DegP functions in another pathway. DegP elicits two main functions: a chaperone function and a protease function, switching between these functions in a temperature-dependent manner (Spiess, *et al*., 1999). In the Δ*bamB* TraDIS dataset, a conditionally essential region was identified in the gene *degP*. In contrast, the *skp* gene was not conditionally essential in the Δ*bamB* TraDIS library. Thus, the importance of DegP might

be due to its protease function as both Skp and DegP have chaperone functions, while only DegP elicits a protease function.

The DegP monomer (1425 bp / 474 a.a.) consists of an N-terminal protease domain (residues 1–259) and two PDZ domains (PDZ1, residues 260–358; PDZ2, residues 359–448) (fig. 3.9) (Krojer, *et al*., 2002). Monomers of DegP associate to form a hexameric complex consisting of a staggered dimer of trimers. Hexamer formation requires side-by-side interactions between the PDZ1-PDZ2 domains (Jomaa, *et al*., 2007; Iwanczyk, *et al*., 2007; Meltzer, *et al.,* 2008). In the Δ*bamB* TraDIS dataset, mutants with transposon insertions in the PDZ2 domain of *degP* were viable (fig. 3.9). Although devoid in the hexameric configuration of DegP, these mutants would still be able to form DegP trimers, allowing DegP to function as both a protease and a chaperone (Jomaa, *et al.,* 2007).

The PDZ1 domain is not required for the chaperone function of DegP. However, it is required for the protease activity of DegP, by binding to unfolded proteins and anchoring the substrate, facilitating its presentation to the proteolytic domain (Iwanczyk, *et al.*, 2007). In the Δ*bamB* TraDIS dataset, no mutants with transposon insertions in the PDZ1 domain were recovered, which implies that the protease function of DegP is required for the survival of the Δ*bamB* mutant. Charlson *et al.* (2006) has confirmed the synthetic lethality between *degP* and *bamB*. Loss of BamB results in decreased levels of OMPs in the OM, while loss of DegP leads to the accumulation of misfolded or mislocalised OMPs (Krojer *et al.,* 2008; Wu *et al*., 2005). Therefore, these results support the hypothesis that DegP is required in a Δ*bamB* mutant to degrade the mislocalized and unfolded OMPs that accumulate. Loss of both proteins leads to toxic OMP aggregation and eventually cell death. In summary, there is a region of *degP* that is conditionally essential in a Δ*bamB* mutant. Without visually inspecting the data, this information would have been lost.

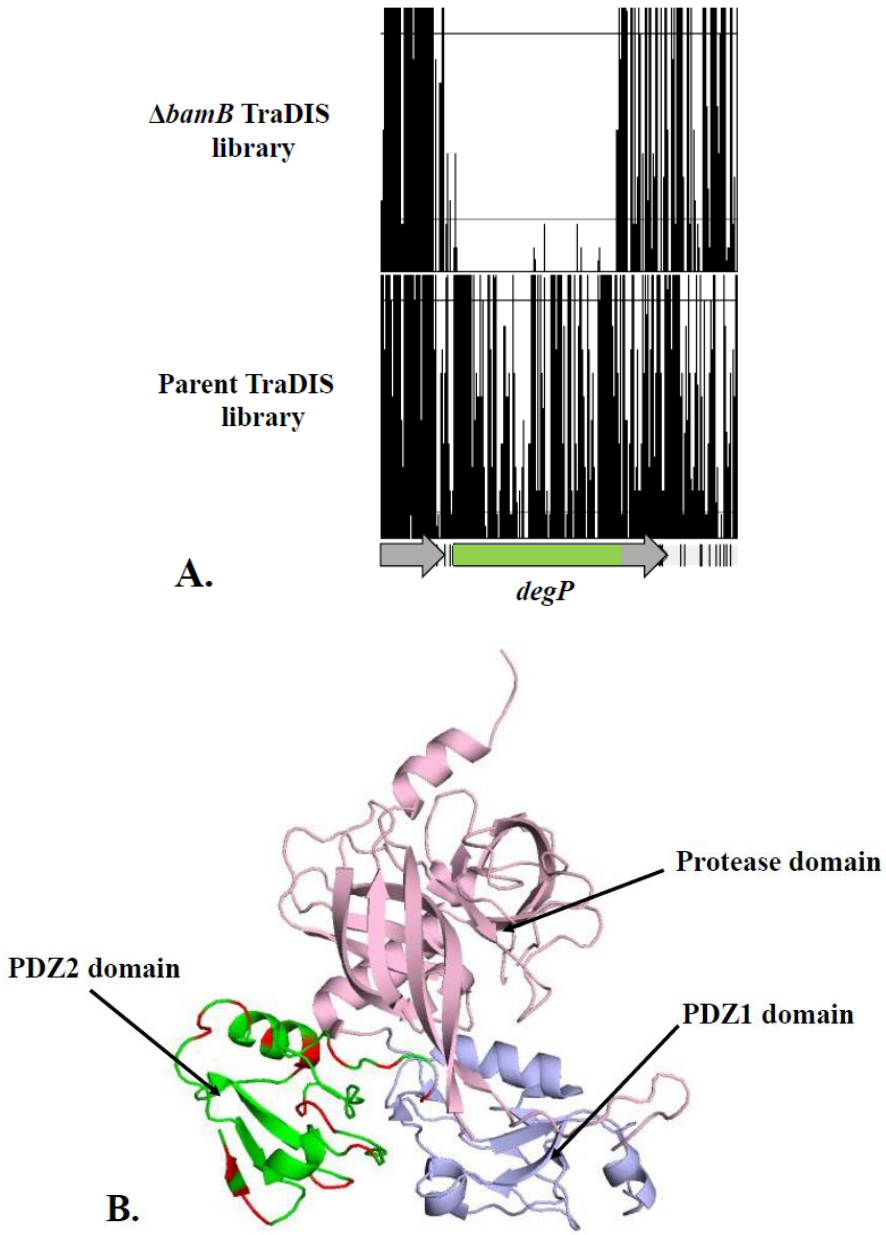


Fig. 3.9 The region in *degP* that is conditionally essential in a Δ*bamB* mutant. (A) In the Δ*bamB* TraDIS library, only mutants with transposon insertions in the C-terminus of *degP* were viable, while in the parent library, mutants were recovered with transposon insertions throughout the length of the gene. (B) In the Δ*bamB* TraDIS dataset, the transposon insertions identified in the *degP* gene were translated onto the crystal structure of a DegP monomer (1KY9), which consists of a protease domain (pink), PDZ1 domain (light blue) and a PDZ2 domain (green) (Krojer, *et al*., 2002). The transposon insertions sites (shown in red) all mapped to the PDZ2 domain.

3.2.10. The role of post-translational modification, protein turnover and chaperones in a Δ*bamB* mutant

In addition to *degP*, a further three genes of interest were assigned to the GO category 'post-translation modification, turnover and chaperones.' In the Δ*bamB* TraDIS library, there were fewer mutants recovered with transposon insertions in the genes *degS* and *lon* than in the parent TraDIS library. The gene *dnaK* was identified as conditionally essential. DegS is a membrane anchored protease, which alongside RseP cleaves RseA under stress conditions to ultimately activate the σ^E stress response (Kanehara *et al*., 2002, Alba *et al*., 2002). Lon is a cytosolic protease that degrades misfolded proteins and a number of regulatory proteins, such as the cell division regulator SulA and the capsule synthesis regulator RcsA (Laskowska *et al.*, 1996; Schoemaker *et al.*, 1984; Torres-Cabassa and Gottesman, 1987). DnaK is a heat shock chaperone that is involved in a number of cytoplasmic cellular processes including rescue of misfolded proteins (Deuerling *et al.,* 1999; Schroder *et al.,* 1993; Skowyra *et al.,* 1990; Teter *et al.,* 1999). Loss of periplasmic proteases such as DegS leads to the toxic aggregation of proteins in the periplasm. Loss of proteases/chaperones such as Lon and DnaK leads to protein aggregation in the cytoplasm, which has downstream negative effects on protein aggregation in the periplasm. Both are unfavourable to a Δ*bamB* mutant. Although the importance of DegP is more direct, as it is a member of the BAM pathway, these proteins are also functionally important to a Δ*bamB* mutant.

3.2.11. Replication, recombination and repair

In the Δ*bamB* dataset, seven out of the 46 genes of interest were assigned to the GO category 'replication, recombination and repair,' which includes: *seqA*; *recA*; *priA*; *holC/D*; *hda*; and *dnaT* (fig. 3.10A). RecA and PriA are involved in repair of double-stranded DNA (dsDNA) breaks. HolC and HolD are subunits of DNA polymerase III. SeqA and Hda are both negative regulators of replication initiation (Lu *et al.,* 1994; Kato and Katayama, 2001).

Breaks in dsDNA can occur, for example, due to exposure to chemical agents or ionizing radiation. This generates a requirement for replication fork restart (Kowalczykowski, 2000). Homologous recombination is one of the major mechanisms of dsDNA break repair, which can proceed via one of two recombination pathways. Both pathways require RecBCD and RecA, but one is RuvABC dependent, while the other relies on RecG (Meddows *et al.*, 2004).

In the Δ*bamB* TraDIS library, there was a slight decrease in the number of mutants recovered with transposon insertion in the genes *recA, recB* and *recC* than in the parent TraDIS library (fig. 3.10B). RecB, RecC and RecD form a complex that recognises and promotes the repair of breaks in dsDNA. The enzyme unwinds the DNA until it encounters a χ sequence (5′-GCTGGTGG-3′). It then cuts the χ containing strand, generating single stranded DNA (ssDNA) (Ponticelli *et al.*, 1985).

RecA is recruited and loaded onto the ssDNA by RecB. RecA catalyses the formation a D-loop between the homologous dsDNA donor and the recipient strand (fig. 3.10C). The D-loop is recognized by PriA, which directs assembly of the replication restart primosome, recruiting PriB, DnaT, DnaB, DnaC and DnaG, which together transfers DnaB to the lagging-strand template. This results in synthesis of the lagging-strand and restart of the stalled replication fork (Marians, 2000). In the Δ*bamB* TraDIS library, the genes, *priA* and *dnaT* were identified as conditionally essential, compared to the parent TraDIS library. These results suggests that certain genes involved in recombination and repair are functionally more important in the Δ*bamB* mutant than in the parent strain. This is the first report of a functional connection between the BAM pathway and recombination and repair.

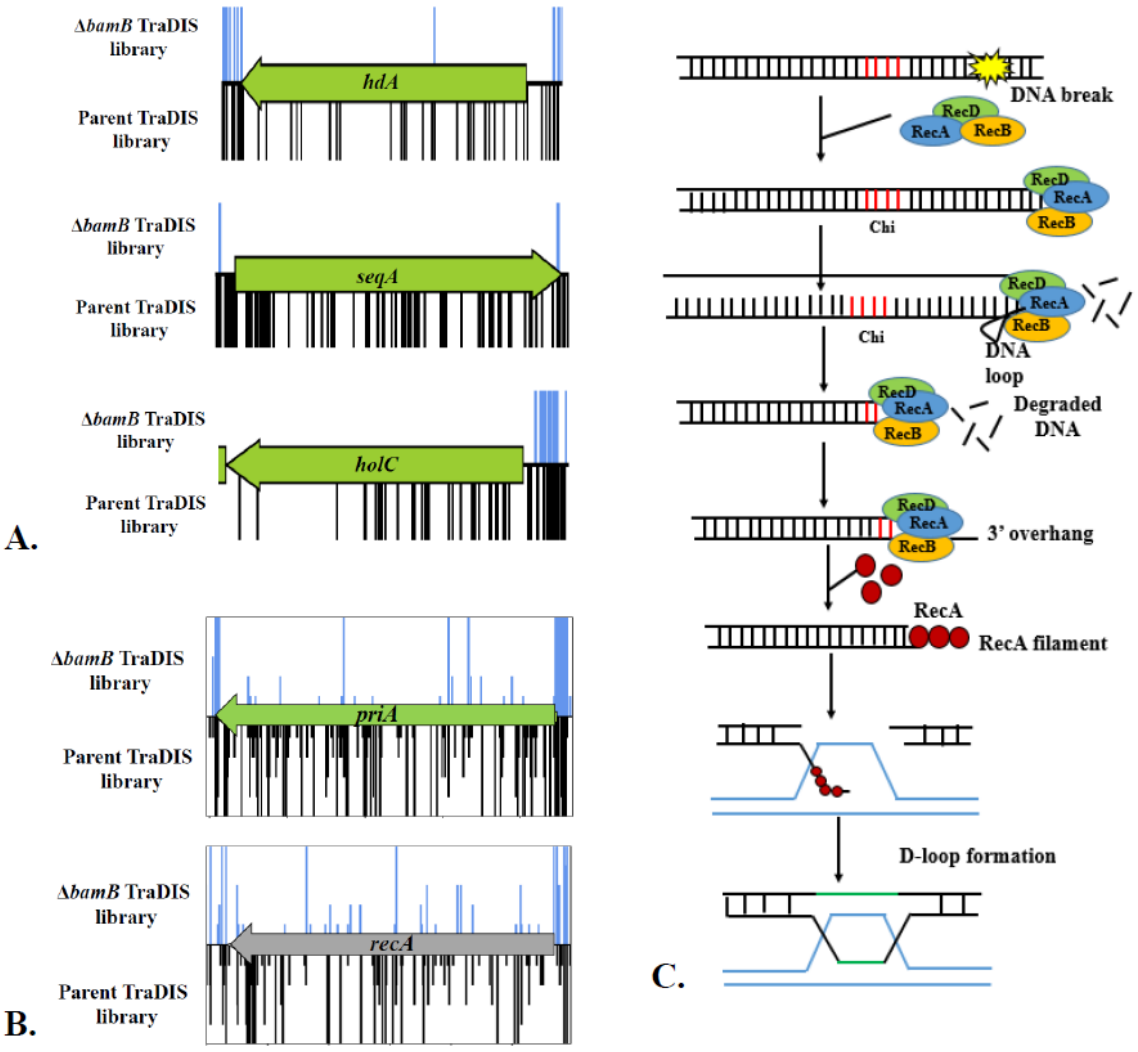


Fig. 3.10 Double-stranded DNA break repair. (A-B) In Δ*bamB* TraDIS library, *priA*, *hdA*, *seqA*, and *holC* were identified as conditionally essential. In the Δ*bamB* TraDIS library, less mutants were recovered with transposon insertions in the gene *recA* than in the parent TraDIS library. The relative transposon cut off for A and B was one and ten, respectively. (C) The RecBCD complex recognises dsDNA breaks and unwinds the DNA until the χ site is reached. The complex then cuts the strand, creating an overhang, which recuits RecA. A D-loop is formed between the donor and the recipient stands. PriA recognises the D-loop and recruits PriB, DnaT, DnaB, DnaC and DnaG, resulting in the downstream restart of the stalled replication fork.

3.2.12. The coordination of lipopolysaccharide synthesis with OMP biogenesis

In the Δ*bamB* TraDIS dataset, of the 46 genes identified as functionally important, a number of these genes elicited roles in cell envelope biogenesis. More specifically, these genes were involved in the synthesis of lipopolysaccharide (LPS). Although this chapter focuses on the Δ*bamB* TraDIS library, the results regarding LPS will be discussed in detail in chapter six. Fewer mutants with transposon insertions in LPS assembly genes were recovered in the Δ*bamB* TraDIS library than in the parent TraDIS library.

The synthesis of heptose was functionally more important in the Δ*bamB* mutant than in the parent strain. Heptose is a core component of LPS and its synthesis consists of five main steps (fig. 3.11A). Sedoheptulose 7-phosphate isomerase, the gene product of *gmhA*, converts D-sedoheptulose 7-phosphate to D-*glycero*-D-*manno*-heptose 7-phosphate, which is then phosphorylated by the N-terminal kinase of HldE to produce D-*glycero*-D-*manno*-heptose-1β, 7-phosphate (Kneidinger *et al.*, 2001; McArthur *et al.*, 2005). GmhB removes the C(7) phosphate group to form D-*glycero*-D-*manno*-heptose-1β-phosphate and the C-terminal domain of HldE catalyses the transfer of AMP from ATP to the C(1) phosphate, leading to the formation of D-*glycero*-D-*manno*-heptose-1β-phosphate-APP. Lastly, WaaD catalyses epimerization of D-*glycero*-D-*manno*-heptose-1β-phosphate-APP to the L-*glycero* epimer (Raetz *et al.*, 2009).

In the Δ*bamB* TraDIS library, *gmhA* was classified as conditionally essential, due to the requirement of this gene for heptose production. Similarly, the number of mutants recovered with transposon insertions in *hldE* was lower than in the parent (fig. 3.11B). Valvano *et al.* (2000) demonstrated that a transposon insertion in the *hldE* gene produced a mutant with a heptoseless LPS phenotype. Thus, *hldE* is functionally more important in a Δ*bamB* mutant

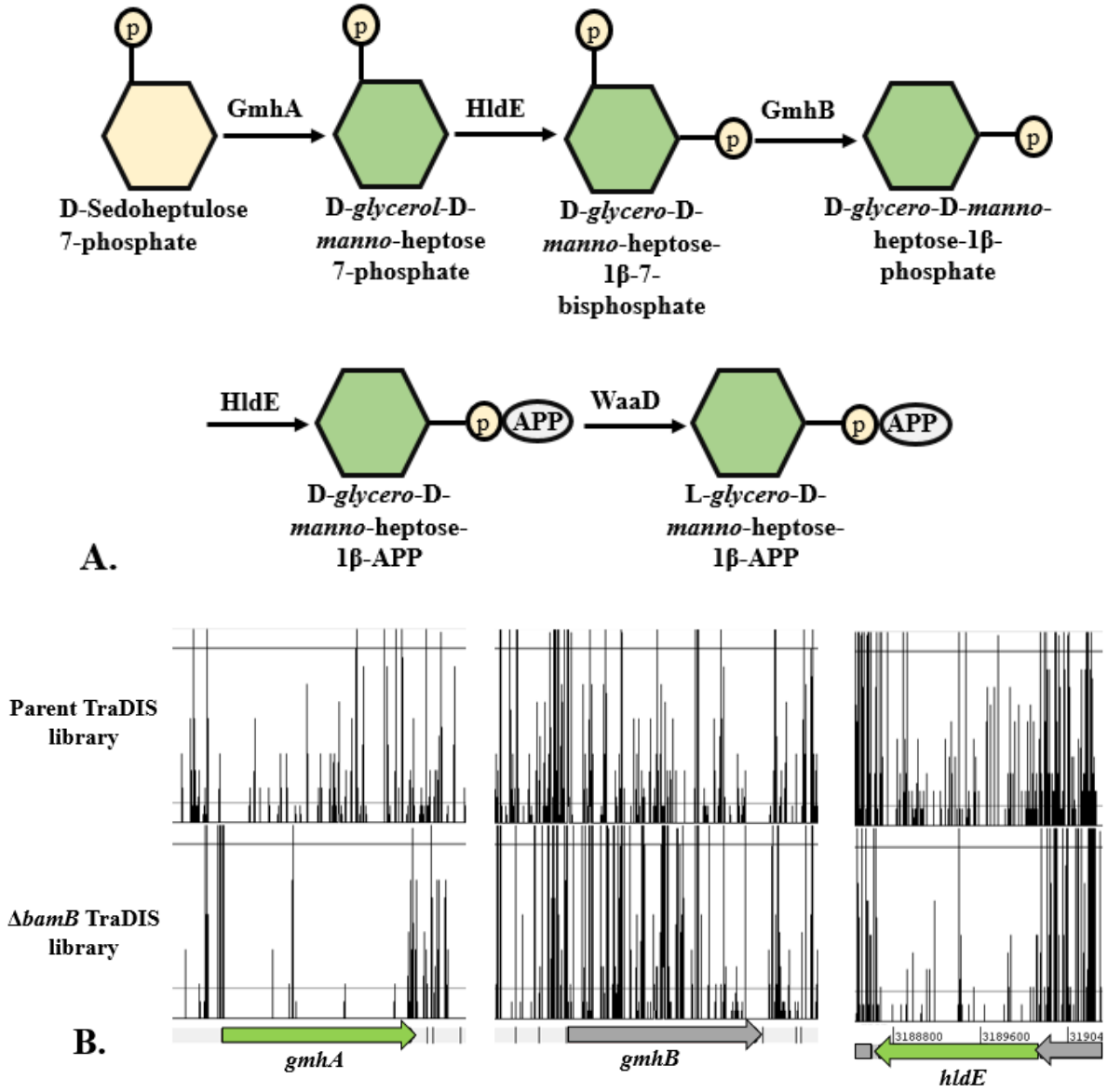


Fig. 3.11 The importance of the synthesis of heptose in a Δ*bamB* mutant. (A) The synthesis of heptose consists of the conversion of D-sedoheptulose 7-phosphate to L-*glycero*-D-*manno*-heptose-1β-APP. (B) Transposon insertions in the genes *gmhA*, *gmhB* and *hldE* in the parent TraDIS library and the Δ*bamB* TraDIS library. The transposon cut-off was set to 10. In the Δ*bamB* TraDIS library, fewer mutants were recovered with a transposon insertion in *gmhA* and *hldE* than in the parent TraDIS library.

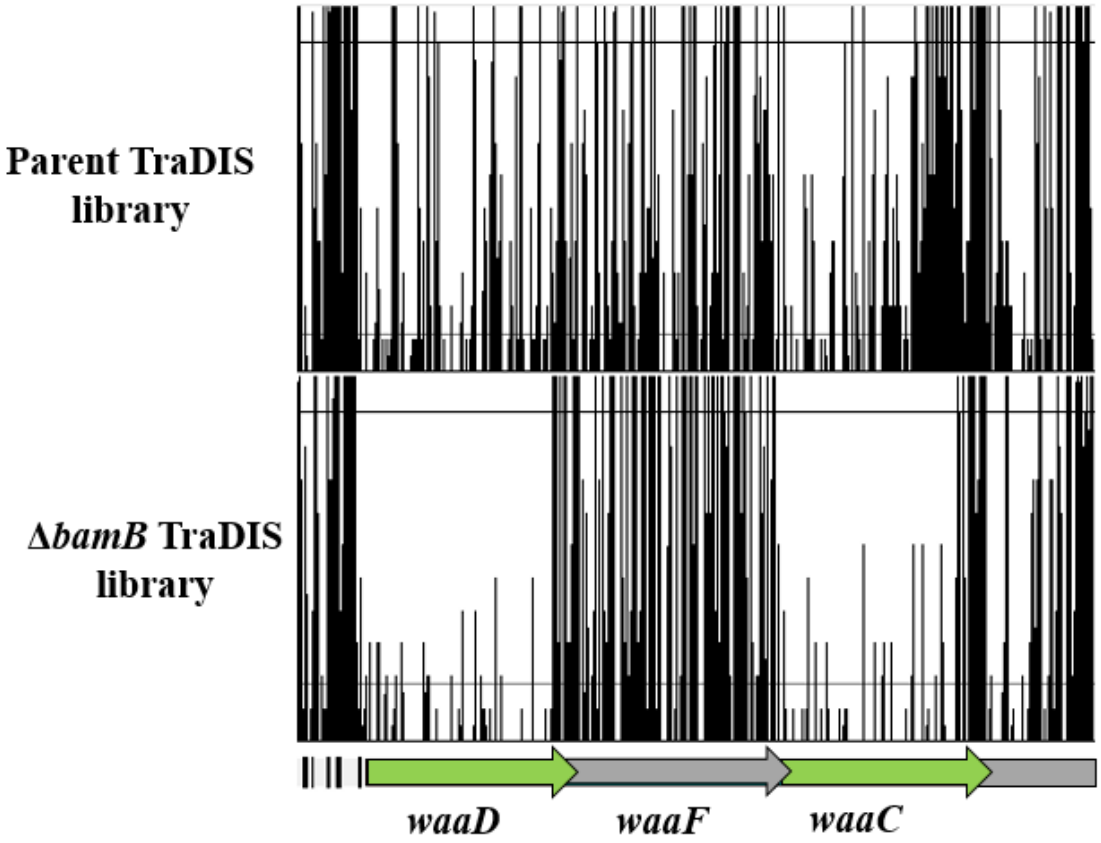


Fig. 3.12 The importance of the incorporation of heptose in a Δ*bamB* mutant. Transposon insertions into the genes *waaD*, *waaF*, and *waaC* in the parent TraDIS library and the Δ*bamB* TraDIS library. The transposon cut-off was set to 10. In the Δ*bamB* TraDIS library, fewer mutants were recovered with a transposon insertion in *waaD* and *waaC* than in the parent TraDIS library. WaaC transfers the first heptose sugar onto the Kdo_2 moiety of the LPS inner core.

than in the parent due to its key role in heptose synthesis. Conversely, the gene *gmhB* was classified as non-essential in the Δ*bamB* mutant. A Δ*gmhB* mutant is partially defective in LPS core synthesis, but is not completely devoid of heptose (Kneidinger *et al.,* 2001). Thus, the Δ*bamB* mutant can survive without *gmhB*.

Heptose production does not require WaaD. In a Δ*waaD* mutant, the stereoisomer D-*glycero*-D-*manno*-heptose-1β-phosphate-APP acts as a substrate for LPS assembly instead of the usual end product L-*glycero*-D-*manno*-heptose-1β-phosphate-APP. The catalytic efficiency with the stereoisomer as the substrate is only 10% that of L-*glycero*-D-*manno*-heptose (Gronow and Brade, 2001). In the Δ*bamB* TraDIS library, fewer mutants were recovered with a transposon insertion in *waaD* than in the parent (fig. 3.12). Interestingly, Storek *et al.* (2019) demonstrated that *waaD* and *bamB* are synthetically lethal. Therefore, the Δ*bamB* mutant requires the higher catalytic efficiency that occurs with L-*glycero*-D-*manno*-heptose.

The incorporation of heptose into the LPS structure was also functionally important. In the Δ*bamB* mutant, fewer mutants were recovered with transposon insertions in *waaC* than in the parent (fig. 3.12). WaaC transfers the first heptose sugar onto the Kdo_2 moiety of the LPS inner core. WaaF and WaaQ add the second and third heptoses, respectively. Other modifications occur, including the addition of phosphates to heptose I and heptose II by WaaP and WaaY, respectively (Gronow and Brade, 2001). The genes *waaP*, *waaY* and *waaF* were shown not to be functionally important in the Δ*bamB* mutant. In summary, in a Δ*bamB* mutant, heptose production and incorporation into LPS is functionally more important than in the parent strain.

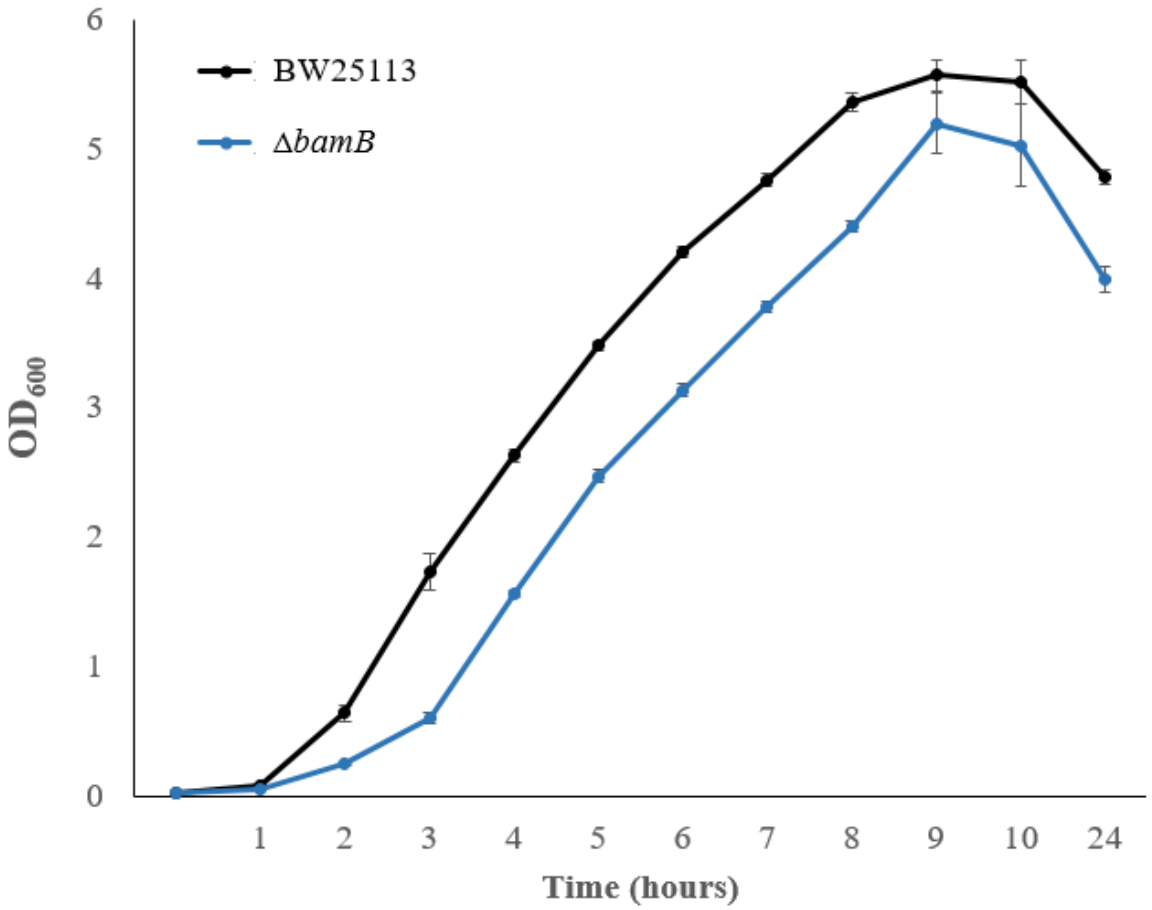


Fig. 3.13 Growth kinetics of the Δ*bamB* mutant. Three replicates of the parent (black) and the Δ*bamB* mutant (blue) were grown in 50 ml of LB in a 250 ml flask. Optical density (OD_{600}) was recorded hourly for 10 h and an additional reading was taken at the 24 h time point. The mean and standard deviation were plotted, with optical density on the Y-axis and time in hours on the X-axis. The Δ*bamB* mutant has a slow growing phenotype compared to the parent strain.

3.2.13. Conditionally non-essential genes in the Δ*bamB* TraDIS library

In the Δ*bamB* TraDIS library, 54 genes were identified to be conditionally non-essential (S. table 3.1). However, Phan *et al.* (2013) acknowledged that the IIS analysis can fail to recognise certain genes as essential. Consequently, the insertion profiles of all 54 conditionally non-essential gene candidates were inspected individually. Genes can be wrongly classified as non-essential for a number of reasons. For example, the occurrence of an insertion free region (IFR), which leads to the classification of a non-essential gene as essential. This especially needs to be taken into consideration for small genes. In the Δ*bamB* TraDIS dataset, 11% of the conditionally non-essential genes identified encoded proteins between 14 and 58 amino acids. A further potential problem is that a suppressor mutation might have inadvertently been introduced during the construction of the original *bamB* mutation. This might allow genes that are usually essential to become non-essential.

The slow growth rate of a Δ*bamB* mutant compared to the parent strain might also complicate the identification of conditionally non-essential genes (fig. 3.13). For example, in the Δ*bamB* TraDIS dataset, the gene *mreC* was classified as conditionally non-essential. A deletion mutant defective in *mreC* is viable but grows very slowly compared to the parent strain, which would result in a low IIS for this mutant in the parent TraDIS library (Bendezu and Boer, 2008). However, the IIS of a Δ*mreC*Δ*bamB* double mutant would be higher because it would be able to compete more effectively against the slow growing Δ*bamB* mutant. Similarly, other transposon mutants with slow growing phenotypes might be outcompeted in the parent TraDIS library and consequently the IIS of the disrupted genes would be low. However, with the longer recovery time that was utilised in the construction of the Δ*bamB* TraDIS library, these mutants should not be outcompeted, which should allow the correct classification of these non-essential genes.

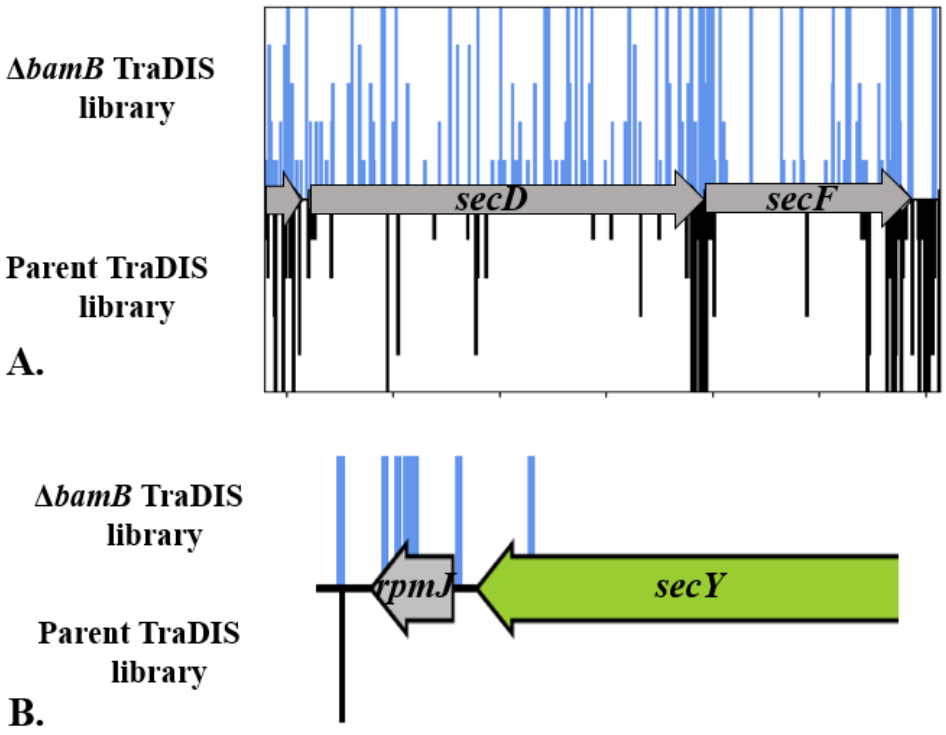


Fig. 3.14 The role of the SEC machinery in the Δ*bamB* mutant. (A) In the Δ*bamB* TraDIS library (blue), numerous mutants were recovered with transposon insertions in the genes *secD* and *secF*. Conversely, these genes were classified as essential in the parent TraDIS library. (B) In the Δ*bamB* TraDIS library (blue), mutants were recovered with transposon insertions in the gene *rpmJ*, which is directly upstream of *secY*, while in the parent TraDIS library, no mutants were recovered with transposon insertions in this gene.

Conversely, in the absence of BamB, certain essential genes might become non-essential for biological reasons. For example, members of the Sec machinery were conditionally non-essential. The Sec machinery transports OMPs across the IM into the periplasm and it consists of a membrane-embedded translocation complex formed from SecY, SecE and SecG; a peripheral SecA subunit; a periplasmic chaperone SecB; and an accessory complex formed from SecD, SecF, YajC and YidC (Mori and Ito, 2001). In the parent TraDIS library, the genes *secD* and *secF*, which are required for efficient protein export, were classified as essential. However, in the Δ*bamB* TraDIS library, more mutants were recovered with transposon insertions in these genes and consequently these genes were classified as non-essential (fig. 3.14A). A similar result occurred in the gene *rpmJ* (fig. 3.14B). Deletion of *rpmJ* decreases the expression of the gene *secY*, reducing the efficiency of Sec mediated transport (Ikegami *et al*., 2005).

Upon loss of BamB, a defect in the BAM complex occurs, resulting in a reduction in OMPs in the OM and an accumulation of OMPs in the periplasm (Ruiz *et al*., 2005; Wu *et al*., 2005). A mutation in *rpmJ, secD* and *secF* could be favourable in a Δ*bamB* mutant as it decreases the efficiency of OMP transport across the IM, reducing periplasmic OMP accumulation.

It is important to note that no single statistical method would fully identify every essential and non-essential gene correctly within a TraDIS dataset. Consequently, manual inspection of data is crucial. In summary, in the Δ*bamB* TraDIS library, genes are conditionally non-essential for a number of reasons. Some might be artefacts, while others are due to biological reality.

3.2.14. Identification of mutations that supress the effects of Δ*bamB*

Suppressor mutations are secondary mutations that can restore the phenotype of a specific mutant to that of the original parent strain. These mutations are useful for understanding biological pathways and functionally interacting molecules. This study attempted to identify potential suppressors of *bamB.* The Δ*bamB* mutant is more susceptible than the parent to a range of antibiotics including vancomycin. Consequently, vancomycin was tested as a potential suppressor screen for the Δ*bamB* mutant.

3.2.14.1. Screening of the Δ*bamB* TraDIS library. The Δ*bamB* mutant was exposed to a range of concentrations of vancomycin to determine the minimum concentration required to inhibit the growth of this mutant that did not inhibit the growth of the parent strain. These results revealed only an approximate inhibitory concentration of vancomycin and did not determine the exact MIC. Growth of the Δ*bamB* mutant was inhibited on LB agar supplemented with 20 µg/ml of vancomycin, while growth of the parent was not affected (fig. 3.15A). Consequently, the Δ*bamB* TraDIS library was screened (2.5×10^6) on LB agar supplemented with 40 µg/ml of vancomycin, double the minimum concentration required to inhibit the growth of the Δ*bamB* mutant (fig. 3.15B). The potential suppressor mutants were collected, pooled and sequenced to identify transposon insertion sites. These suppressor mutants contained a transposon insertion that either restores the phenotype produced by loss of *bamB* or a mutation that makes the cell more resistant to vancomycin. Consequently, the parent TraDIS library was also exposed to vancomycin in an attempt to separate natural suppressors of vancomycin from suppressors of Δ*bamB.* Gene-deletion suppressors of vancomycin refer to mutants that were recovered at high levels in both TraDIS libraries. Examples include numerous members of the Mla pathway *mlaA, mlaC, mlaD*, *mlaE*, *mlaF* and numerous genes involved in LPS synthesis: *waaG*; *waaP*; *waaQ*; *waaR*; and *waaB*.

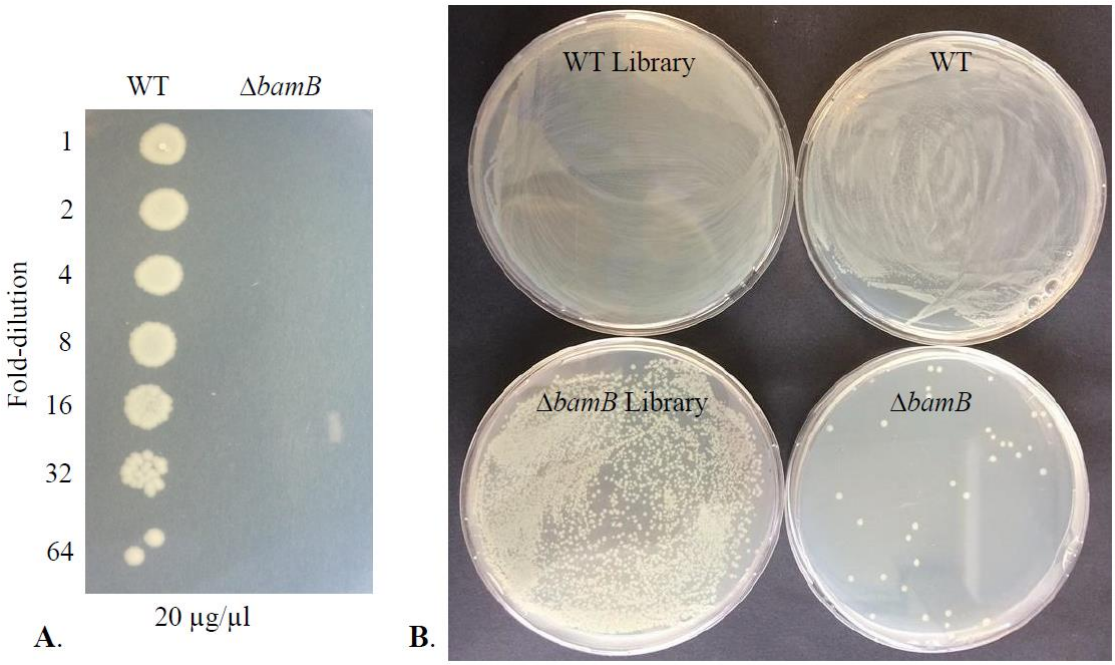


Fig. 3.15 Exposure of the Δ*bamB* mutant and the Δ*bamB* TraDIS library to inhibitory concentrations of vancomycin. (A) Growth of the Δ*bamB* mutant was inhibited on LB agar supplemented with 20 µg/ml of vancomycin, while growth of the parent was not affected. (B) The Δ*bamB* TraDIS library, the parent TraDIS library, the background Δ*bamB* mutant and the *bamB*+ parent (WT) were exposed to LB agar supplemented with 40 µg/ml of vancomycin. The parent TraDIS library and the background parent strain formed a lawn of colonies. Only sparse growth of suppressors occurred on the plates inoculated with the Δ*bamB* mutant. However, a significantly higher number of colonies were formed on the agar plate inoculated with the Δ*bamB* TraDIS library.

Table 3.4 Potential suppressors specific to the Δ*bamB* TraDIS library

Name of Gene	Gene Function
slt	A lytic murein transglycosylase that cleaves the β-1,4 glycosidic bond between N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) residues of PG (Yunck *et al.*, 2016).
mltB	The gene *mltB* encodes a lytic murein transglycosylase that cleaves the β-1,4 glycosidic bond between GlcNAc and MurNAc residues of PG (Yunck *et al.*, 2016).
yceG (*mltG*)	MltG is an endolytic murein transglycosylase that might function in the termination of nascent PG synthesis (Yunck *et al.*, 2016).
opgH	OpgH is a glycosyltransferase involved in the synthesis of osmoregulated periplasmic glucans (OPGs).
opgG	OpgG is involved in synthesis of OPGs.
dacA	The gene *dacA* encodes penicillin-binding protein 5 (PBP5), which cleaves the carboxy-terminal D-alanine from PG pentapeptides (Matsuhashi *et al*., 1979).
ybbY	A putative purine IM transporter.
ompR	A cytoplasmic response regulator.

*Where no reference was given, the functions were taken from the Ecocyc website: https://ecocyc.org/

Transposon insertion sites with fewer than 10 mapped reads were disregarded to filter for noise. Alternatively, genes with 10 or more mapped reads per insertion site along the length of the gene, which contained little to no transposon insertions in the parent TraDIS library, were considered to be potential suppressor mutants of Δ*bamB* (table 3.4). Of the genes identified, four are involved in the synthesis and remodelling of peptidoglycan: *slt*; *mtlB*; *mltG*; and *dacA*. Slt and MltB are two of seven lytic transglycosylases (LTs) that cleave the MurNAc-(1→4)-GlcNAc linkages in PG. This mediates the remodelling and recycling of PG (van Heijenoort, 2011). The IM protein MltG associates with PG synthetic complexes to cleave nascent polymers and terminate their elongation (Yunck *et al*., 2016). The gene *dacA* encodes a membrane-bound DD-carboxypeptidase referred to as penicillin-binding protein 5 (PBP5). PBP5 cleaves the carboxy-terminal D-alanine from peptidoglycan pentapeptides, removing excess pentapeptide in new PG (Matsuhashi *et al*., 1979).

The main mode of action of vancomycin is to inhibit PG synthesis by preventing the polymerisation of PG. Loss of *slt*, *mltB*, *mltG* or *dacA* reduces breakdown of PG. Consequently, the Δ*slt*, Δ*mltB*, Δ*mltG* and Δ*dacA* mutants were screened on 250 μg/ml of vancomycin to determine their level of susceptibility to vancomycin, relative to the parent strain. These mutants grew on vancomycin concentrations that inhibit the growth of the parent (fig. 3.16A). Thus, deletion of these genes arise vancomycin resistance and do not suppress the Δ*bamB* phenotype. Consequently, these genes were not further investigated.

Of the genes identified, two are involved in the synthesis of osmoregulated periplasmic glucans (OPG). OPGs are oligosaccharides with 8 to 10 glucose units in a highly branched structure, which elicit key roles in monitoring osmotic pressure and in maintaining proper arrangement of the IM and OM (Kennedy, 1982). The genes *opgG* and *opgH* when disrupted conferred resistance to 250 μg/ml of vancomycin (fig. 3.16B). Thus, deletion of these genes

arise vancomycin resistance and do not suppress the Δ*bamB* phenotype. A mutant defective in these genes displays a larger periplasmic space than the parent strain, suggesting that loss of OPGs reduce permeability of the cell envelope to vancomycin (Holtje *et al*., 1988).

The genes *ompR* and *ybbY* were also identified as potential suppressor candidates of *bamB*. YbbY is a putative purine transporter found in the IM, while OmpR regulates the expression of major OM porins OmpF and OmpC, in an osmolarity dependent manner (Kawaji *et al*., 1979; Van Alphen and Lugtenberg, 1977). As the osmolarity increases, the *ompC* gene is preferentially activated, while *ompF* is repressed. Alternatively, as the osmolarity decreases, the *ompF* gene is favourably activated and the gene *ompC* is repressed (Aiba *et al*., 1989). Loss of the genes *ompR* and *ybbY* did not restore the growth of the parent strain on 250 μg/ml of vancomycin. Consequently, the double mutants Δ*bamB*Δ*ompR* and Δ*bamB*Δ*ybbY* were screened on a range of vancomycin concentrations: 5 μg/ml; 10 μg/ml; 15 μg/ml; and 20 μg/ml. However, loss of *ybbY* or *ompR* did not restore the growth of the Δ*bamB* mutant (fig. 3.17A-B). This suggests that in the Δ*bamB* suppressor TraDIS dataset, the mutants recovered with transposon insertions in *ybbY* or *ompR* were satellite colonies, which are small colonies that do not supress the condition tested but instead grow in close proximity to resistant colonies that break down the antibiotic.

In summary, this study failed to identify any gene-deletion suppressors of the Δ*bamB* mutant. Of the suppressor candidates identified, six conferred resistance to vancomycin and did not restore the phenotype produced by loss of BamB, while mutants recovered with transposon insertions in *ybbY* or *ompR* were probably satellite colonies. However, the lack of identification of gene-deletion suppressors further suggests that the gene *bamB* is a good target in the development of potential anti-bacterials.

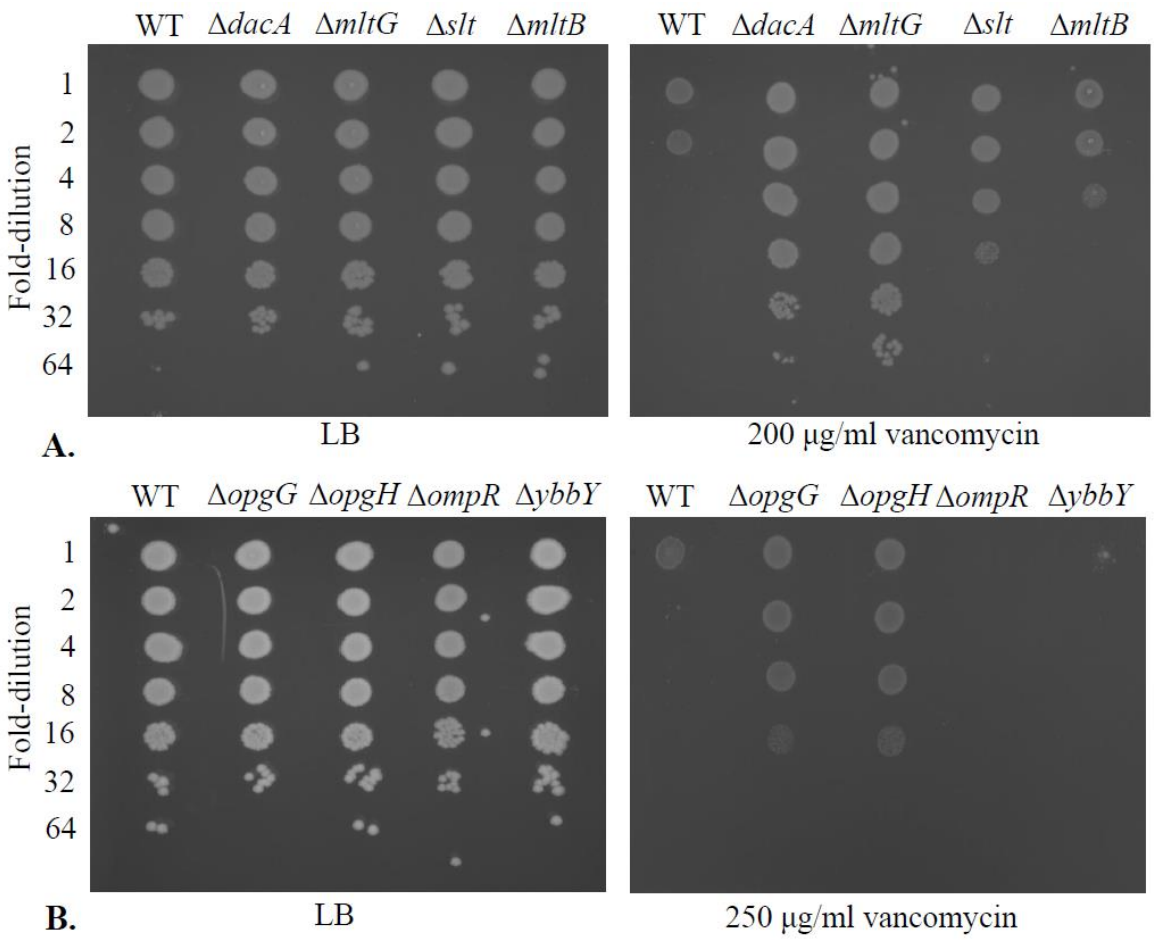


Fig. 3.16 Screening of potential suppressors on concentrations of vancomycin that inhibit the growth of the parent strain. Strains were grown overnight, diluted to an OD_{600} of 1.00 and 2 µl of 10-fold serially-diluted strains were inoculated onto agar plates with and without vancomycin. Mutants were screened on 200 and 250 µg/ml of vancomycin. (A) The mutants Δ*dacA*, Δ*mltG*, Δ*slt* or Δ*mltB* conferred resistance to vancomycin on both of the concentrations tested. (B) Loss of *opgH* or *opgG* also restored the growth of the parent strain on vancomycin, while deletion of *ompR* or *ybbY* did not restore the growth of the parent strain on vancomycin.

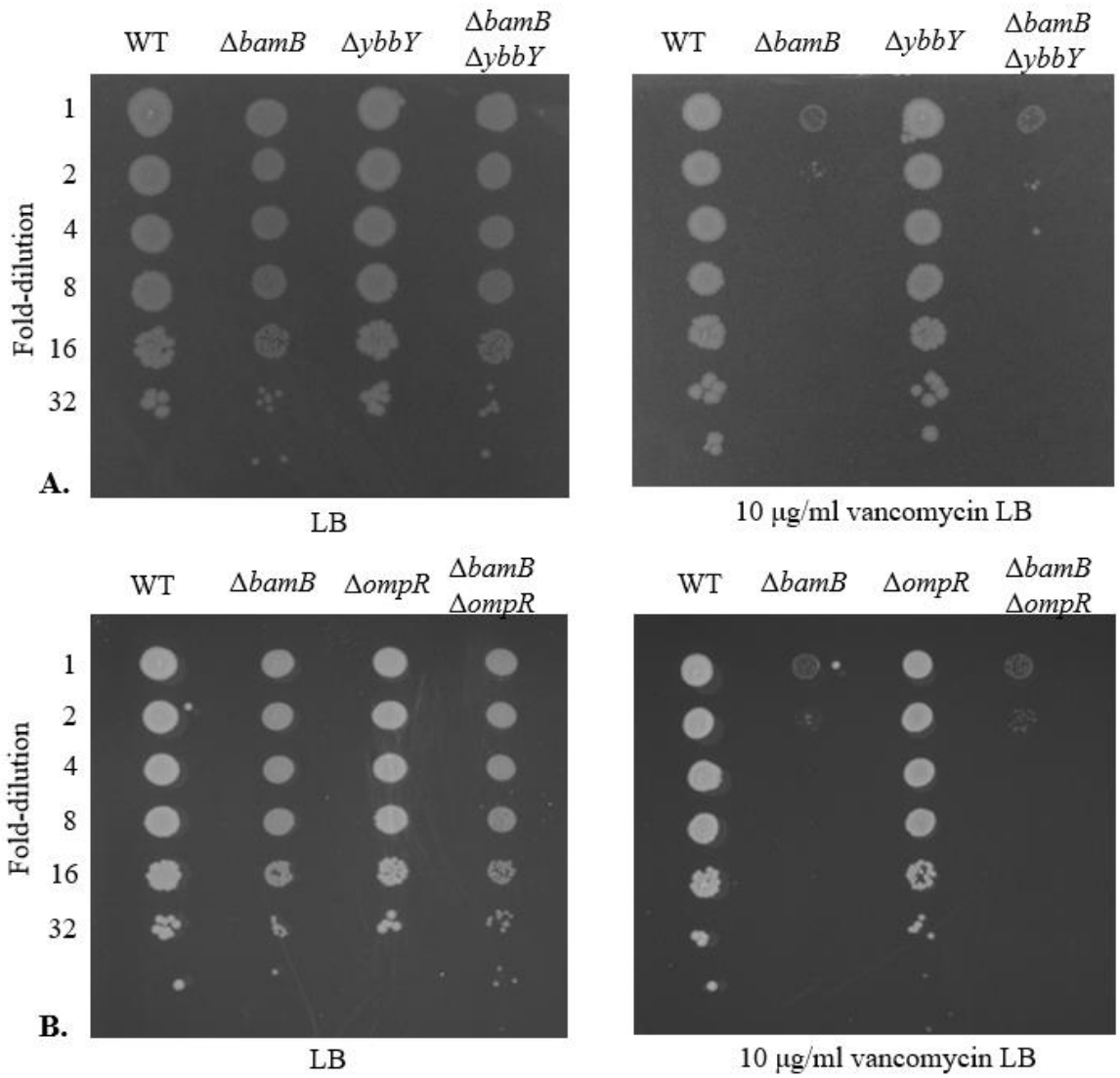


Fig. 3.17 Screening potential suppressors *ompR* and *ybbY*. Strains were grown overnight, diluted to an OD_{600} of 1.00 and 2 µl of 10-fold serially-diluted strains were inoculated onto agar plates with and without vancomycin. Double mutants Δ*bamB*Δ*ompR* and Δ*bamB*Δ*ybbY* were screened on a number of vancomycin concentrations. (A) Loss of *ybbY* did not restore the growth of the Δ*bamB* mutant on 10 µg/ml of vancomycin. (B) Deletion of *ompR* did not restore the growth of Δ*bamB* on 20 µg/ml of vancomycin, which suggests that loss of *ompR* does not suppresses loss of BamB.

3.3. Conclusion

A TraDIS library was constructed in a Δ*bamB* mutant and compared to the parent TraDIS library to identify genes required for the survival of the Δ*bamB* mutant. Twenty-nine conditionally essential genes were identified by the statistical parameters, while an additional 17 genes of interest were identified by manual inspection. Some of these genes were involved in post-translational modification and transport; recombination and repair; and LPS biogenesis. More specifically a number of genes involved in the synthesis and incorporation of heptose into the LPS structure were functionally more important in the Δ*bamB* mutant than in the parent strain. Lastly, no gene-deletion suppressors of Δ*bamB* were identified.

CHAPTER 4

Identification of conditionally essential genes in mutants devoid of *bamC* or *bamE*

4.1. Introduction

The BAM complex consists of sub-complexes: BamA:B and BamC:D:E. BamC and BamE are of particular interest because they do not interact with BamA directly and instead assemble into the BAM complex only through interactions with BamD (Malinverni *et al.*, 2006). Loss of BamC results in destabilization of these sub-complexes and increased protease susceptibility of BamB and BamD. Loss of BamC or BamE results in disruption of BamA–BamD interactions to the same degree, which suggests an overlapping function between these proteins in the stabilization of BamA-BamD interactions (Rigel *et al.*, 2012). However, the precise functions of the non-essential BAM components BamC and BamE remain unresolved. Only minor β-barrel protein assembly defects occur in a Δ*bamE* mutant, while no known growth or phenotypic effect occurs in a Δ*bamC* mutant. Furthermore, there are only modest phenotypic differences in terms of the number of OMPs in the OM and antibiotic susceptibility between double mutants Δ*bamB*Δ*bamC* and Δ*bamC*Δ*bamE* and the single deletion mutants. Recently, Anwari *et al.* (2012) observed that BamC is highly conserved in bacteria, which suggests that the role of BamC is crucial to the cell. However, despite its conservation the role of BamC is difficult to determine.

In this study, TraDIS was used to identify synthetic lethal partners of *bamC* and *bamE* that might help determine the roles of these proteins in the cell. TraDIS libraries were constructed in Δ*bamC* and Δ*bamE* mutants and the TraDIS libraries were processed using the statistical criteria for essential gene prediction that was discussed in chapter three. This predicts gene essentiality depending on the likelihood of a gene belonging to either the essential or non-essential mode. The mutant TraDIS libraries were compared to the parent TraDIS library to identify conditionally essential and non-essential genes. In addition, TraDIS was also utilised in an attempt to identify potential gene-deletion suppressors of *bamC* and *bamE*.

4.2. Results

4.2.1. Construction and sequencing of the Δ*bamC* and Δ*bamE* TraDIS libraries

In the construction of the Δ*bamC* TraDIS library, over 800,000 Δ*bamC* transposon mutants were pooled. The analysis checked for the presence of an inline index barcode, followed by the transposon sequence, removed short sequence reads and mapped sequenced reads to the *E. coli* K-12 BW25113 genome, as previously discussed in chapter three. Analysis of the Δ*bamC* TraDIS library identified ~7 million reads and over 708,000 unique transposon insertion sites, which equates to a transposon insertion site approximately every 6.5 base pairs throughout the genome. In the construction of the Δ*bamE* TraDIS library, over 800,000 Δ*bamE* transposon mutants were pooled to produce a highly saturated library. In addition, the analysis identified ~6 million reads and over 660,000 unique transposon insertion sites, which suggests that a transposon insertion site occurred approximately every 6.9 base pairs. Data recovered from the Δ*bamC* and Δ*bamE* TraDIS libraries are summarized in table 4.1. and the frequency of insertion index scores were plotted to produce bimodal histograms (appendix 4.1).

For an internal measure of quality control two replicates of each transposon library were sequenced. The gene insertion index scores were compared to ensure that there was a high correlation within datasets. The R-squared value acts as a measure of quality control. The closer this value is to one, the higher the similarity between replicates. The respective R-squared values for the Δ*bamC* and Δ*bamE* TraDIS libraries were 0.97 and 0.95, respectively, indicating a high correlation between the two replicates (fig. 4.1). As a result, data from replicates were pooled and the sequenced transposon insertion sites for the Δ*bamC* and Δ*bamE* TraDIS libraries were mapped to the reference *E. coli* BW25113 genome (fig. 4.2). In the mutant TraDIS libraries, the transposon insertion sites were evenly distributed around the BW25113 genome, with the exception of a bias to the origin as expected.

Table 4.1 The number of reads at each stage of the data processing pipeline for technical replicates of the Δ*bamC* and Δ*bamE* TraDIS libraries

Library	Sample	Tn1 check	Tn2 check	Mapped reads	UIS
Δ*bamC*	Rep 1	2,828,303	2,772,869	2,433,310	491,868
	Rep 2.	5,533,454	5,317,471	4,502,496	553,496
	Combined	8,361,757	8,090,340	6,935,811	708,256
Δ*bamE*	Rep 1	2,859,591	2,775,724	2,388,876	403,520
	Rep 2.	5,152,751	5,011,453	3,956,132	523,140
	Combined	8,012,342	7,787,177	6,345,013	667,793

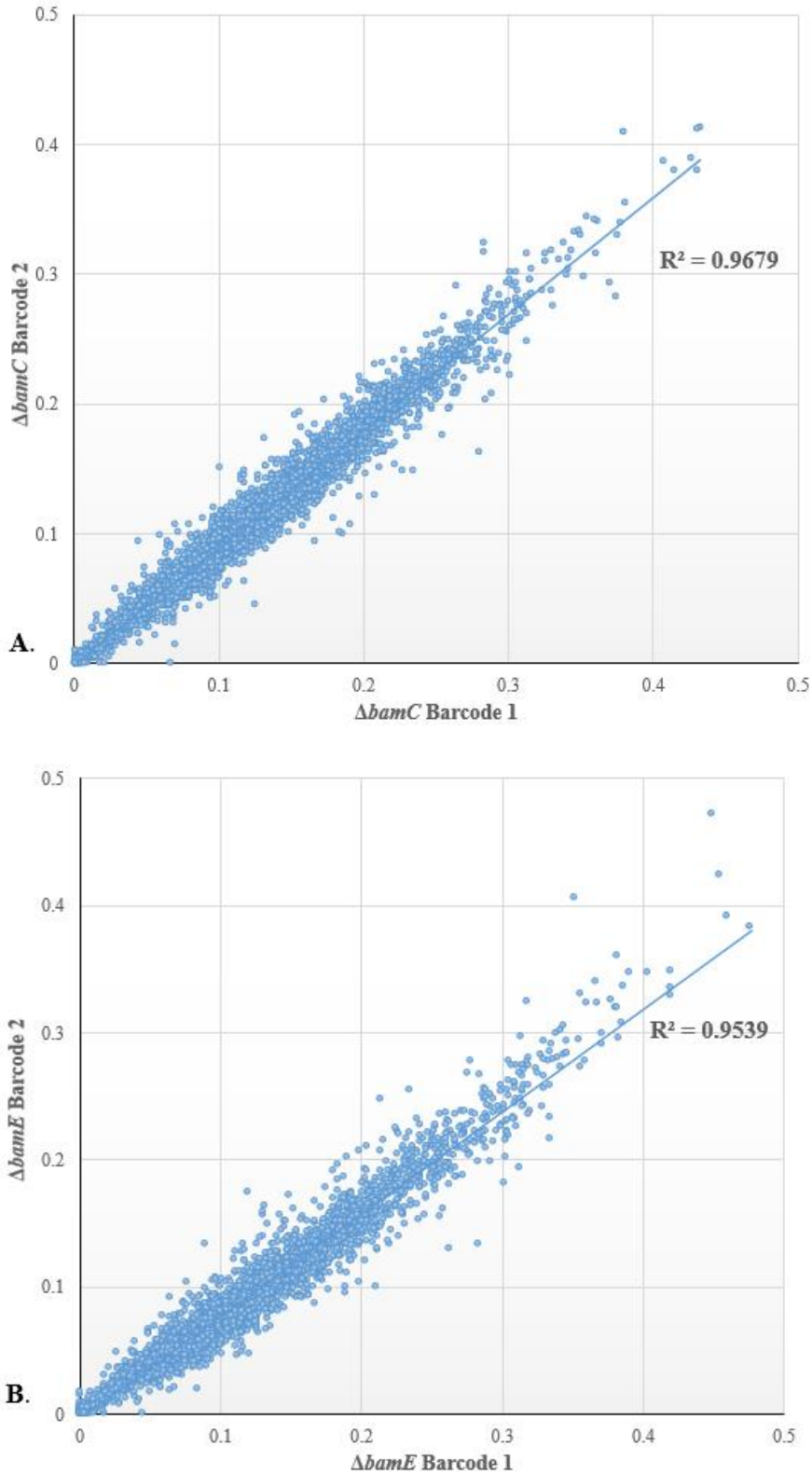


Fig. 4.1 Sequencing of two independent replicates. The correlation between the gene insertion index scores for sequenced replicates of the (A) Δ*bamC* TraDIS library and (B) Δ*bamE* TraDIS library.

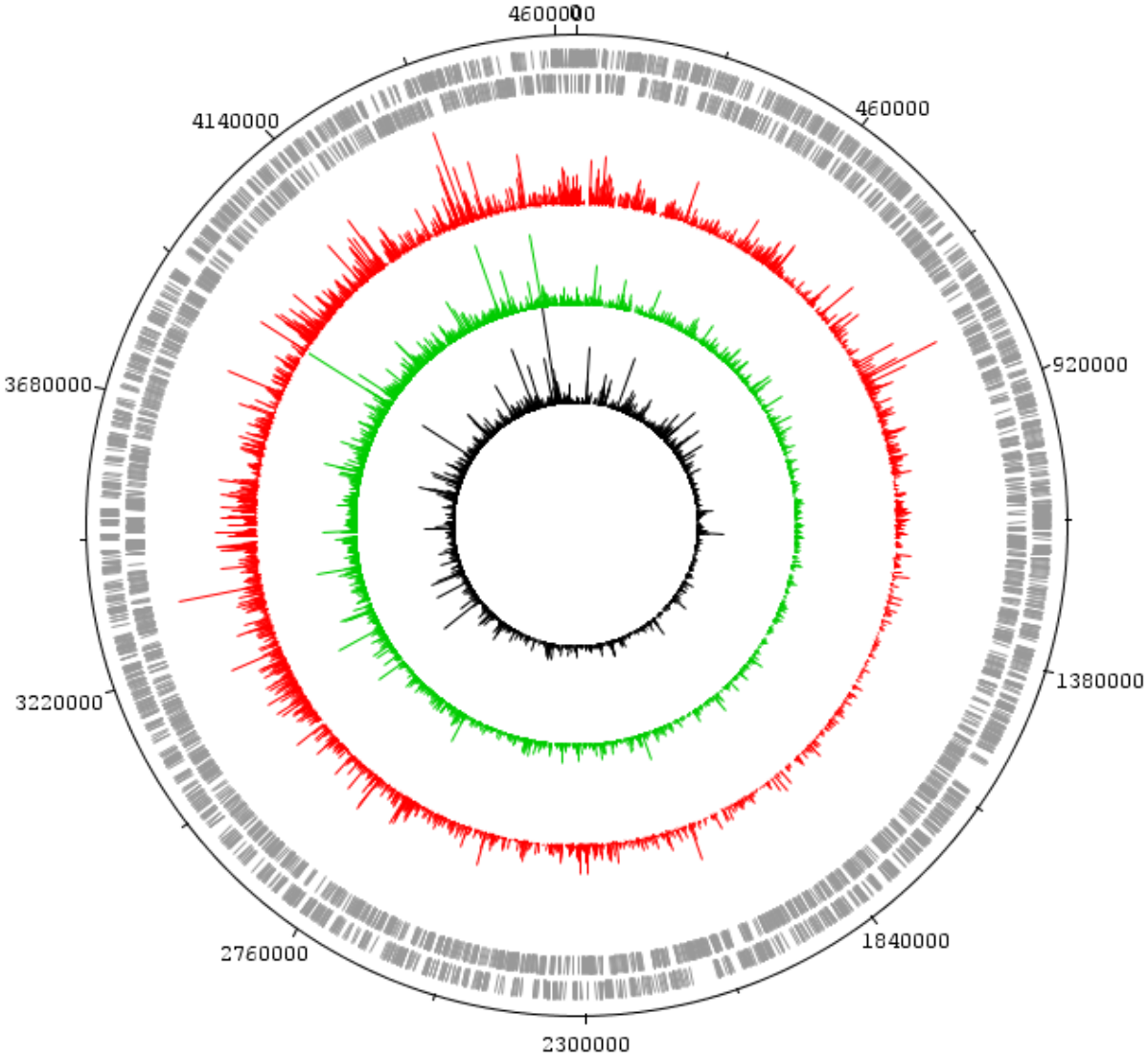


Fig. 4.2 Frequency and location of transposon insertion sites throughout the Δ*bamC* and Δ*bamE* TraDIS libraries. The outer track marks the BW25113 genome in base pairs, starting at the annotation origin. The next two inner tracks correspond to the sense and antisense CDS, respectively (grey). The inner circles correspond to the frequency and location of transposon insertion sites in the Δ*bamC* TraDIS library (red), the Δ*bamE* TraDIS library (green) and the parent TraDIS library (black), mapped to the BW25113 genome (CP009273.1). This figure was created using DNAPlotter.

4.2.2. Identification of genes that are synthetically lethal with *bamE*

In the Δ*bamE* TraDIS library, the 334 genes identified as essential were compared to the essential genes identified in the parent TraDIS library. Both datasets shared 317 essential genes, which were not investigated further (fig. 4.3). In the Δ*bamE* TraDIS library, 17 genes were identified as essential that were not required for survival of the parent (table 4.2). In the parent TraDIS library, 24 genes were classified as essential that were not required for the survival of the Δ*bamE* mutant (S. table 4.1). All 24 genes were manually inspected and the ratio of transposon insertions between the mutant and the parent were compared to identify genes of interest (table 4.3). A number of these genes were involved in iron-sulfur cluster formation and the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS).

Iron-sulfur clusters elicit important physiological roles in metabolic reactions, electron transfer and transcriptional regulation. Two systems are utilised in the assembly of iron-sulfur clusters, the Isc system and the Suf system. In the Ics system, IcsS and IscA respectively deliver sulfane sulfur and iron to the scaffold protein IscU (fig. 4.4A). IscU forms a complex with HscA and HscB, which help facilitate iron-sulfur cluster assembly and the transfer to ferredoxin (Blanc *et al.*, 2015). In the Δ*bamE* TraDIS library, a higher number of mutants were recovered with transposon insertions in the genes *hscA*, *hscB*, *iscA*, *iscU* and *iscS*, compared to the parent TraDIS library (fig. 4.4B). In contrast, the Suf system is required under iron starvation or oxidative stress conditions (Outten *et al.*, 2004). However, in the Δ*bamE* and parent TraDIS libraries, a similar number of mutants were recovered with transposon insertions in the *suf* genes: *sufE*; *sufS*; *sufD*; *sufC*; *sufB* and *sufA* (fig. 4.4C). Thus, iron-sulfur cluster formation through the Isc system was functionally less important in the Δ*bamE* mutant than in the parent strain. One possibility is that loss of BamE induces oxidative stress conditions, which increases the importance of the Suf system.

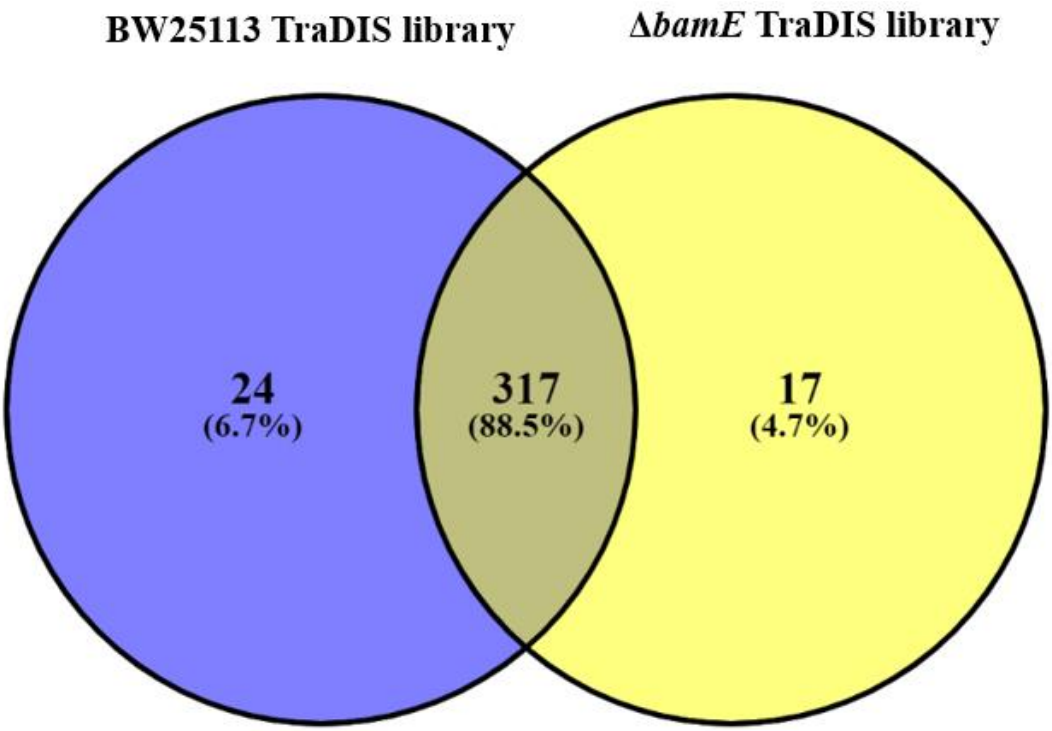


Fig. 4.3 Comparison of essential gene candidates shared between the Δ*bamE* and parent TraDIS datasets. Both the parent and Δ*bamE* TraDIS libraries shared 317 essential genes (88.5% of essential genes were shared). In the Δ*bamE* TraDIS library, 17 conditionally essential genes and 24 conditionally non-essential genes were identified.

Table 4.2 Conditionally essential genes identified in the Δ*bamE* TraDIS library

Name of Gene	Gene Function
cmK	Cytidylate kinase (Cmk) rephosphorylates CMP and dCMP produced by the turnover of nucleic acids and CDP diglycerides.
degS	A protease that senses and reacts to damaged and mislocalised proteins, ultimately activating the σ^E mediated stress response system (Alba *et al.*, 2002).
dicC	DicC negatively regulates the expression of the gene *dicB*, which is involved in inhibition of cell division (Aldea *et al*., 1988).
glmS	L-glutamine:D-fructose-6-phosphate aminotransferase (GFAT) catalyses the first step in hexosamine biosynthesis.
higA	Antitoxin of the mRNA interferase toxin HigB (Christensen-Dalsgaard, 2010).
pbl	Transglycosylase that is a remnant of a type III secretion system (Ren *et al*., 2004).
rnpA	A ribonuclease P holoenzyme component that processes tRNA precursor molecules and the stable 4.5 sRNA precursor (Chang and Carbon, 1975; Bothwell *et al*., 1976).
secB	A cytoplasmic chaperone that forms part of the Sec pathway.
yccE	Function unknown.
yciG	The gene *yciG* is part of the σ^S regulon (Weber *et al*., 2005).
ydcX (ortT)	An IM toxin that damages the cell membrane and reduces the intracellular ATP level (Islam *et al*., 2015).
ygeF	Remnant of an ETT2 (type III secretion system) pathogenicity island with an unknown function (Ren *et al*., 2004).
ygeL	Function unknown.
ygeN	Gene of unknown function that forms part of a putative type III secretion system.
ymfD	Function unknown.
ymgC	Function unknown.
zwf	Glucose-6-phosphate dehydrogenase is an enzyme involved in the pentose phosphate pathway.

*Where no reference was given the functions were taken from the Ecocyc website: https://ecocyc.org/

Table 4.3 Genes of interest classified as conditionally non-essential in the Δ*bamE* TraDIS library

Name of gene	Gene Function
crp	A DNA-binding transcriptional regulator.
hicA	Toxin of the HicA-HicB toxin-antitoxin system.
hscA	An iron-sulfur cluster biosynthesis chaperone.
iscS	A cysteine desulfurase involved in iron-sulfur cluster assembly.
iscU	A scaffold protein involved in iron-sulfur cluster assembly.
lpd	The *lpd* gene encodes the E3 component of three enzyme complexes: pyruvate dehydrogenase; the 2-oxoglutarate dehydrogenase complex; and the glycine cleavage system.
ptsH	A phosphoenolpyruvate-protein phosphotransferase.
ptsI	A phosphoenolpyruvate-protein phosphotransferase.
tonB	A component of the Ton system, which is involved in the harness of energy for the transport of B12 and iron siderophore complexes across the OM.
ubiE	The gene *ubiE* encodes a C-methyltransferase that catalyses reactions in both ubiquinone and menaquinone biosynthesis.

*These functions were taken from the Ecocyc website: https://ecocyc.org/

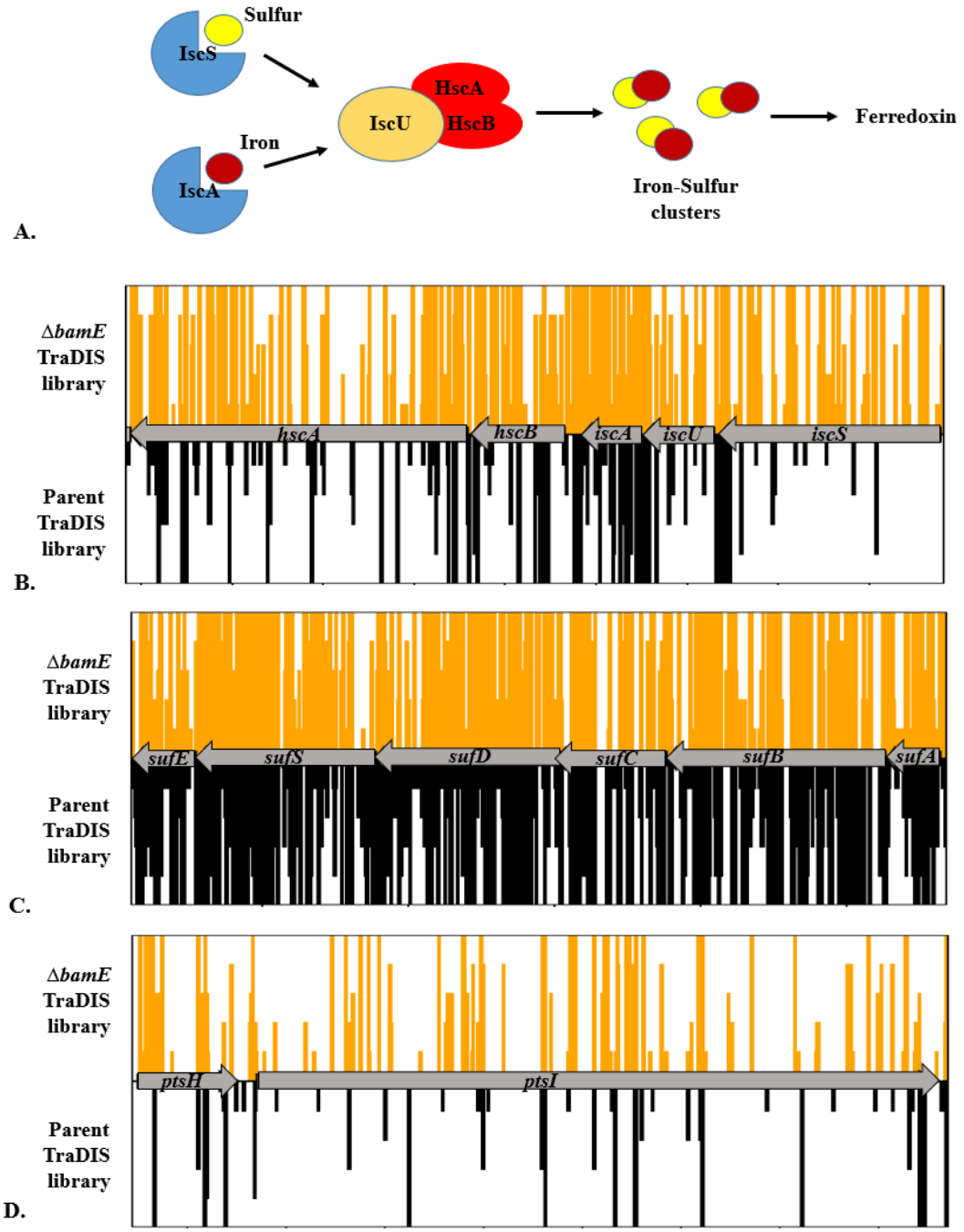


Fig. 4.4 Iron-sulfur cluster formation through the Isc system. (A) Sulfur and iron are transported to IscU, which alongside HscA and HscB leads to the formation and transfer of iron-sulfur clusters to ferredoxin. (B) In the parent TraDIS library (black), fewer mutants were recovered with transposon insertions in the genes *hscA*, *hscB*, *iscU* and *iscS* than in the Δ*bamE* TraDIS library (orange). (C) In the parent and Δ*bamE* TraDIS libraries, a similar number of mutants were recovered with transposon insertions in the genes: *sufA*; *sufB*; *sufC*; *sufD*; *sufS*; and *sufE*. (D) In the Δ*bamE* TraDIS library, more mutants were recovered with transposon insertions in the genes *ptsH* and *ptsI*, compared to the parent counterpart.

Consequently, the Suf system elicits iron-sulfur cluster assembly in the cell, decreasing the importance of the Isc system.

Iron-sulfur cluster assembly was one of two pathways that were more important to the fitness of the parent strain than to the Δ*bamE* mutant. In the Δ*bamE* TraDIS library, more mutants were recovered with transposon insertions in the genes *ptsH* and *ptsI* than in the parent TraDIS library (fig. 4.4D). These genes encode two proteins that form part of the phosphoenolpyruvate (PEP): carbohydrate phosphotransferase system (PTS), which is a sugar uptake method where sugar substrates are transported and phosphorylated at the expense of PEP. It is possible that mutations in these genes compete more effectively against the other mutants recovered during the production of the Δ*bamE* TraDIS library. For example, these mutants could grow faster because they remove a factor that in the presence of the Δ*bamE* mutant is deleterious. In summary, mutations in genes involved in the iron-sulfur cluster assembly through the Isc system and mutations in the genes involved in the PEP: PTS were more advantageous to the fitness of the Δ*bamE* mutant than to the parent strain.

4.2.3. Identification of genes that are synthetically lethal with *bamC*

In the Δ*bamC* TraDIS library, 359 essential gene candidates were identified and compared to the parent TraDIS library. Both datasets shared 312 essential genes, while 47 conditionally essential gene candidates were identified (table 4.4). In addition, 29 genes that were required for the survival of the parent strain were classified as non-essential in the Δ*bamC* TraDIS library (S. table 4.2). All of the non-essential gene candidates were inspected individually and the ratio of transposon insertions between the mutant and the parent were compared to identify genes of interest (table 4.5). In the Δ*bamC* TraDIS library, no mutants were recovered with transposon insertions in a prophage operonic region with an unknown

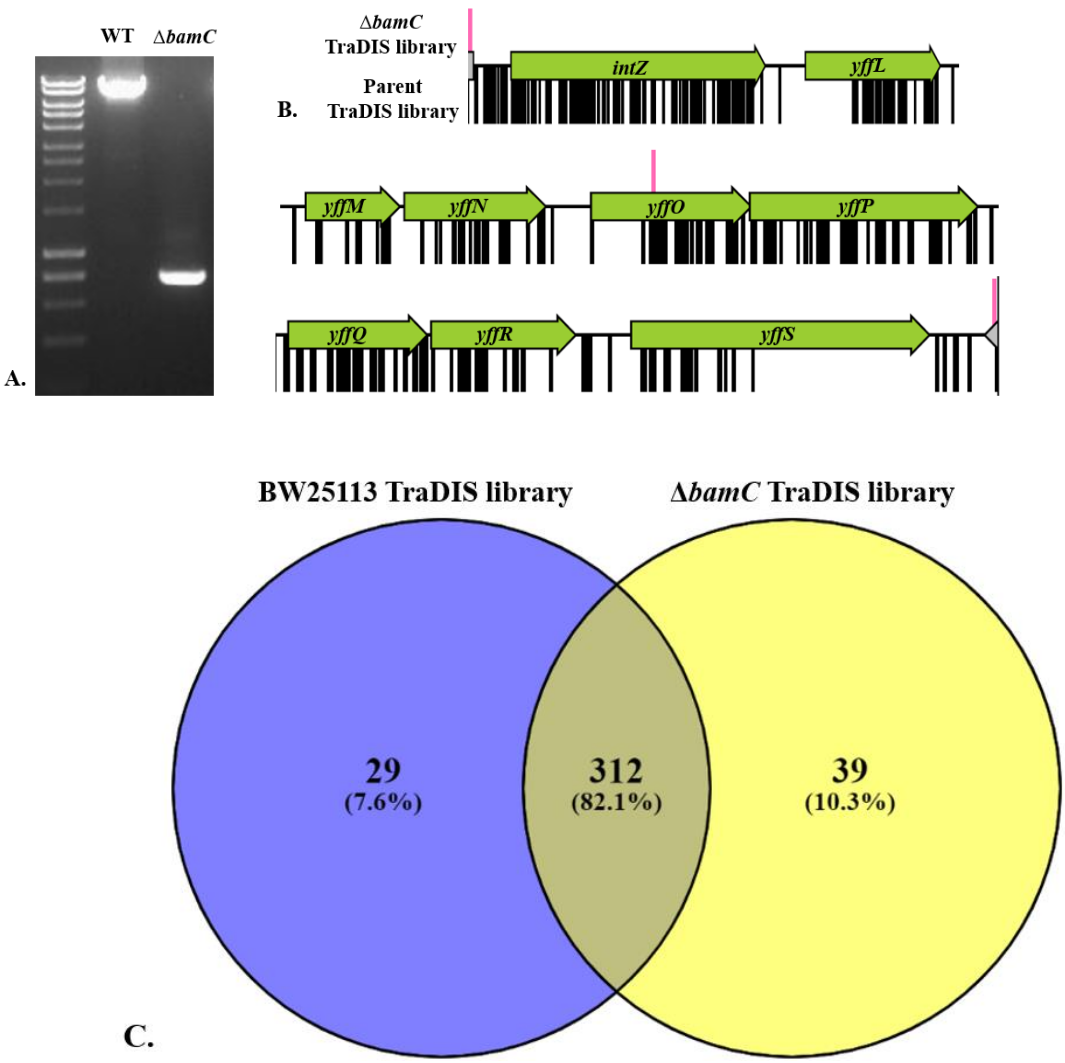


Fig. 4.5 Classification of conditionally essential genes in the Δ*bamC* TraDIS library. (A) The presence/absence of the *yff* genes in the parent (WT) and the Δ*bamC* mutant. This region was present in the parent strain (~8 kb). However, in the Δ*bamC* mutant, this operonic region was absent (~1 kb). (B) In the Δ*bamC* TraDIS library (pink), no mutants were recovered with transposon insertions in the *yff* operonic region, while in the parent TraDIS library (black), numerous mutants were recovered with transposon insertions in this region. (C) Essential gene candidates shared between the Δ*bamC* and parent TraDIS libraries. Both libraries shared 312 essential gene candidates. In the Δ*bamC* TraDIS library, 39 genes were identified as conditionally essential and 29 genes as conditionally non-essential.

Table 4.4 Conditionally essential genes identified in the Δ*bamC* TraDIS library

Name of Gene	Gene Function
csrA	CsrA is a regulator of carbohydrate metabolism (Sabnis *et al.*, 1995; Yang *et al.*, 1996).
dapF	DapF is involved in the biosynthesis of lysine.
degS	DegS is a protease that senses and reacts to damaged and mislocalised proteins.
elaD	A deubiquitinating protease.
glmS	GlmS catalyses the first step in the biosynthesis of hexosamine.
gnsA	Function unknown.
kilR	Overexpression of KilR inhibits cell division and leads to morphological defects (Burke *et al.*, 2013; Conter *et al.*, 1996).
lipB	LipB catalyses the first step of *de novo* lipoate biosynthesis (Jordan and Cronan, 1997; Jordan and Cronan, 2003).
pbl	Transglycosylase that forms part of an ETT2 pathogenicity island (Ren *et al.*, 2004).
rclB	Function unknown.
rnpA	A component of the ribonuclease P holoenzyme, which processes tRNA precursor molecules and the stable 4.5 sRNA precursor (Chang and Carbon, 1975; Bothwell *et al.*, 1976).
secB	A cytoplasmic chaperone that forms part of the SEC machinery.
sucB	SucB is involved in the conversion of 2-oxoglutarate to succinyl-CoA and carbon dioxide in the TCA cycle.
ybcK	Function unknown.
ybcV	Function unknown.
ybfB	Function unknown.
ybfC	Function unknown.
yccE	Function unknown.
yceQ	Function unknown.
yciE	Function unknown.
yciG	Function unknown.
ydeO	DNA-binding transcriptional dual regulator that activates genes involved in cellular responses to acid resistance (Ma *et al.*, 2004; Masuda *et al.*, 2002; Masuda *et al.*, 2003).
yehC	A putative fimbrial chaperone (Zhai *et al.*, 2002).
yehD	A putative fimbrial chaperone (Korea *et al.*, 2010).
yfbN	Function unknown.
yfdF	Function unknown.

yfjW	Function unknown.
ygeF	Unknown function, remnant of a type III secretion system.
ygeG	Unknown function, remnant of a type III secretion system.
ygeI	A gene with an unknown function that forms part of a remnant of a type III secretion system.
ygeK	A putative DNA-binding transcriptional regulator.
ygeN	Unknown function, remnant of an ETT2 (type III secretion system) pathogenicity island (Ren *et al*., 2004).
yjbL	Function unknown.
yjbS	Function unknown.
ymfD	Function unknown.
yncI	Function unknown.
ypjC	Function unknown.
yqeJ	Function unknown, a remnant of an ETT2 (type III secretion system) pathogenicity island (Ren *et al.*, 2004).
yqeK	A gene with an unknown function that forms part of a type III secretion system (Ren *et al*., 2004).

*Where no reference was given the functions were taken from the Ecocyc website: https://ecocyc.org/

Table 4.5 Genes of interest classified as conditionally non-essential in the Δ*bamC* TraDIS library

Name of gene	Gene function
crp	DNA-binding transcriptional dual regulator.
dcd	Dcd catalyses a step in the synthesis of deoxythymidine triphosphate.
hicA	Toxin of the HicA-HicB toxin-antitoxin system.
iscS	Cysteine desulfurase involved in iron-sulfur cluster assembly.
ptsI	Phosphoenolpyruvate-protein phosphotransferase.
rpsI	The gene *rpsI* encodes the S9 protein, which is a component of the 30S ribosome subunit.
ubiE	The gene *ubiE* encodes a C-methyltransferase, which catalyses reactions in both ubiquinone and menaquinone biosynthesis.
ubiH	The gene *ubiH* encodes 2-octaprenyl-6-methoxyphenol hydroxylase, which is involved in the ubiquinone biosynthesis pathway.
ybeY	Endoribonuclease involved in maturation of 16S rRNA and in transcription anti-termination of an rRNA promoter.
ygfZ	A gene of unknown function that might play a role in iron-sulfur cluster assembly.

*The functions were taken from the Ecocyc website: https://ecocyc.org/

function, which consisted of eight genes: *intZ*; *yffL*; *yffM*; *yffN*; *yffO*; *yffP*; *yffQ*; and *yffR* (fig. 4.4B). It was hypothesised that these genes were conditionally essential or absent in the Δ*bamC* mutant. A check PCR confirmed that in the Δ*bamC* mutant, this region was not present (fig. 4.4A). Consequently, these genes were no longer considered as conditionally essential, which resulted in the new number of 39 conditionally essential gene candidates (fig. 4.4C). A new Δ*bamC* mutant was constructed and the presence of the *yff* operon was confirmed by PCR. This mutant was used to confirm synthetic lethal candidates of interest.

Yamamoto *et al.* (2009) demonstrated that a bacteriophage randomly inserted the *yff* operonic region into the bacterial genome. Thus, it is likely that this operonic region was excised randomly in the Δ*bamC* mutant and that these genes are not functionally important in *E. coli*. In addition, little to no information was found in the literature about this operon and it is absent from the Keio collection. However, to ensure that the conditionally essential genes identified was due to loss of *bamC* and not due to loss of the *yff* operon, the synthetic lethal partner of interest *dapF* was validated (section 4.2.6).

4.2.4. Classification of Δ*bamC* and Δ*bamE* conditionally essential genes based on Gene Ontology

To help understand why the Δ*bamC*/Δ*bamE* conditionally essential genes are required for the survival of their respective mutants, the conditionally essential genes were classified based on function and the literature was searched for explanations. The majority of genes in the *E. coli* genome can be assigned to one or more Gene Ontology (GO) categories, based on the gene products role in a specific pathway or cellular process. In the Δ*bamC* and Δ*bamE* TraDIS libraries, the genes classified as conditionally essential were processed using the online Gene Ontology (GO) database (Ashburner *et al.*, 2000; The Gene Ontology Consortium, 2019).

In the Δ*bamE* dataset, of the 17 conditionally essential genes identified, nine were genes of unknown function. The remainder of the genes were involved in the Gene Ontology (GO) categories: ‘carbohydrate transport and metabolism;’ ‘post-translational modification, protein turnover and chaperones;’ ‘nucleotide transport and metabolism;’ 'intracellular trafficking, secretion and vesicular transport;’ ‘translation, ribosomal structure and biogenesis;’ and ‘cell envelope biogenesis.’

In the Δ*bamC* dataset, of the 39 conditionally essential gene candidates, 25 were genes of unknown function. The other 13 genes were involved in the Gene Ontology (GO) categories: 'intracellular trafficking, secretion and vesicular transport;’ ‘post-translational modification, protein turnover and chaperones;’ ‘replication, recombination and repair;’ ‘cell cycle control, cell division and chromosome partitioning;’ ‘transcription;’ ‘signal transduction mechanism;’ ‘translation, ribosomal structure and biogenesis;’ ‘cell envelope biogenesis;’ coenzyme transport and metabolism;’ and ‘energy production and conversion.’

The proportion of proteins involved in a specific pathway or biological process can be quantified, relative to the entire genome. The PANTHER (Protein Analysis Through Evolutionary Relationships) overrepresentation test assigned a suitable GO biological process to each of the genes. The expected proportion of genes that belongs to a GO category were compared to the actual number of genes identified per GO category to calculate a fold enrichment score with an associated p-value. The fold enrichment score identifies GO categories that are statistically more often in the input list than expected by chance (Mi *et al.,* 2017). In the Δ*bamE* dataset, no GO categories were enriched to a statistically significantly level, while in the Δ*bamC* dataset; only one GO category was significantly enriched (2.83 fold enrichment, with a false discovery p-value of less than 0.05). This result

suggests that the group ‘unclassified’ is functionally more important to a Δ*bamC* mutant than the parent. Thus, the genes of unknown function were investigated further.

4.2.5. The importance of genes of unknown function to a Δ*bamC* mutant

Over 65% of the genes identified as conditionally essential in the Δ*bamC* mutant were genes of unknown function. These genes were first classified based on the location of the encoded gene products in the cell (fig. 4.6A). SignalP 5.0 was utilised to search protein sequences for signal peptides that allow a protein to cross the IM (Armenteros *et al.*, 2019). Thus, if a protein lacks a signal sequence it is most likely cytoplasmic. Protein sequences were also screened by TMHMM server v2.0 for the presence of a transmembrane domain, which allows a protein to reside in the IM or OM (Moller *et al.*, 2001).

Genes were also grouped according to genomic position as genes that form part of the same operon usually elicit related functions. Eight genes formed part of a remnant of an ETT2 (type III secretion system) pathogenicity island: *yqeJ*; *yqeK*; *ygeG*; *ygeF*; *ygeI*; *pbl*; *ygeK*; and *ygeN* (fig. 4.6B). The majority of proteins encoded by this operonic region are cytoplasmic: YqeF; YqeH; YqeK; YgeF; YgeG; YgeH; YgeI; YgeK; YgeN; and YgeO. However, the proteins YqeG, YqeJ and Yqel are embedded or partially embedded in the IM and Pbl is periplasmic. In addition, Pbl is a transglycosylase with a peptidoglycan binding domain. YqeI, YqeH, YgeH and YgeK are putative DNA-binding transcriptional regulators, while YgeG contains a tetratricopeptide repeat (TPR), which is a structural motif that is utilised to mediate protein-protein interactions, suggesting this protein acts as a chaperone (Ren *et al.*, 2004).

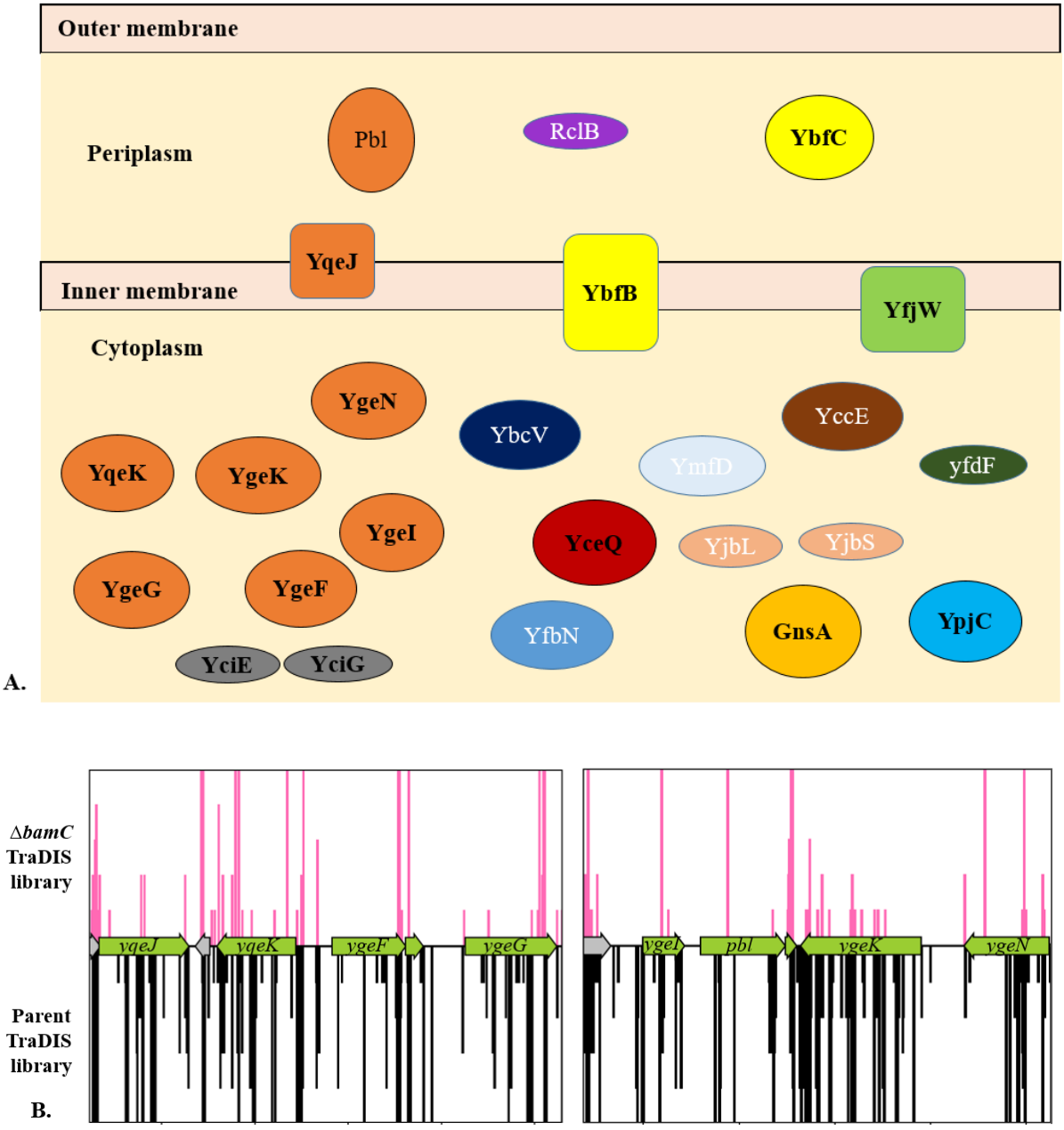


Fig. 4.6 Classification of genes of unknown function. (A) Genes were grouped according to location of the gene product in the cell, by screening the protein sequences for the presence of transmembrane domains (membrane embedded) and signal sequences (periplasmic proteins). Genes belonging to the same operon or operonic region were given the same colour. (B) A number of conditionally essential genes identified in the Δ*bamC* TraDIS library were genes belonging to an ETT2 (type III secretion system) pathogenicity island.

This genomic region is fully present in pathogenic strains such as *E. coli* O157:H7 (Ren *et al.,* 2004). The tool xBASE was utilised to determine if the presence of this pathogenicity island was conserved amongst *E. coli* phylogroups (Chaudhuri *et al.,* 2007). The pathogenicity island was at least partially present in the *E. coli* phylogroups A, B1, D1, D2 and E. However, it was absent from the B2 phylogroup, which consists of *E. coli* CFT073, *E. coli* LF82 and *E. coli* O83 H1 NRG 857C (Chaudhuri *et al.,* 2012). This operonic region was further discussed in section 4.2.7.

The genes *yjbL* and *yjbS* form another operon of interest. However, no information was found regarding this operon in the literature. In addition, the genes *ybfB* and *ybfC* encode a 327 bp IM protein and a 570 bp periplasmic protein, respectively. These genes occur in neighbouring operons with the genes *rhsC* and *ybfO*. Furthermore, the conditionally essential genes *yciE* and *yciG* form an operonic region with *yciF*. These genes are cytoplasmic stress response genes that are induced under osmotic stress conditions (Weber *et al.,* 2006).

Some of these uncharacterised genes and operons might be associated with the function of BamC. However, further characterisation is required to determine the functional connection between *bamC* and these genes of unknown function. Nevertheless, these results indicate that even though loss of BamC elicits no measurable phenotypic effect on the cell, it instead produces a significant genomic effect. This suggests that BamC elicits a key function in the cell, which was overlooked in previous studies.

4.2.6. *dapF* is a synthetic lethal partner of *bamC*

The gene *dapF* is conditionally essential in a mutant devoid of BamC (fig. 4.7A). This gene encodes the product diaminopimelate epimerase, which catalyses the stereoinversion of LL-diaminopimelate (LL-DAP) to *meso*-diaminopimelate (*meso*-DAP), the penultimate step in lysine biosynthesis. *Meso*-DAP is either decarboxylated by LysA to produce L-lysine or it

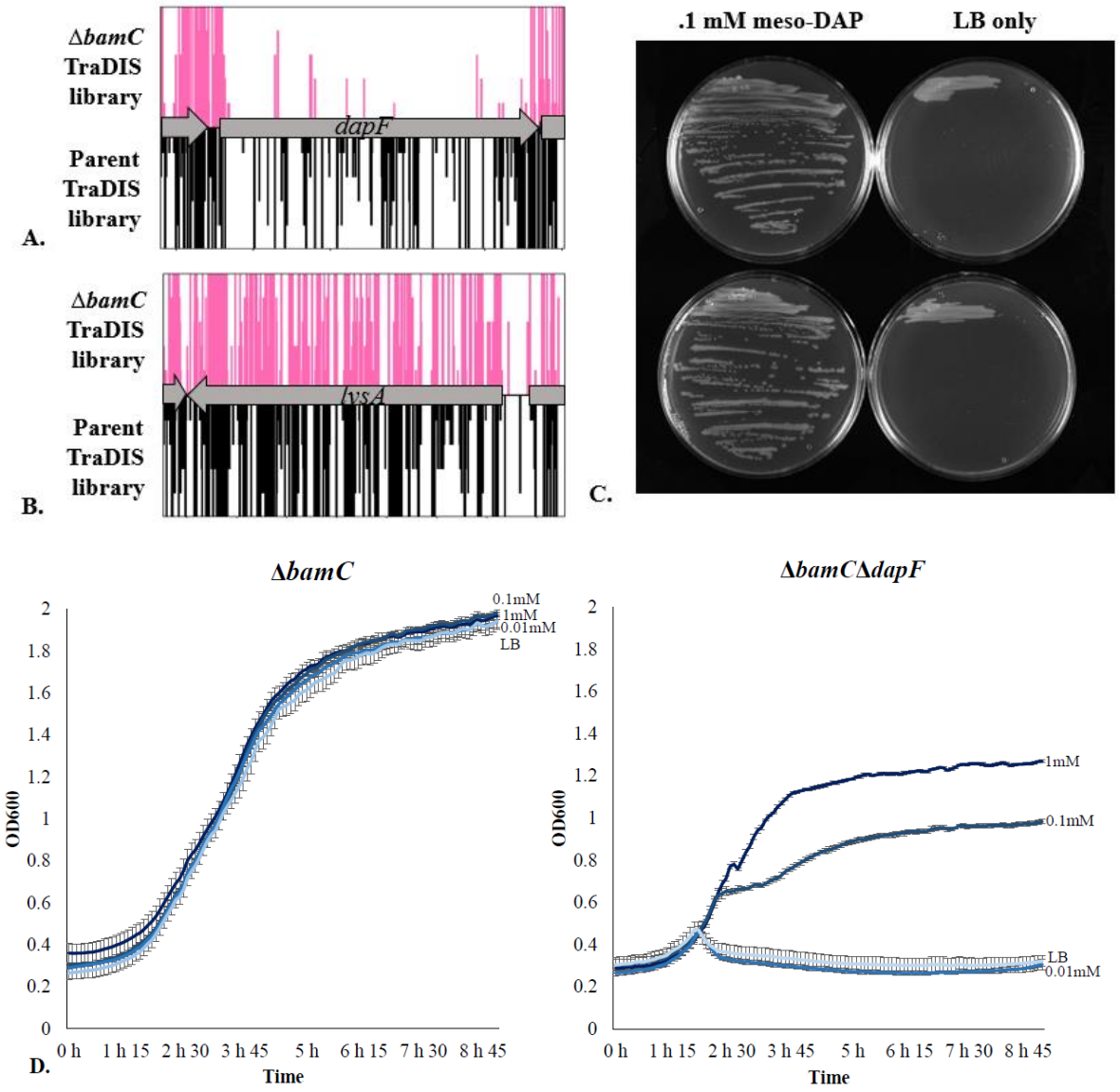


Fig. 4.7 The gene *dapF* is a synthetic lethal partner of *bamC*. (A) In the Δ*bamC* TraDIS library, the gene *dapF* was identified as conditionally essential. (B) The gene *lysA* was not required for the survival of the Δ*bamC* mutant. (C) The Δ*bamC*Δ*dapF* double mutant grew on LB agar supplemented with 0.1 mM *meso*-DAP. In the absence of *meso*-DAP, the Δ*bamC*Δ*dapF* mutant did not grow on LB agar, with the exception of the point of inoculation. (D) The growth of the single Δ*bamC* mutant was not affected by the presence and absence of different concentrations of *meso*-DAP, while the Δ*bamC*Δ*dapF* mutant did not grow in LB media that lacked *meso*-DAP. The growth of the Δ*bamC*Δ*dapF* mutant was partially restored in LB supplemented with *meso*-DAP.

is utilised in the biosynthesis of peptidoglycan (Dewey and Work, 1952; Richaud *et al.*, 1987). However, the gene *lysA* was non-essential in the Δ*bamC* TraDIS library (fig. 4.7B). Consequently, the gene *dapF* is required for the survival of the Δ*bamC* mutant due to its role in the synthesis of peptidoglycan.

A Δ*bamC*Δ*dapF* double mutant was constructed in the presence of 1 mM *meso*-DAP and confirmed by PCR. To ensure that the growth of this double mutant was restored by *meso*-DAP, the mutant was re-streaked onto LB agar and LB agar supplemented with 0.1 mM *meso*-DAP. The Δ*bamC*Δ*dapF* mutant grew on the entire plate supplemented with *meso*-DAP, while in the absence of *meso*-DAP, growth occurred only at the point of inoculation (fig. 4.7C). This study also determined the effect of various concentrations of *meso*-DAP on the growth of the Δ*bamC*Δ*dapF* mutant. A plate-reader was used to monitor the growth of the Δ*bamC* and Δ*bamC*Δ*dapF* mutants in the presence and absence of various concentrations of *meso*-DAP: 1 mM *meso*-DAP; 0.1 mM *meso*-DAP; or 0.01 mM *meso*-DAP. The growth of the double mutant was partially restored by the *meso*-DAP, in a concentration dependent manner (fig. 4.7D).

Peptidoglycan (PG) usually contains 50% LL-DAP and 50% *meso*-DAP (Mengin-Lecreylx *et al.*, 1988). A Δ*dapF* mutant accumulates LL-DAP and lacks *meso*-DAP, which is not required for growth. However, loss of *meso*-DAP decreases crosslinking of PG (Richaud *et al.*, 1987). Consequently, it can be suggested that PG crosslinks become vital in a Δ*bamC* mutant. The identification of *dapF* as a synthetic lethal partner of *bamC* offers the potential to develop an assay to probe the function of BamC.

4.2.7. Comparison of the essential gene profiles between the BAM subunits

Loss of BamC produces the least physical effect on the cell of all of the BAM subunits. However, the highest number of genes identified as conditionally essential was in the Δ*bamC*

TraDIS library (fig. 4.8). Rigel *et al.* (2012) previously proposed that BamC and BamE elicit overlapping roles in stabilizing the interactions between BamA and BamD. However, genes that encode gene products that share overlapping roles should contain similar synthetic lethal partners and as a result similar conditionally essential gene profiles. Consequently, it is surprising that the number of conditionally essential genes identified by the Δ*bamC* and Δ*bamE* TraDIS libraries was quite different (fig. 4.8). In addition, the contents of these essential gene lists were quite different (fig. 4.9). These results suggest that these proteins elicit functionally distinct roles in the cell.

Furthermore, the lack of similarities between the Δ*bamB* and Δ*bamE* datasets suggests that there is no functional redundancy between these genes (fig. 4.9). Similarly, there was also a lack of similarities between the Δ*bamB* and Δ*bamC* datasets. However, all three TraDIS libraries shared five conditionally essential gene candidates: *ygeF*; *pbl*; *ygeN*; *secB*; and *rnpA*. Of these five genes, *ygeF*, *pbl* and *ygeN* belong to the ETT2 (type III secretion system) pathogenicity island, which is discussed in section 4.2.5 As mentioned in chapter three, over reporting of essential genes can occur when non-essential genes have low insertion index scores. In the parent TraDIS library, very few mutants were recovered with transposon insertions in the ETT2 pathogenicity island. This suggests that the lack of transposon insertions in all of the BAM mutant TraDIS libraries might be due to inaccessibility of the operonic region to transposition. This could be due to exclusion by DNA-binding proteins or due to unusual DNA structure that are not easily available for transposon insertion (Goodall *et al.,* 2018). Thus, it is unlikely that this operonic region elicits a downstream role or effect on OMP biogenesis.

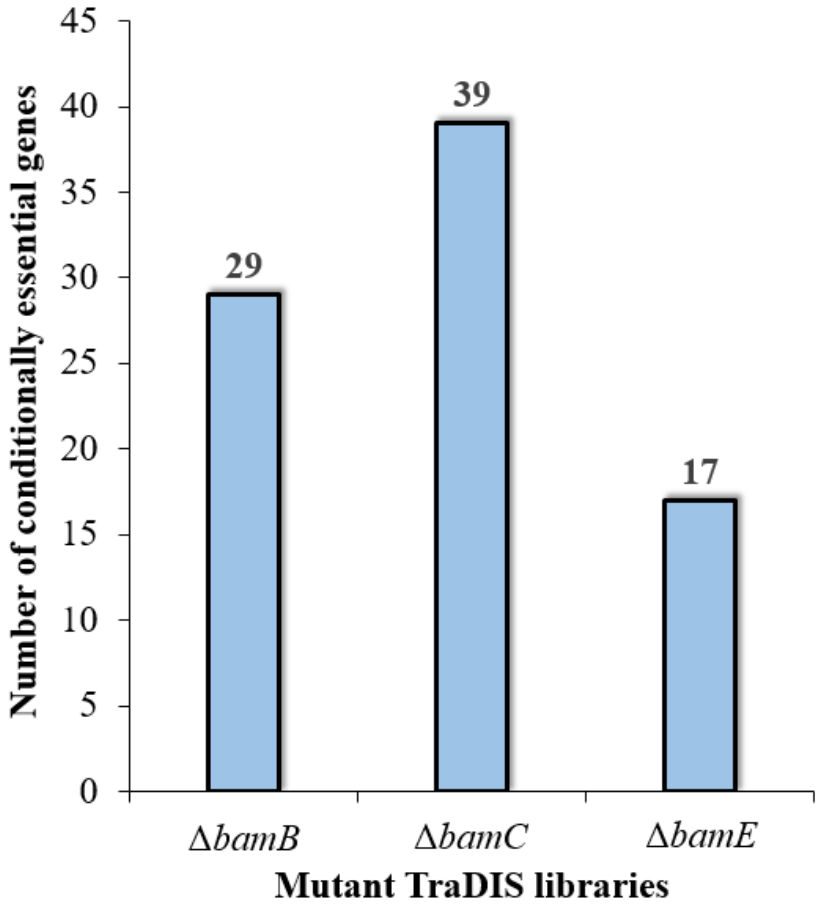


Fig. 4.8 Conditionally essential genes identified by the Δ*bamB,* Δ*bamC and* Δ*bamE* TraDIS libraries. The number of conditionally essential genes identified by each of the mutant TraDIS libraries**.** In the Δ*bamB* TraDIS library, 29 genes were identified as conditionally essential. In the Δ*bamC* TraDIS library, 39 genes were identified as conditionally essential, while in the Δ*bamE* TraDIS library, 17 genes were identified as conditionally essential.

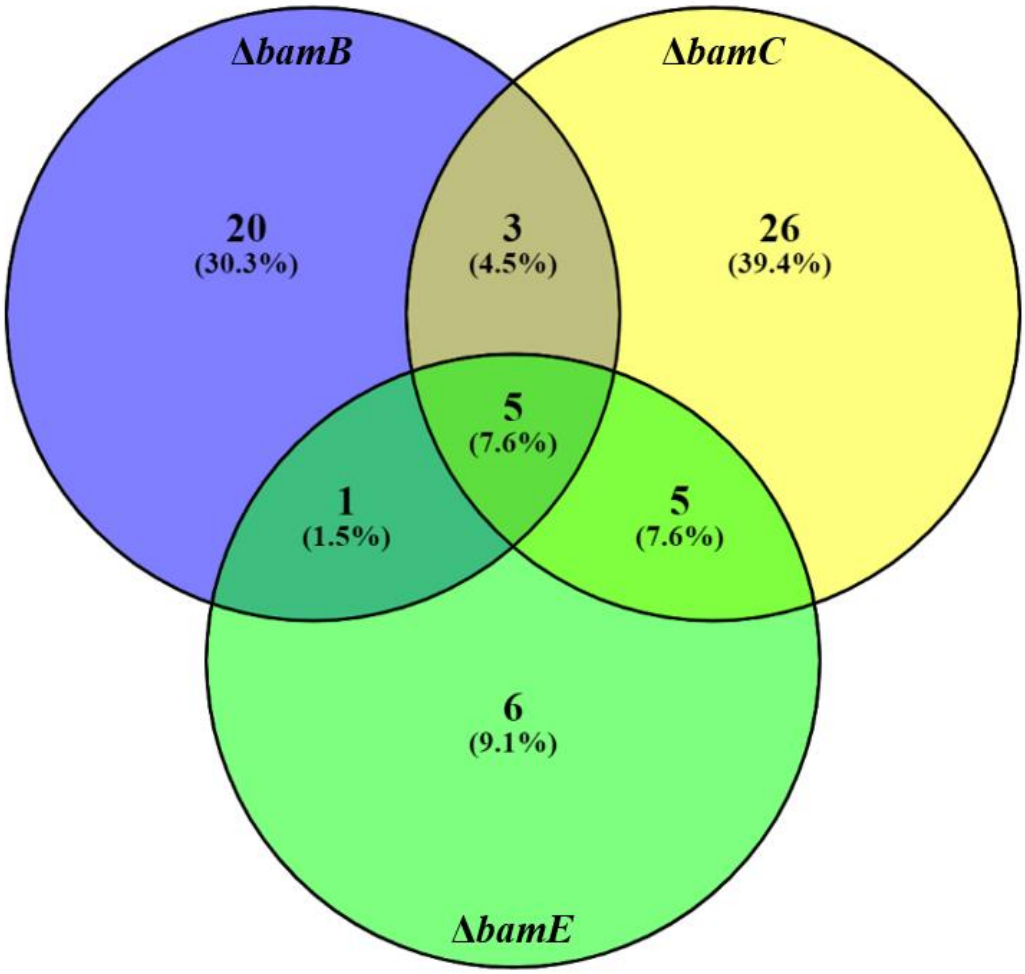


Fig. 4.9 Comparison of essential genes shared between the Δ*bamB*, Δ*bamC and* Δ*bamE* TraDIS datasets. The essential genes were compared between mutant TraDIS libraries to identify overlapping genes. All three datasets shared five essential gene candidates.

4.2.8. Identification of mutations that supress the effects of Δ*bamE*

Suppressor mutations are secondary mutations that can restore the phenotype of a specific mutant to that of the original parent strain. This study attempted to identify potential suppressors of *bamC* and *bamE*. Mutants defective in one or several BAM components are more susceptible to a range of antibiotics including vancomycin (Namdari *et al*., 2005; Vuong *et al*., 2008). Consequently, vancomycin was tested as a potential suppressor screen for the mutants of interest.

4.2.8.1. Screening of Δ*bamC* and Δ*bamE* mutants on vancomycin. The Δ*bamC* and Δ*bamE* mutants were exposed to a number of different concentrations of vancomycin to determine the minimum concentration required that inhibited the growth of these mutants but supported the growth of the parent strain. Growth of the Δ*bamE* mutant was inhibited on LB agar supplemented with 120 µg/ml of vancomycin (fig. 4.10A). Conversely, the Δ*bamC* mutant was susceptible to the same concentrations of vancomycin as the parent (fig. 4.10B). Consequently, a number of other conditions were tested, which included SDS, SDS and EDTA, ampicillin, amoxicillin and rifamycin. However, this study did not find a viable screen that could identify potential gene-deletion suppressors of *bamC*. Thus, only the Δ*bamE* TraDIS library was screened for suppressors.

4.2.8.2. Screening of the Δ*bamE* TraDIS library on vancomycin. The Δ*bamE* TraDIS library was screened on two different concentrations of vancomycin: 240 µg/ml (2xMIC) and 180 µg/ml (1.5xMIC). However, bacterial growth was only detected on 180 µg/ml of vancomycin (fig. 4.10C). The parent TraDIS library was also exposed to vancomycin in an attempt to separate natural suppressors of vancomycin from suppressors of Δ*bamE*. The suppressor mutants were collected, pooled and sequenced to identify transposon insertion sites. Transposon insertion sites with fewer than 10 mapped reads were disregarded to filter for noise.

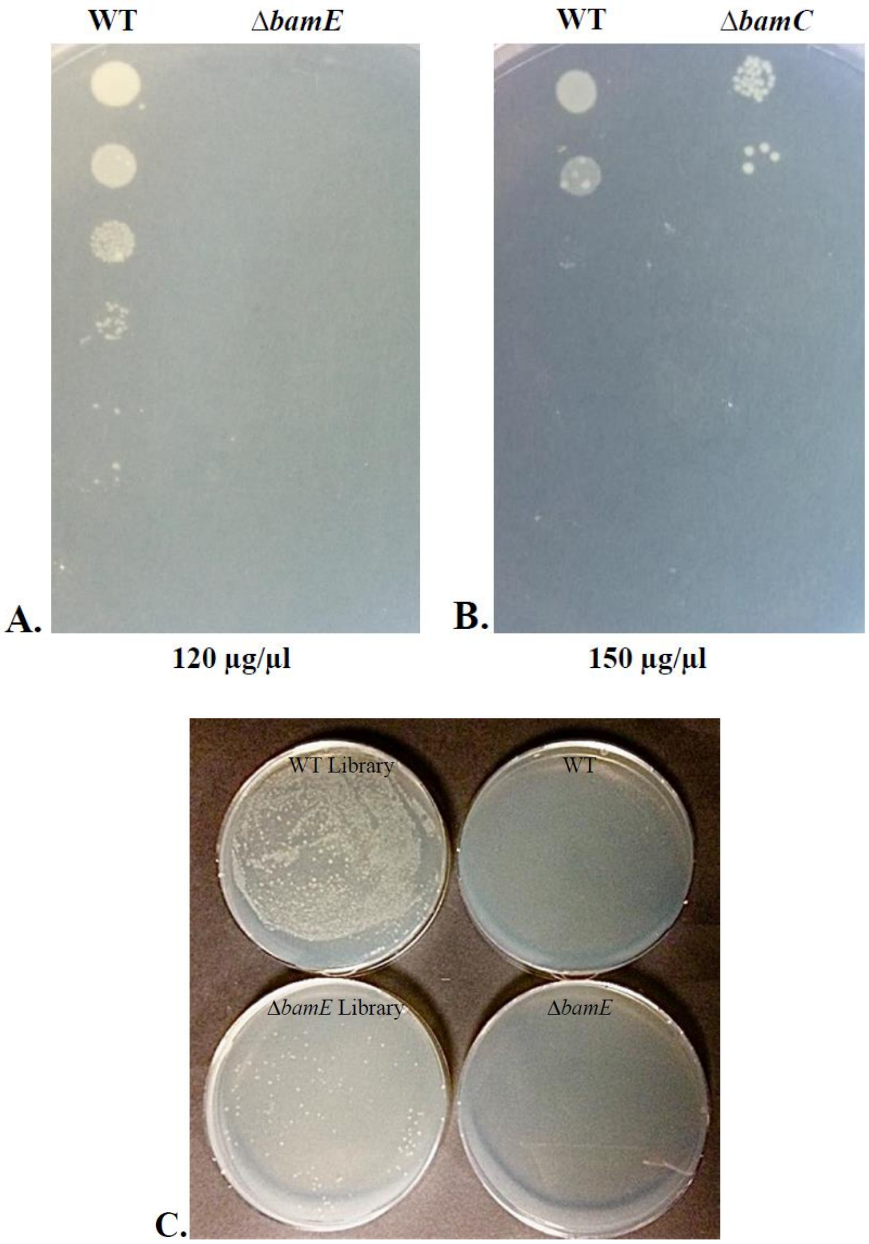


Fig. 4.10 Screening of the Δ*bamE* and Δ*bamC* mutants and the Δ*bamE* TraDIS library on vancomycin. (A) Growth of the Δ*bamE* mutant was inhibited on 120 µg/ml of vancomycin. (B) Growth of the Δ*bamC* mutant and the parent occurred on the same concentrations of vancomycin. (C) The Δ*bamE* TraDIS library, the parent TraDIS library, the background Δ*bamE* mutant and the parent were exposed to LB agar supplemented with 180 µg/ml of vancomycin. Suppressor colonies formed on the Δ*bamE* TraDIS library and the parent TraDIS library plates, while minimum to no growth occurred on the plates inoculated with the Δ*bamE* mutant or the parent strain.

Alternatively, genes with 10 or more mapped reads per insertion site along the length of the gene, which contained little to no transposon insertions in the parent TraDIS library, were considered to be potential gene-deletion suppressors of Δ*bamE*. All suppressor mutants recovered from the Δ*bamE* TraDIS library were also recovered from the parent TraDIS library. Thus, this study failed to identify any potential gene-deletion suppressors of Δ*bamE*. The lack of identification of suppressors suggests potential anti-bacterials that target the *bamE* gene would have low levels of bacterial resistance. Thus, the gene *bamE* is a good target in the development of potential anti-bacterials and has applications in the development of dual inhibitors and combination therapy, which are discussed in chapter seven.

4.3. Conclusion

BamC and BamE form part of a BAM sub-complex with BamD. To help determine the roles of BamC and BamE in the cell, the synthetic lethal partners of *bamC* and *bamE* were identified using TraDIS. Clear differences were identified between the Δ*bamC* and Δ*bamE* TraDIS libraries in respect to the number of conditionally essential genes identified and the contents of these essential gene lists. This clearly demonstrates that BamC and BamE elicit different functions, contrary to conclusions by Rigel *et al.* (2012). In the Δ*bamE* TraDIS library, 17 genes were identified as conditionally essential and 24 genes were identified as conditionally non-essential. In contrast, in the Δ*bamC* TraDIS library, 39 conditionally essential gene candidates and 29 conditionally non-essential genes were identified.

Loss of BamC produces the least physical effect on the cell out of all of the BAM subunits. However, in the Δ*bamC* TraDIS library, the highest number of conditionally essential gene candidates were identified. Over 65% of the genes identified as conditionally essential were genes of unknown function. These genes were classified based on location of the gene product in the cell and genomic position, which lead to the identification of a number of operonic regions of interest including a remnant of an ETT2 pathogenicity island. In addition, in the

Δ*bamC* TraDIS library, the synthetic lethal partner *dapF* was identified. Lastly, this study attempted to identify potential suppressors of Δ*bamC* and Δ*bamE*. No viable screen was found that could identify potential gene-deletion suppressors of Δ*bamC,* while no gene-deletion suppressors of the Δ*bamE* mutant were identified.

CHAPTER 5

The chaperones of the BAM complex

5.1. Introduction

Outer membrane proteins perform various essential functions, for example, they act as transporters, receptors, porins and enzymes (Fairman *et al.,* 2011). To fulfil these roles the OMPs require transportation and insertion into the OM. However, OMPs are prone to aggregation and must be maintained in a folding competent state during their translocation through the periplasm to the BAM complex (Ruiz *et al.,* 2006). The transport of OMPs to the BAM complex is not yet fully understood.

The three quality control factors that chaperone OMPs across the periplasm are SurA, Skp and DegP. Single mutants Δ*surA*, Δ*skp* and Δ*degP* are viable. However, double mutants Δ*surA*Δ*skp* and Δ*surA*Δ*degP* are inviable, which suggests functionally redundancy exists between SurA and Skp and between SurA and DegP (Rizzitello *et al.*, 2001). Sklar *et al.* (2007) proposed that SurA is the primary chaperone pathway to the BAM complex, while Skp and DegP together form a secondary pathway that is amplified in stress conditions. *E. coli* can withstand the loss of one pathway. However, loss of both pathways results in synthetic lethal phenotypes.

However, there is no molecular evidence to suggest that the functions of SurA and Skp are redundant. Some studies have even identified functional distinctions between SurA and Skp. The periplasm requires a steady state of holdase occupancy. A holdase is a form of molecular chaperone that binds to folding intermediates and prevents their aggregation but without directly refolding them. Chum *et al.* (2019) proposed that Skp acts as holdase by binding to unfolded OMPs and preventing their aggregation in the periplasm. Li *et al.* (2018) identified functional distinctions between Skp and SurA, including differences in the conformations of OmpC recognised and differences in their stoichiometries of binding. In addition, Skp is capable of converting OmpC aggregates into the monodisperse form, while SurA lacks this ability.

Consequently, despite extensive studies on the biogenesis of OMPs, the redundancy between these chaperones remains unclear. This chapter focuses on understanding the roles of these periplasmic chaperones in OM biogenesis and their mutual synthetic lethality. TraDIS libraries were constructed in Δ*surA*, Δ*skp* and Δ*degP* mutants to identify genes functionally connected to these mutants. The genes were classified as essential and non-essential based on methods discussed in chapters two and three. In addition, the identification and understanding of synthetic lethal partners is key in the recognition of bacterial pathways that could potentially be inhibited by combinational therapy. Combinational therapy is a proposed alternative to antibiotics, which utilises a mixture of compounds to block synthetically lethal partners simultaneously, leading to demise of the cell.

5.2. Results

5.2.1. Construction and sequencing of the Δ*surA*, Δ*skp* and Δ*degP* TraDIS libraries

Declaration: A previous student Karl Dunne constructed the Δ*surA* and Δ*skp* TraDIS libraries, while the author constructed the Δ*degP* TraDIS library discussed in this Thesis. Following TraDIS library construction, the author prepared and sequenced the samples. In addition, the author completed all of the analysis of the TraDIS libraries and any subsequent experiments, unless otherwise stated.

In the construction of the TraDIS libraries, transposon mutants were collected, pooled and sequenced. As previously discussed in chapter three, following sequencing, the data were checked for the presence of an inline index barcode. Short sequenced reads were removed and surviving reads were mapped onto the *E. coli* K-12 BW25113 genome. Analysis of the Δ*skp* TraDIS library identified over 368,000 unique insertion sites, which equates on average to a

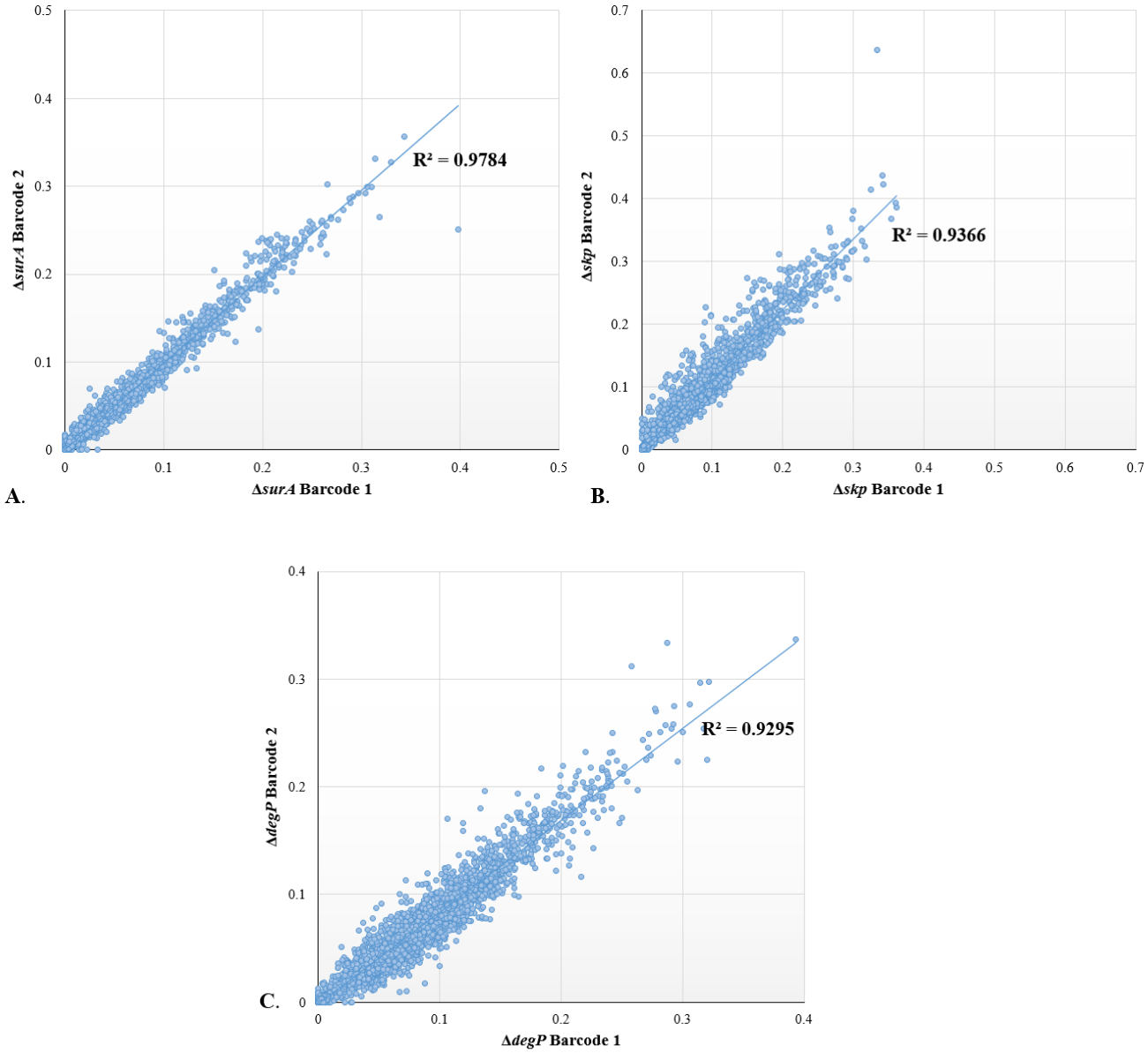


Fig. 5.1 Sequencing of independent replicates. The correlation between the gene insertion index scores for sequenced replicates of the (A) Δ*surA* TraDIS library, (B) Δ*skp* TraDIS library and (C) Δ*degP* TraDIS library. The respective R-squared values for the Δ*surA*, Δ*skp* and Δ*degP* TraDIS libraries were 0.98, 0.94 and 0.93, respectively. Thus, there was a high correlation between replicates and as a result, replicate data were pooled.

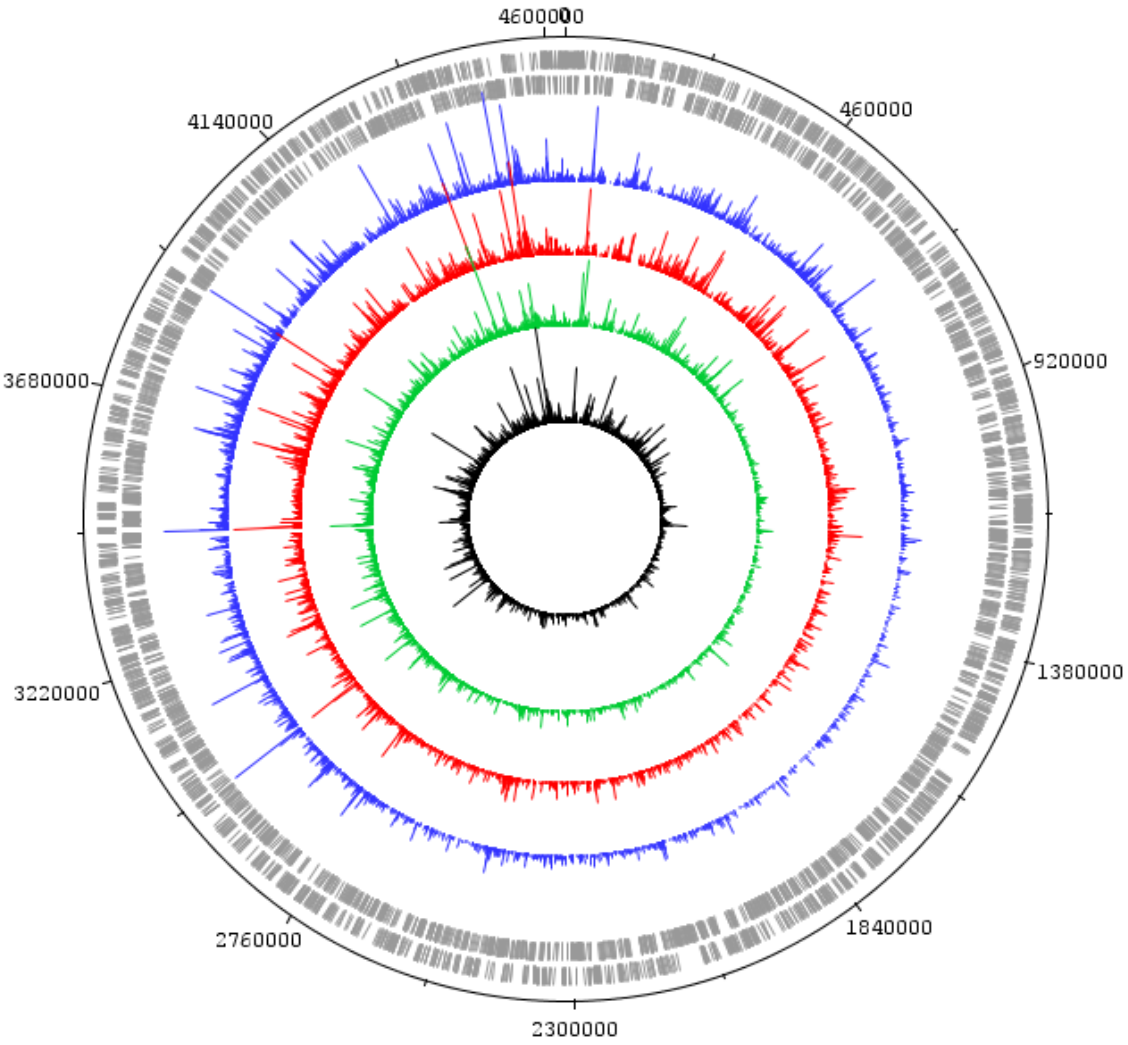


Fig. 5.2 The frequency and location of transposon insertion sites throughout the Δ*surA*, Δ*skp*, Δ*degP* and parent TraDIS libraries generated using DNAPlotter. The outer track marks the BW25113 genome in base pairs, while the next two inner tracks correspond to sense and antisense CDS (grey). The four inner tracks correspond to the frequency and location of transposon insertions in the Δ*surA* TraDIS library (blue), Δ*skp* TraDIS library (red), Δ*degP* TraDIS library (green) and the parent TraDIS library (black), mapped to the BW25113 genome (CP009273.1).

transposon insertion site occurring every 12.6 base pairs. The Δ*degP* TraDIS library comprised of ~1 million mutants and the analysis identified 667,793 unique transposon insertion sites, which corresponds to a transposon insertion site approximately every 6.9 base pairs throughout the genome**.** Analysis of the Δ*surA* TraDIS library identified over 257,000 unique transposon insertion sites, which equates to a transposon insertion site approximately every 18 base pairs throughout the genome.

For an internal measure of quality control, independent replicates of each TraDIS library were sequenced and the gene insertion index scores were compared between replicates. The correlation coefficients (R-squared value) for the Δ*surA*, Δ*skp* and Δ*degP* TraDIS libraries were 0.98, 0.94 and 0.93, respectively (fig. 5.1). This indicates that there was a high correlation between replicates. Consequently, replicate data were pooled and the sequenced transposon insertion sites for the Δ*surA*, Δ*skp* and Δ*degP* TraDIS libraries were mapped to the reference *E. coli* BW25113 genome. With the exception of an increased density occurring around the origin, the transposon insertion sites were mapped evenly throughout the genome (fig. 5.2). In addition, the frequency of insertion index scores were plotted as bimodal histograms for each mutant TraDIS library (appendix 5.1).

5.2.2. Identification of conditionally essential genes in the Δ*skp*, Δ*degP* and Δ*surA* TraDIS libraries

Mutant TraDIS libraries were compared to the parent TraDIS library to identify conditionally essential genes. There was an overlap of 270 essential genes between the Δ*skp* and the parent TraDIS dataset (fig. 5.3A). These genes were not investigated further. In the Δ*skp* TraDIS library, nine genes were classified as conditionally essential (table 5.1). Of the nine genes, two act as regulators (*csrA* and *mgrB*), one was *surA* and six were genes of unknown function.

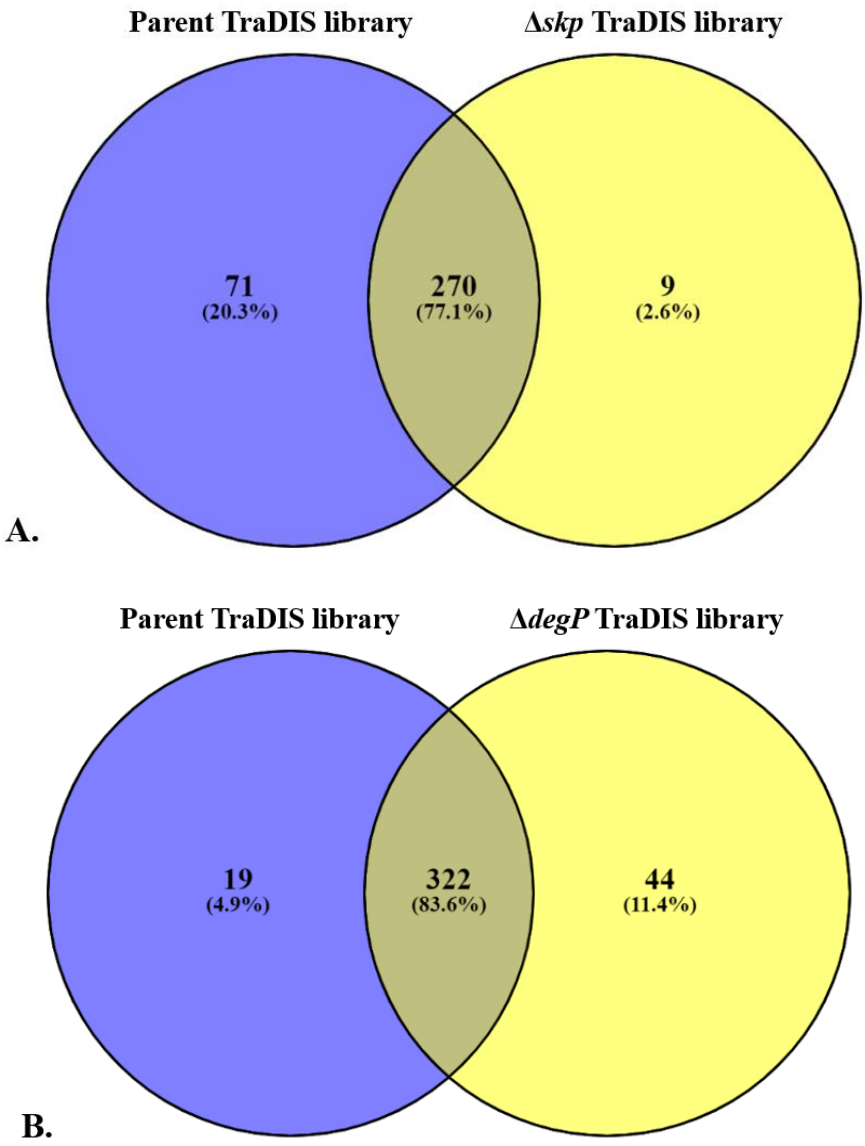


Fig. 5.3 Comparison of essential gene candidates shared between the Δ*skp*/Δ*degP* and parent TraDIS datasets. The essential genes identified in the Δ*skp*/Δ*degP* TraDIS library and the parent TraDIS library were compared. (A) The Δ*skp* and the parent TraDIS datasets shared 270 essential genes. In the Δ*skp* TraDIS library, an additional nine genes were identified as essential, while in the parent TraDIS library, an additional 71 genes were classified as essential. (B) The Δ*degP* and the parent TraDIS datasets shared 322 essential gene candidates. In the Δ*degP* TraDIS library, 44 conditionally essential genes and 19 conditionally non-essential genes were identified.

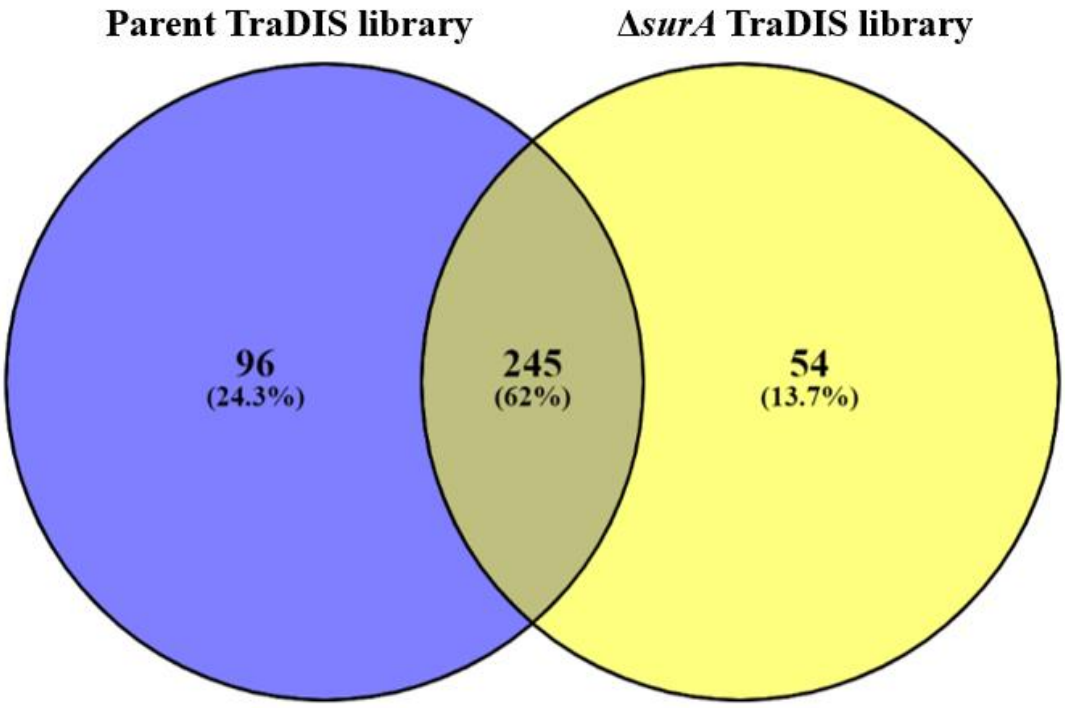


Fig. 5.4 Conditionally essential genes in the Δ*surA* TraDIS library compared to the parent TraDIS library. The essential genes identified in the Δ*surA* and the parent TraDIS libraries were compared (A). Both datasets shared 245 essential genes. In the Δ*surA* TraDIS library, 54 genes were identified as conditionally essential and 96 genes were identified as conditionally non-essential.

Table 5.1 Conditionally essential genes identified in the Δ*skp* TraDIS library

Name of Gene	Gene Function
csrA	A regulator of carbohydrate metabolism that affects glycogen biosynthesis, gluconeogenesis, glycolysis and glycogen degradation (Sabnis *et al.*, 1995; Yang *et al.*, 1996).
iroK	Function unknown.
mgrB	An IM protein that negatively regulates the activity of PhoQ.
surA	A periplasmic chaperone that transports OMPs to the BAM complex.
ydfR	Function unknown.
ydiE	This gene belongs to the Fur regulon and might play a role in heme trafficking (Nishimura *et al.*, 2015; McHugh *et al.*, 2003)
ydiH	Function unknown.
yebG	A DNA damage-inducible gene that forms part of the SOS regulon (Lomba *et al.*, 1997).
yncJ	Function unknown.

*Where no reference was given the functions were taken from the Ecocyc website: https://ecocyc.org/

Table 5.2 Genes identified as conditionally essential in the Δ*degP* TraDIS library

Name of Gene	Gene Function
aceF	AceF is the core component of the pyruvate dehydrogenase multienzyme complex (Angelides *et al.*, 1979).
arcA	A cytosolic transcription factor of the ArcAB two-component system.
bamB	A non-essential subunit of the BAM complex.
cmk	Cmk rephosphorylates CMP and dCMP produced by the turnover of nucleic acids and CDP diglycerides.
csrA	A regulator of carbohydrate metabolism (Sabnis *et al.*, 1995; Yang *et al.*, 1996).
degS	DegS senses and reacts to damaged and mislocalised proteins.
fur	A DNA-binding transcriptional dual regulator.
galU	GalU catalyses the synthesis of UDP-D-glucose from UTP and α-D-glucose 1-phosphate (Hossain *et al.*, 1994; Weissborn *et al.*, 1994).
glmS	GlmS catalyses the first step in hexosamine biosynthesis.
gmhA	GmhA catalyses the first step in the heptose synthesis pathway.
gmhB	GmhB catalyses an intermediate reaction in the heptose synthesis pathway.
higA	The antitoxin of the toxin HigB (Christensen-Dalsgaard *et al.*, 2010).
hldD (*waaD*)	HldD catalyses the last step in the heptose synthesis pathway (Kneidinger *et al.*, 2002).
hldE	HldE catalyses two intermediate reactions in the heptose synthesis pathway.
holD	DNA polymerase III subunit **Ψ.**
mgrB	A small protein that negatively regulates the activity of PhoQ (Lippa and Goulian, 2009).
minE	MinE forms part of the MinC/D/E system, which directs septation to the central site in diving cells (Boer *et al.*, 1989).
pbl	A transglycosylase that forms part of a remnant of an ETT2 (type III secretion system) pathogenicity island (Ren *et al*., 2004).
pgm	Pgm is involved in the breakdown of glycogen and metabolism of galactose and maltose (Lasserre *et al.*, 2006).
pnp	Polynucleotide phosphorylase (PNPase) is involved in general mRNA degradation.
prc	A periplasmic protease involved in the processing and degradation of a number of proteins (Park *et al.*, 1988).
proQ	A RNA chaperone that elicits RNA duplexing and strand exchange activity.
psd	Phosphatidylserine decarboxylase catalyses the formation of the phospholipid phosphatidylethanolamine.
secB	The periplasmic chaperone component of the SEC machinery.
sucA	SucA is involved in the conversion of 2-oxoglutarate to succinyl-CoA and CO_2 in the tricarboxylic acid (TCA) cycle.

sucB	SucB is involved in the conversion of 2-oxoglutarate to succinyl-CoA and CO_2 in the TCA cycle.
surA	A periplasmic OMP chaperone.
tolA	A component of the Tol-Pal system that interacts with TolQ and TolR (Derouiche *et al.*, 1995).
tolB	A periplasmic component of the Tol-Pal system (Derouiche *et al.*, 1995).
tolQ	An IM component of the Tol-Pal system (Derouiche *et al.*, 1995).
tolR	An IM component of the Tol-Pal system.
waaC	WaaC transfers the first heptose into the inner core of LPS (Gronow *et al.*, 2000).
waaF	WaaF transfers the second heptose into the inner core of LPS (Gronow *et al.*, 2000).
yajC	YajC forms a complex with SecD, SecF and YidC, which together stabilize the insertion of SecA (Nouwen and Driessen, 2002).
yceQ	Function unknown.
yciG	Part of the σ^S regulon (Weber *et al*., 2005).
yciM (*lapB*)	LapB might be involved in LPS biosynthesis and transport.
yciS (*lapA*)	LapA might be involved in the transport of LPS (Klein *et al.*, 2014).
yciU	Function unknown.
yddM	A putative DNA-binding transcriptional regulator (Gao *et al.*, 2018).
yecJ	Function unknown.
ygeF	Forms part of a remnant of an ETT2 pathogenicity island (Ren *et al*., 2004).
ygeN	Unknown function, remnant of an ETT2 (type III secretion system) pathogenicity island.
zwf	The gene *zwf* encodes glucose-6-phosphate 1-dehydrogenase, which is involved in the pentose phosphate pathway.

*Where no reference was given the functions were taken from the Ecocyc website: https://ecocyc.org/

Table 5.3 Conditionally essential genes identified in the Δ*surA* TraDIS library

Name of Gene	Gene Function
azoR	AzoR is involved in the protection against thiol-specific stress (Liu *et al.*, 2009).
bhsA	BhsA is involved in biofilm formation and stress response (Zhang *et al.*, 2007).
cmk	Cmk rephosphorylates CMP and dCMP.
def	Def releases the formyl group from the amino terminal methionine residue of most nascent proteins (Meinnel and Blanquet, 1995).
degS	DegS is a protease that senses and reacts to damaged and mislocalised proteins (Alba *et al.*, 2002).
fadR	FadR is a multifunctional dual regulator, regulating fatty acid biosynthesis and fatty acid degradation (DiRusso *et al.*, 1993).
gloA	GloA catalyses the first two steps in the conversion of methylgloxal to D-lactate.
gmhA	GmhA catalyses the first step in the synthesis of heptose.
hldD	HldD catalyses the last step in the synthesis of heptose (Kneidinger *et al.*, 2002).
hldE	HldE catalyses two reactions in the heptose synthesis pathway.
ihfA	Function unknown.
insP	Function unknown.
iroK	Function unknown.
kilR	The overexpression of KilR inhibits cell division and leads to morphological defects (Burke *et al.*, 2013; Conter *et al.*, 1996).
lpxM	LpxM is involved in the synthesis of Kdo_2-lipid A.
lsrR	LsrR regulates the expression of genes involved in stress responses, host invasion and biofilm formation (Li *et al.*, 2007).
nusA	NusA is involved in the prevention and enhancement of transcription termination.
pbl	Transglycosylase that is a remnant of a type III secretion system (Ren *et al.*, 2004)
pgm	Pgm is involved in the breakdown of glycogen and metabolism of galactose and maltose (Lasserre *et al.*, 2006).
prc	A tail-specific protease involved in the processing and degradation of a number of proteins (Park *et al.*, 1988).
pspC	PspC activates the expression of the *psp* operon (Brissette *et al.*, 1991).
racC	Function unknown.
rlmH	A methyltransferase.
rsfS	A ribosomal silencing factor that inhibits assembly of the 70S ribosome.
ruvB	RuvB mediates branch migration of Holliday junctions.
secB	A cytoplasmic chaperone that forms part of the SEC pathway.

skp	A periplasmic chaperone to the BAM complex.
smrA	SmrA binds dsDNA and elicits endonuclease activity (Gui *et al.*, 2011).
sodC	SodC is a superoxide dismutase that converts superoxide radicals to hydrogen peroxide and water (Groote *et al.*, 1997).
tpr	Function unknown.
waaC	WaaC transfers the first heptose into inner core of LPS (Gronow *et al.*, 2000).
wecB	WecB is involved in the synthesis of a building block of enterobacterial common antigen (ECA).
wecC	WecC is involved in the ECA biosynthesis pathway.
wecD	WecD is involved in the ECA biosynthesis pathway.
wecE	WecE is involved in the synthesis of Fuc4NAc, a component of ECA.
wecF	WecF is involved in the ECA biosynthesis pathway.
wzxE	WzxE flips the ECA molecules across the IM.
yajC	YajC forms a complex with SecD, SecF and YidC, which together stabilize the insertion of SecA (Nouwen and Driessen, 2002).
yciM (lapB)	LapB might be involved in LPS biosynthesis and transport.
yciS (lapA)	LapA might be involved in LPS transport (Klein *et al.*, 2014).
yciU	Function unknown.
ydaV	Function unknown.
ydcX (ortT)	A toxin that damages the cell membrane and reduces the intracellular ATP level (Islam *et al*., 2015)
yddM	Putative DNA-binding transcriptional regulator (Gao *et al.*, 2018).
ydfR	Function unknown.
ydhL	Function unknown.
ydhR	Function unknown.
ydiE	A gene that belongs to the Fur regulon (McHugh *et al*., 2003).
ygeF	Forms part of a remnant of an ETT2 (type III secretion system) pathogenicity island, with an unknown function (Ren *et al*., 2004)
yjbS	Function unknown.
yoaK	Function unknown.
yohO	Function unknown.
yrbN	Function unknown.
zwf	The gene *zwf* encodes glucose-6-phosphate 1-dehydrogenase, which is involved in the pentose phosphate pathway.

*Where no reference was given, the functions were taken from the Ecocyc website: https://ecocyc.org/

Barring *surA*, all of these genes encode proteins that are ≤103 amino acids, with the average being 66 amino acids. This suggests that some of these genes were incorrectly classified as essential due to stochastic gaps in the library. In addition, in the Δ*skp* TraDIS library, 71 genes were classified as conditionally non-essential (S. table 5.1). The essential genes identified in the Δ*degP* TraDIS library were compared to the essential genes identified in the parent TraDIS library. There was an overlap of 322 essential genes between the two datasets (fig. 5.3B). In the Δ*degP* TraDIS library, 44 genes were identified as conditionally essential (table 5.2), while 19 genes were classified as conditionally non-essential (S. table 5.2). In contrast, there was an overlap of 245 essential genes between the Δ*surA* and the parent TraDIS datasets (fig. 5.4). In the Δ*surA* TraDIS library, the remaining 54 essential genes were identified as conditionally essential (table 5.3). In addition, 96 genes were classified as conditionally non-essential (S. table 5.3).

5.2.3. Comparison of the essential gene profiles between the BAM chaperones

Contrary to Sklar *et al.* (2007), there no molecular evidence to suggest that the functions of SurA and Skp are redundant. To help understand this synthetic lethality, the number of conditionally essential genes and the contents of these essential gene lists were compared between the Δ*surA*, Δ*skp* and Δ*degP* TraDIS datasets. The TraDIS data confirmed the synthetic lethality pairings between *surA* and *skp* and between *surA* and *degP* (fig. 5.5A-B). However, different numbers of conditionally essential genes were identified in each mutant TraDIS library. Of the three chaperones investigated, the highest number of conditionally essential genes were identified in the Δ*surA* TraDIS library (54), while the number of conditionally essential genes identified in the Δ*degP* and Δ*skp* TraDIS libraries were 44 and 9, respectively (fig. 5.5C).

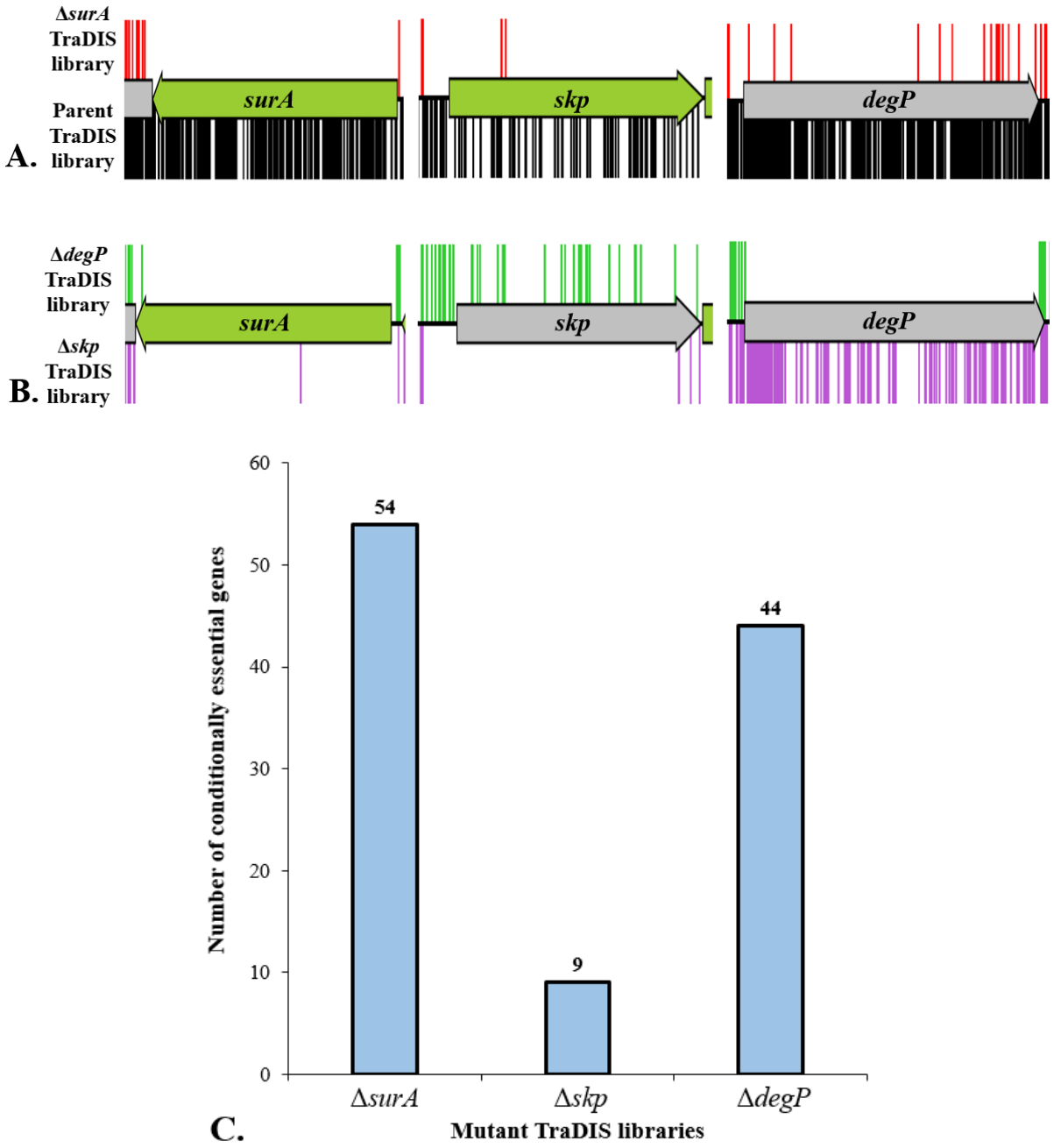


Fig. 5.5 Synthetic lethality between the BAM chaperones. (A) In the Δ*surA* TraDIS library, the gene *skp* was conditionally essential. In the Δ*surA* TraDIS library, the analysis failed to identify the gene *degP* as conditionally essential due to the occurrence of a number of transposon insertions in the N-terminal of this gene. In the parent TraDIS library, the genes *surA*, *skp* and *degP* were non-essential. The transposon cut-off was set to one. (B) In the Δ*skp* and Δ*degP* TraDIS libraries, the gene *surA* was conditionally essential. (C) The number of conditionally essential genes identified in the Δ*surA*, Δ*skp* and Δ*degP* TraDIS libraries.

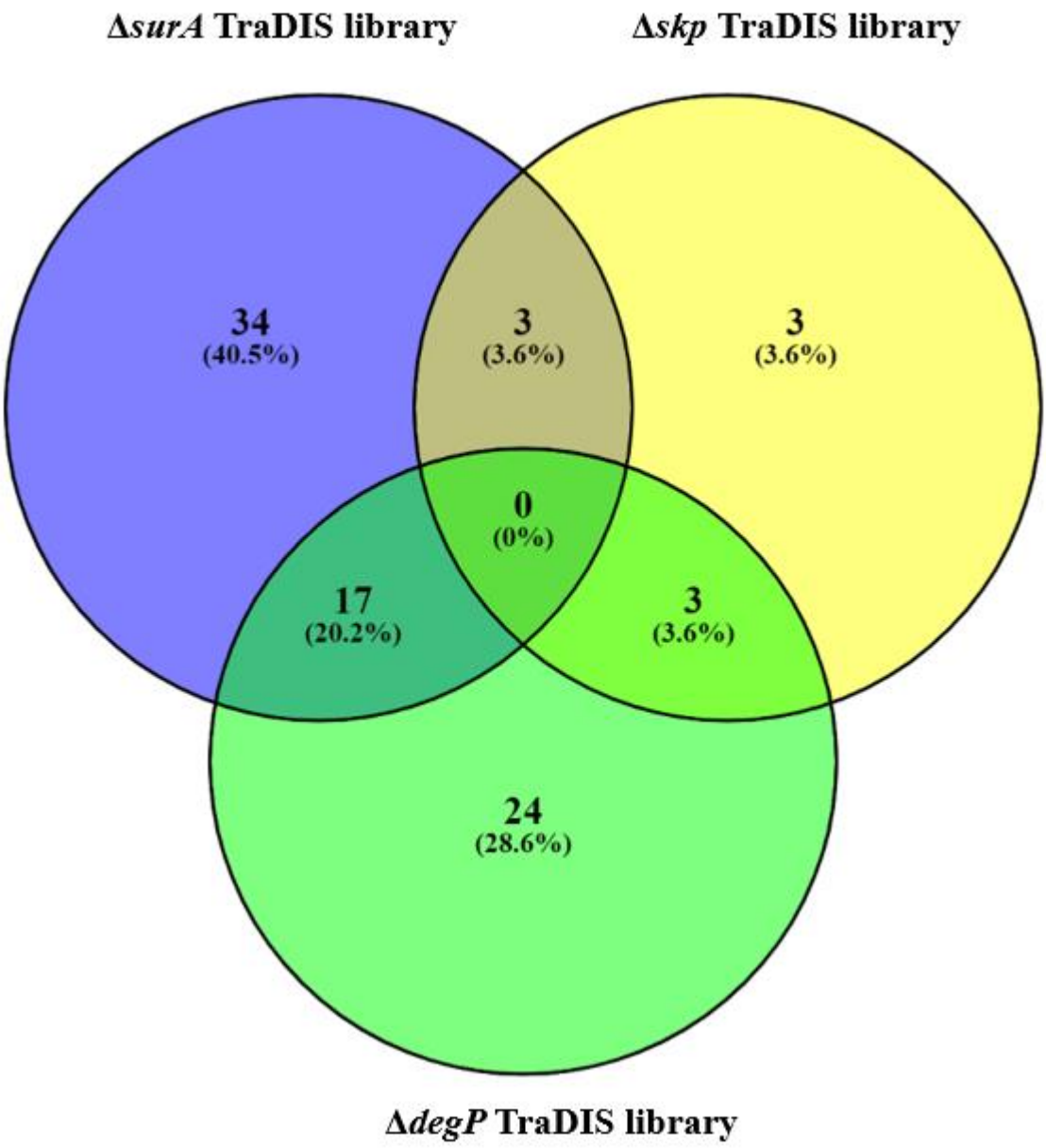


Fig. 5.6 Conditionally essential genes shared between the Δ*surA*, Δ*skp* and Δ*degP* TraDIS datasets. The conditionally essential genes identified by each of the mutant TraDIS libraries were compared to identify overlapping genes. No conditionally essential genes were shared by all three datasets. The Δ*surA* and Δ*degP* datasets shared 17 conditionally essential genes, while the Δ*surA* and Δ*skp* datasets shared only three conditionally essential gene candidates.

In addition, there were a number of differences in the contents of the essential gene lists between the Δ*surA* and Δ*skp* datasets and between the Δ*degP* and Δ*skp* datasets (fig. 5.6). The Δ*surA* and Δ*skp* datasets shared only three conditionally essential genes, while the Δ*skp* and Δ*degP* datasets also only shared three conditionally essential genes. A number of genes that were essential in both the Δ*surA* and Δ*degP* datasets were non-essential in the Δ*skp* mutant (17 genes). For example, genes involved in the synthesis of the inner core of LPS (*gmhA, hldE, hldD* and *waaC*); genes that form part of the SEC machinery (*yajC* and *secB*); genes that encode LPS assembly proteins (*lapA* and *lapB*); and genes involved in carbohydrate transport and metabolism (*pgm* and *zwf*) (S. table 5.4). The high number of shared conditionally essential genes between the Δ*surA* and Δ*degP* datasets suggests that a partially overlapping function exists between these two proteins. In contrast, the number of differences between the Δ*surA* and the Δ*skp* TraDIS libraries suggests that the functions of the chaperones SurA and Skp are separate and distinct as functionally redundant proteins or proteins that make up parallel pathways should contain similar synthetic lethal partners.

5.2.4. Gene ontology classification of conditionally essential genes

The Gene Ontology (GO) database was utilised as discussed in section 4.2.4 to quantify the proportion of proteins involved in a specific pathway or biological process, relative to the entire genome (Ashburner *et al.,* 2000; The Gene Ontology Consortium, 2019). In the Δ*skp,* Δ*degP* and Δ*surA* TraDIS datasets, the genes identified as conditionally essential were assigned suitable GO categories and fold enrichment scores were calculated.

In the Δ*skp* dataset, no GO categories were significantly enriched. In the Δ*degP* dataset, four GO categories were significantly enriched, with a false discovery p-value of <0.05. The GO categories functionally important to a Δ*degP* mutant were ‘heptose biosynthetic pathway’; ‘protein import’; ‘bacteriocin transport’; and ‘LPS core region biosynthetic process’ (fig.

5.7A). Furthermore, some proteins were reported in more than one GO category, which results in overlapping contents and some of the categories identified were functionally related, for example 'heptose biosynthetic pathway' and 'LPS core region biosynthetic process.' In the Δ*surA* TraDIS dataset, two GO categories were significantly enriched: 'enterobacterial common antigen biosynthetic process' and 'lipopolysaccharide process' (fig. 5.7B). The contents of the enriched GO categories were further investigated.

5.2.5. Characterisation of the synthetically lethal genes involved in the Tol-Pal system

In the Δ*degP* TraDIS dataset, the GO categories 'protein import' and 'bacteriocin transport' were functionally important. These GO categories contained a number of genes that are involved in the Tol-Pal system, which consists of a cytoplasmic protein YbgC; three IM proteins TolA, TolR, and TolQ; two periplasmic proteins TolB and YbgF; and a peptidoglycan-associated OM lipoprotein Pal (fig. 5.8A) (Isnard *et al.,* 1994; Lazzaroni and Portalier, 1992). TolQ spans the IM three times, while TolR and TolA are comprised of a transmembrane domain and a periplasmic component (Kampfenkel and Braun, 1993; Vianney *et al.,* 1994).

In the Δ*degP* TraDIS library, four members of the Tol-Pal system were identified as conditionally essential: *tolQ, tolR, tolA* and *tolB* (fig. 5.8B). The Tol-Pal system is involved in the maintenance of OM integrity. However, its precise function remains largely unknown. OM defects occur in each of the *tol-pal* mutants including the external release of periplasmic proteins (Lazzaroni *et al.,* 1999; Suzuki *et al.,* 1978). In addition, TolB and TolA interact with trimeric porins. Consequently, the Tol-Pal system might be involved in OMP assembly and/or the regulation of porin activity. TolA might drive newly synthesised OM components across the periplasm, while TolQ-TolR function as a motor energizing TolA (Cascales *et al.,* 2000; Cascales *et al.,* 2001; Dérouiche *et al.,* 1996; Dover *et al.,* 2000; Gaspar *et al.,* 2000; Germon

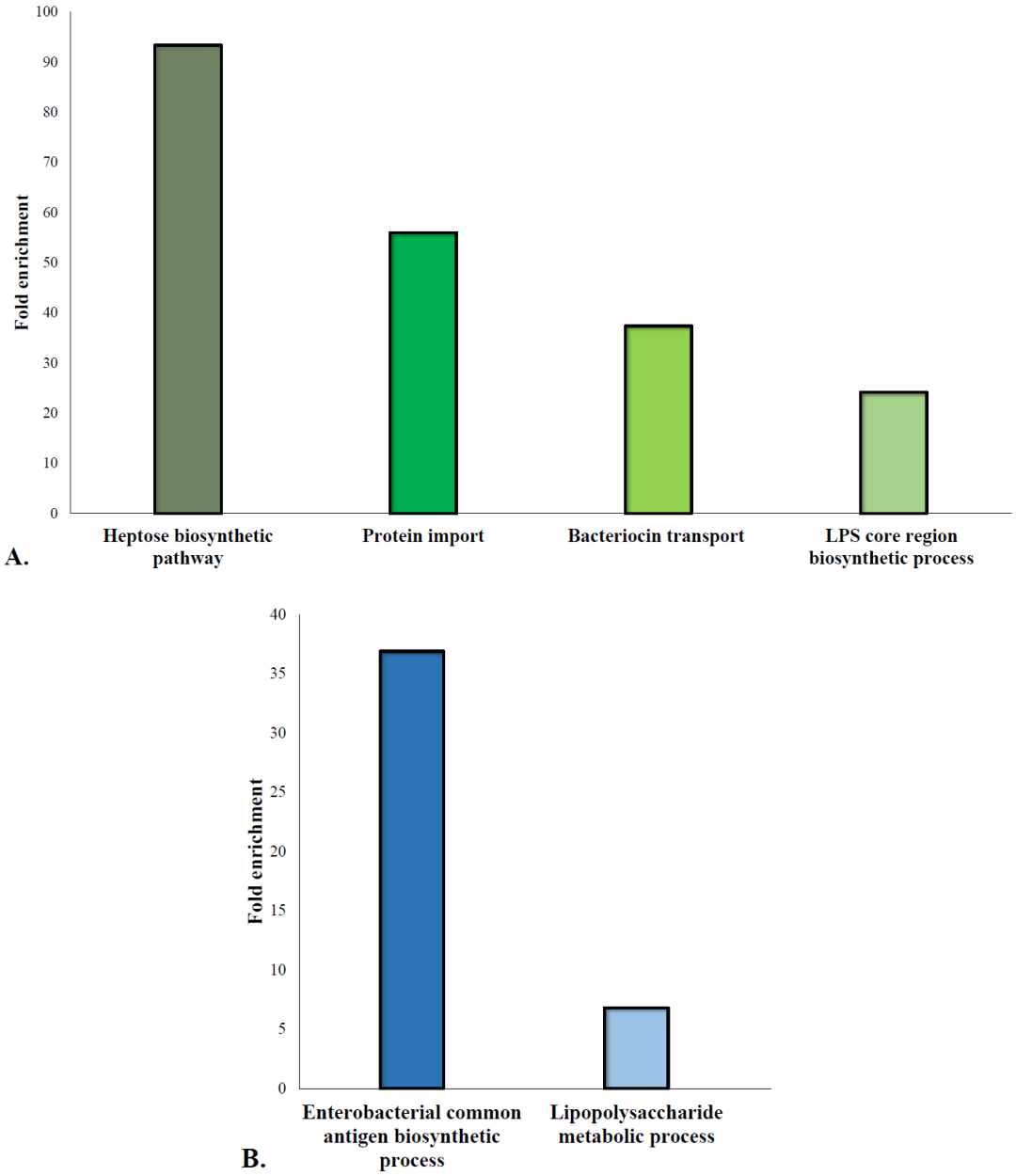


Fig. 5.7 Functional enrichment of GO categories that contain genes that are conditionally essential in the Δ*degP* and Δ*surA* TraDIS libraries. The expected proportion of genes that belongs to a GO category were compared to the actual number of genes identified per GO category. (A) In the Δ*degP* TraDIS dataset, four GO categories were identified as significantly enriched, including the 'heptose biosynthetic pathway;' 'protein import;' 'bacteriocin transport;' and 'LPS core region biosynthetic process.' (B) In the Δ*surA* TraDIS dataset, two GO categories were significantly enriched, including the 'ECA biosynthesis process' and 'LPS metabolic process.'

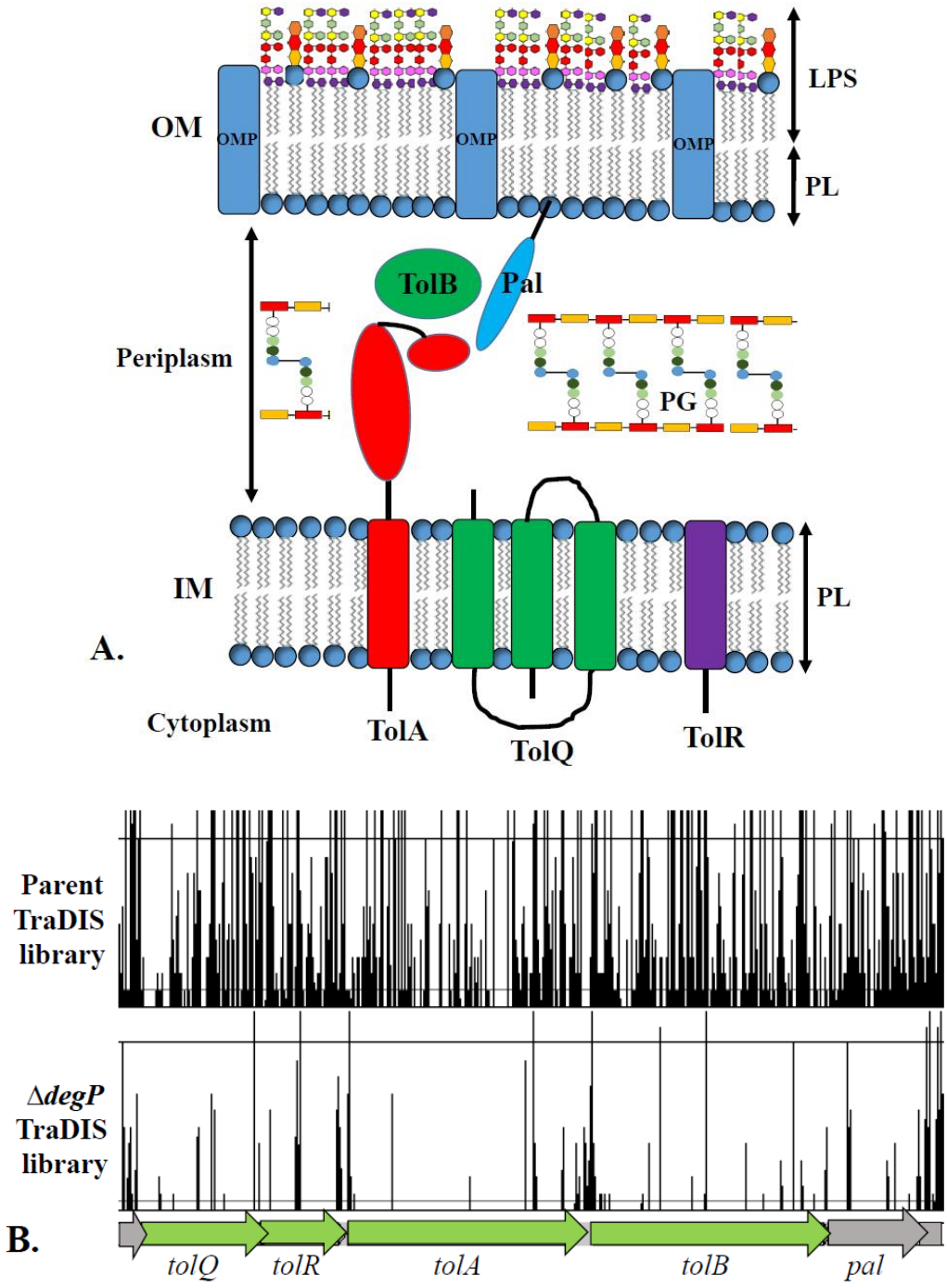


Fig. 5.8 The Tol-Pal system. (A) The Tol-Pal systems consists of IM proteins TolQ and TolR; a protein TolA that contains an IM and periplasmic component; a periplasmic protein TolB; and an OM lipoprotein Pal. (B) In the Δ*degP* TraDIS library, fewer mutants were recovered with transposon insertions in the genes *tolQ*, *tolR*, *tolA* and *tolB* than in the parent TraDIS library.

et al., 2001; Rigal *et al.,* 1997). The synthetically lethality between members of the Tol-Pal system and *degP* further implicates the role of this system in OMP assembly.

5.2.6. The importance of enterobacterial common antigen (ECA) synthesis to OMP biogenesis

In the Δ*surA* TraDIS dataset, the GO category 'enterobacterial common antigen biosynthetic process' was significantly enriched. This GO category contains gene products involved in the synthesis of enterobacterial common antigen (ECA). ECA is a carbohydrate-derived molecule that is found on the external leaflet of the OM and in the periplasm (Kuhn *et al.,* 1988). It is specific to members of the Enterobacteriaceae family and is highly conserved within this family (Kunin, 1963). ECA is composed of three main subunits: GlcNAc (*N*-acetylglucosamine); ManNAcA (*N*-acetyl-D-mannosaminuronic acid); and Fuc4NAc (4-acetamido-4,6-dideoxy-d-galactose) (Lugowski *et al.,* 1983; Männel and Mayer, 1978). ECA is synthesised in the cytoplasm and flipped across the IM into the periplasm. WecA catalyses the first step in this pathway, with the transfer of GlcNAc-1-phosphate to undecaprenyl-phosphate (Und-P). WecB and WecC synthesise ManNAcA, which is attached to Und-P-P-GlcNAc by WecG. RffH, RffG, WecE and WecD together synthesise Fuc4NAc, which is attached to Und-P-P-GlcNAc-ManNAcA by WecF. WzxE flips the ECA molecules across the IM into the periplasm, where WzyE polymerizes the ECA and WzzE controls the length of the ECA chain (Barr *et al.,* 1999; Brade, 1999; Rick *et al.,* 2003).

In the Δ*surA* TraDIS library, the majority of genes involved in the synthesis of ECA were conditionally essential: *wecB*; *wecC*; *wecE*; *wecD*; *wecF*; *wecG*; and *wzxE* (fig. 5.9A). An exception was the gene *wxyE,* which is required for the survival of both the parent and the Δ*surA* mutant. In addition, in the Δ*surA* TraDIS library, fewer mutants were recovered with transposon insertions in *rffH* and *rffG* than in the parent TraDIS library. The gene products RffH and RfbA are iso-enzymes. They catalyse the same reaction, but form part of different

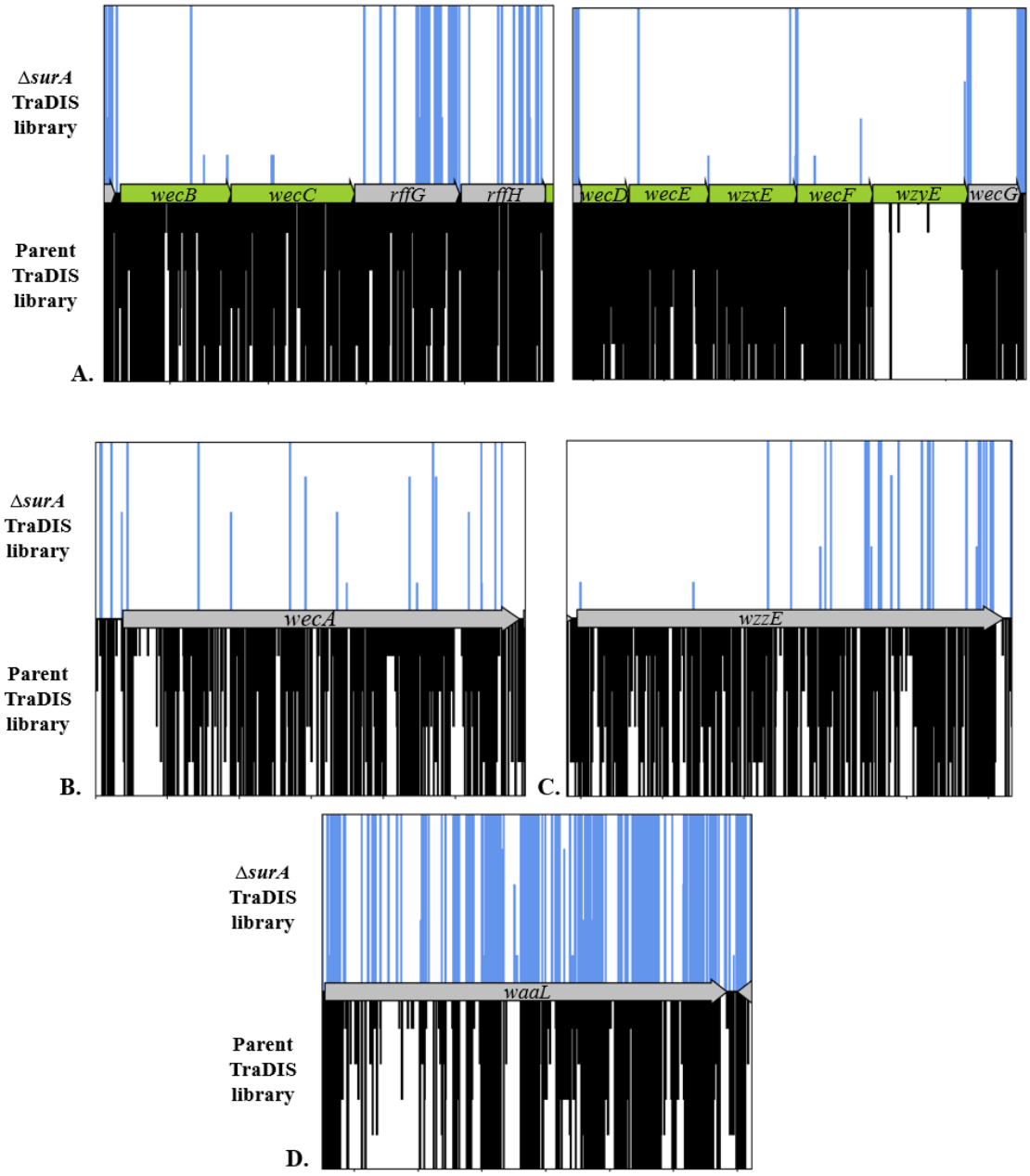


Fig. 5.9 The synthesis of ECA is functionally important to a Δ*surA* mutant. (A) In the Δ*surA* TraDIS library, the genes *wecB*, *wecC*, *wecE*, *wecD*, *wecF* and *wzzE* were identified as conditionally essential by the statistical analysis. Manual inspection of the data identified the gene *wecG* as also conditionally essential. (B-C) In the Δ*surA* TraDIS library, significantly fewer mutants were recovered with transposon insertions in the genes *wecA* and *wzzE* than in the parent TraDIS library. (D) The gene *waaL* is not required for the survival of the Δ*surA* mutant or the parent strain.

operons and function in separate pathways (Sivaraman *et al.*, 2002). Similarly, RfbB catalyses the same reaction as RffG, but functions in the rhamnose synthesis pathway. Marolda *et al.* (1995) demonstrated that *rffG* complemented a defect in the gene *rfbB*, which suggests that loss of *rffG* or *rffH* could be rescued by the genes *rfbB* or *rfbA*, respectively. In summary, ECA biosynthesis is conditionally essential in a Δ*surA* mutant. This result was specific to the Δ*surA* TraDIS library and did not occur in any of the other BAM mutant TraDIS libraries.

5.2.6.1. The form of ECA that is functionally important to the Δ*surA* mutant. ECA exists in three forms: covalently linked to LPS (ECA_{LPS}), covalently linked to the lipid phosphatidylglycerol (ECA_{PG}) and a cyclic form (ECA_{CYC}) (Kuhn *et al.*, 1988). ECA_{LPS} and ECA_{PG} both occur in the OM, while ECA_{CYC} is periplasmic. The same biosynthetic pathway is utilised in the synthesis of all three forms. An aim of this study was to identify the form of ECA that is conditionally essential in the Δ*surA* mutant.

5.2.6.1.1. The importance of the ECA_{LPS} form. WaaL transfers ECA molecules onto the sugar moieties in LPS (Schmidt *et al.*, 1976). In the Δ*surA* TraDIS library, the gene *waaL* was non-essential, which suggests that ECA_{LPS} is not required by the Δ*surA* mutant (fig. 5.9D). To validate this observation a defined Δ*surA*Δ*waaL* double mutant was constructed and confirmed by PCR. The successful isolation of this double mutant strongly indicates that ECA_{LPS} is not required in the Δ*surA* mutant.

5.2.6.1.2. The importance of the ECA_{PG} form. In the formation of ECA_{PG}, an unknown molecule attaches the ECA chains onto the phospholipid PG by a phosphodiester linkage (Kuhn *et al.*, 1983). In an attempt to determine whether ECA_{PG} is conditionally essential in a Δ*surA* mutant, a Δ*pgsA*Δ*surA* mutant was constructed. The gene *pgsA* encodes phosphatidylglycerophosphate synthase, the enzyme that catalyses the first committed step in the biosynthesis of the phospholipid PG. Thus, a Δ*pgsA* null mutant is defective in the anionic phospholipids PG and cardiolipin.

However, loss of *pgsA* is lethal to the cell. This lethal effect is alleviated by loss of the lipoprotein encoded by the *lpp* gene (Kikuchi *et al.,* 2000; Suzuki *et al.,* 2002). In addition, in the Δ*pgsA* mutant, the Rcs system is activated and this activation is reverted to wild-type levels by loss of *rcsF*. To ensure that the potential synthetic lethality between *surA* and ECA_{PG} was not rescued by activation of the Rcs system, the *rcsF::aph* allele was transferred from the Keio library into the parent strain and the kan^R cassette was removed by use of the pCP20 plasmid. The *lpp:aph* allele was then transferred into the Δ*rcsF* mutant and the kan^R cassette was removed by the λ-Red recombination method (Datsenko and Wanner, 2000). Similarly, the *pgsA* gene was replaced by a *pgsA:aph* allele and the kan^R cassette was removed. Lastly, the *surA::aph* allele was transferred from the Keio library into the Δ*rcsF*Δ*lpp*Δ*pgsA* mutant. The Δ*rcsF*Δ*lpp*Δ*pgsA*Δ*surA* quadruple mutant was successfully constructed and confirmed by PCR.

5.2.6.1.3. Comparison of lipid species between the parent and the Δ*rcsF*Δ*lpp*Δ*pgsA*Δ*surA* mutant. Following successful isolation of the mutant, the phospholipid species of this mutant and the parent strain were evaluated. Phospholipids were extracted using the Bligh-Dyer method (1959) and separated by thin layer chromatography with a chloroform:methanol:acetic acid solvent system (65:25:10). The parent strain contained all relevant phospholipids: PG, PE and CL, while only PE was present in the Δ*rcsF*Δ*lpp*Δ*pgsA*Δ*surA* mutant. Thus, this mutant did not contain the phospholipids PG and CL (fig. 5.10). There was an additional phospholipid spot ~1 cm above the origin in the sample extracted from the Δ*rcsF*Δ*lpp*Δ*pgsA*Δ*surA* cells. This spot is likely to be phosphatidic acid (PA) as cells lacking both PG and CL contain higher levels of other anionic lipids including PA (Mileykovskaya *et al.,* 2009; Rowlett *et al.,* 2017). The absence of PG in the Δ*rcsF*Δ*lpp*Δ*pgsA*Δ*surA* mutant suggests that ECA_{PG} was not formed. Thus, the viable nature of this quadruple mutant strongly suggests that the PG form of ECA is not required for the survival of the Δ*surA* mutant.

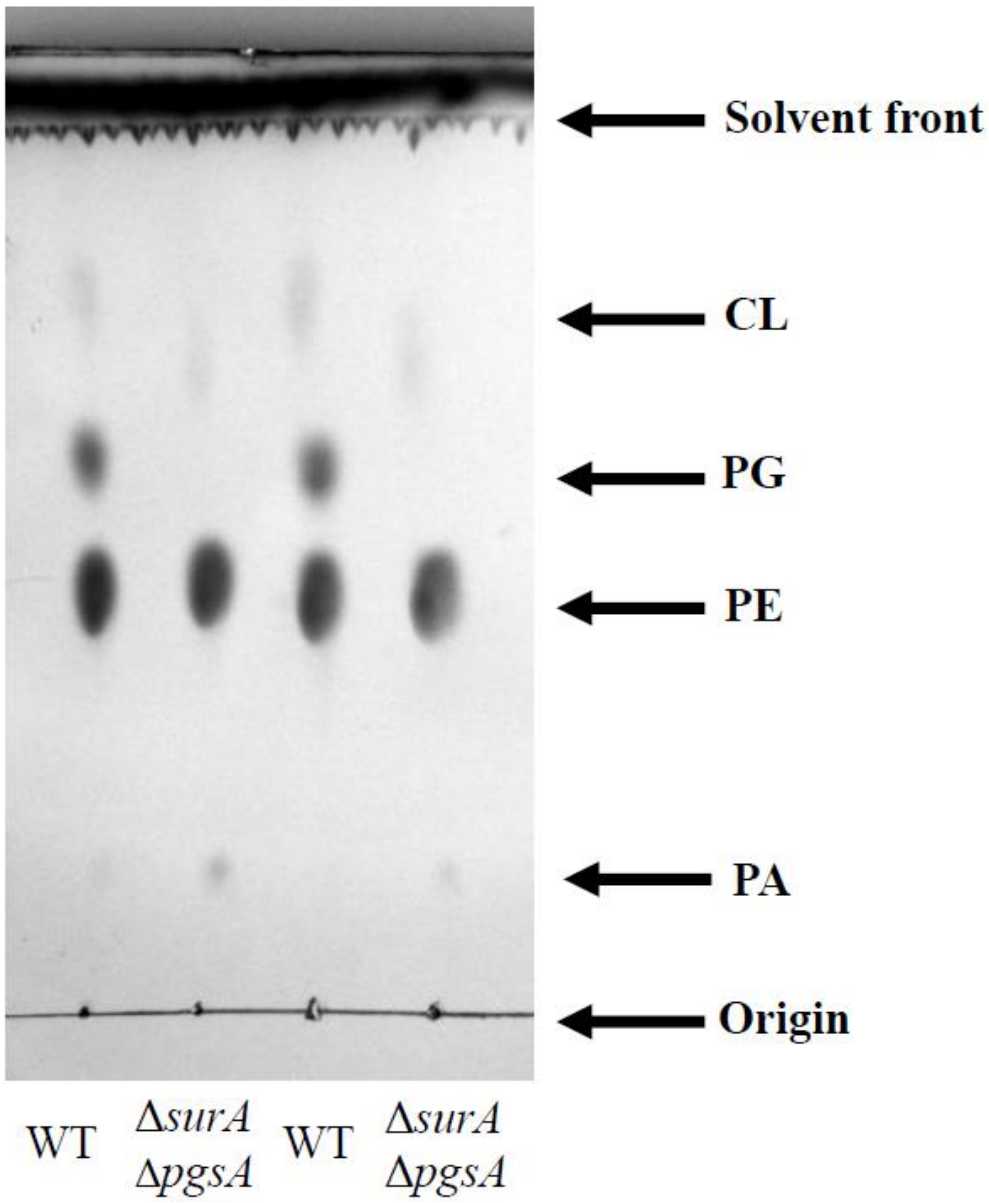


Fig. 5.10 Thin layer chromatography of phospholipids extracted from the parent and the Δ*rcsF*Δ*lpp*Δ*pgsA*Δ*surA* mutant. The phospholipids were extracted by the Bligh-Dyer method, separated by thin layer chromatography with a mobile phase comprised of chloroform:methanol:acetic acid (65:25:10) and were visualised by staining with phosphomolybdic acid. Similar levels of phosphatidylethanolamine (PE) occurred in both the parent and mutant strain. All three phospholipids were present in the parent strain (WT), while only PE was present in the Δ*rcsF*Δ*lpp*Δ*pgsA*Δ*surA* mutant. No phosphatidylglycerol (PG) or cardiolipin (CL) was present in the Δ*rcsF*Δ*lpp*Δ*pgsA*Δ*surA* mutant.

5.2.6.1.4. The importance of the ECA_{CYC} form. In addition, to ensure that loss of both ECA_{PG} and ECA_{LPS} is viable in the Δ*surA* mutant, a Δ*rcsF*Δ*lpp*Δ*pgsA*Δ*surA*Δ*waaL* mutant was constructed and confirmed by PCR. The viable isolation of this mutant suggests that the Δ*surA* mutant can withstand the loss of both ECA_{PG} and ECA_{LPS}. This would suggest that ECA_{CYC} is the form of ECA required for the survival of the Δ*surA* mutant. In addition, Mitchell *et al.* (2018) demonstrated that *wzzE* is not required for the production of ECA_{LPS} or ECA_{PG}, but is required for production of ECA_{CYC}. In the Δ*surA* TraDIS library, the gene *wzzE* contained a conditionally essential region (fig. 5.9C). Therefore, collectively the evidence suggests that ECA_{CYC} is the form of ECA required for the survival of the Δ*surA* mutant.

5.2.7. The importance of ECA in the absence of SurA

Mitchell *et al.* (2018) demonstrated that disruption of ECA synthesis can cause downstream effects on other pathways including O-antigen biosynthesis and peptidoglycan synthesis (Mitchell *et al.,* 2018). Disruption of the intermediate steps in the ECA biosynthetic pathway leads to the accumulation of ECA intermediates that sequester the lipid carrier undecaprenyl phosphate (Und-P) from the finite Und-P pool. Numerous metabolic reactions including the production of peptidoglycan, capsule, O-antigen and membrane derived oligosaccharides compete for Und-P. Consequently, accumulation of ECA intermediates will alter and restrict the peptidoglycan biosynthesis pathway, eliciting stress on this pathway (Jorgenson *et al.*, 2016).

5.2.7.1. Negative implications on downstream pathways. A possibility considered was that ECA_{cyc} might be conditionally essential in a Δ*surA* mutant due to negative implications on other pathways that occur due to sequestration of Und-P. An O-antigen negative strain was utilised in this study. Thus, disruption of ECA biosynthesis did not elicit downstream negative effects on O-antigen biosynthesis. Disruption of *wecA* prevents the synthesis of ECA and increases the pool of sugar precursors and Und-P available for peptidoglycan biosynthesis

(Jorgenson *et al.,* 2015). In the Δ*surA* TraDIS library, significantly fewer mutants were recovered with transposon insertions in the gene *wecA* than in the parent TraDIS library (fig. 5.9B). If ECA was conditionally essential due to negative implications on peptidoglycan synthesis, disruption of *wecA* would be favourable in the Δ*surA* mutant. In order to validate this observation an attempt was made at construction of a Δ*surA*Δ*wecA* double mutant. This double mutant was not successfully isolated. This strongly indicates that the synthesis of ECA is not essential in the Δ*surA* mutant due to downstream negative effects on peptidoglycan biosynthesis.

5.2.7.2. Chaperoning abilities to the BAM complex. The possibility was also considered that ECA_{CYC} might act as a chaperone to the BAM complex, which would alleviate loss of SurA. Storek *et al.* (2019) demonstrated that BAM activity and membrane fluidity are interrelated. An alternative possible explanation is therefore that loss of ECA_{CYC} might increase membrane fluidity, which decreases BAM activity. To determine whether either of these were the case, the activity of the BAM complex was monitored in a number of ECA mutants: Δ*wecA*; Δ*wecD*; Δ*wecF*; Δ*wzzE*; and Δ*wzxE*. BAM activity was quantified via fluorescence in an in vivo OmpT assay, which measured the insertion of OmpT into the OM. The inserted OmpT cleaved a fluorogenic peptide, which produced a fluorescence emission that was recorder on a plate reader. Only minor differences in BAM activity occurred in the ECA mutants compared to the parent strain (fig. 5.11). An independent sample T-test demonstrated that these minor differences were not statistically significant. Thus, ECA_{CYC} does not chaperone OmpT to the BAM complex. Conversely, ECA_{CYC} might act as a chaperone to other OMPs. Thus, further confirmation is required to determine whether ECA_{CYC} can fulfil a chaperone function to the BAM complex.

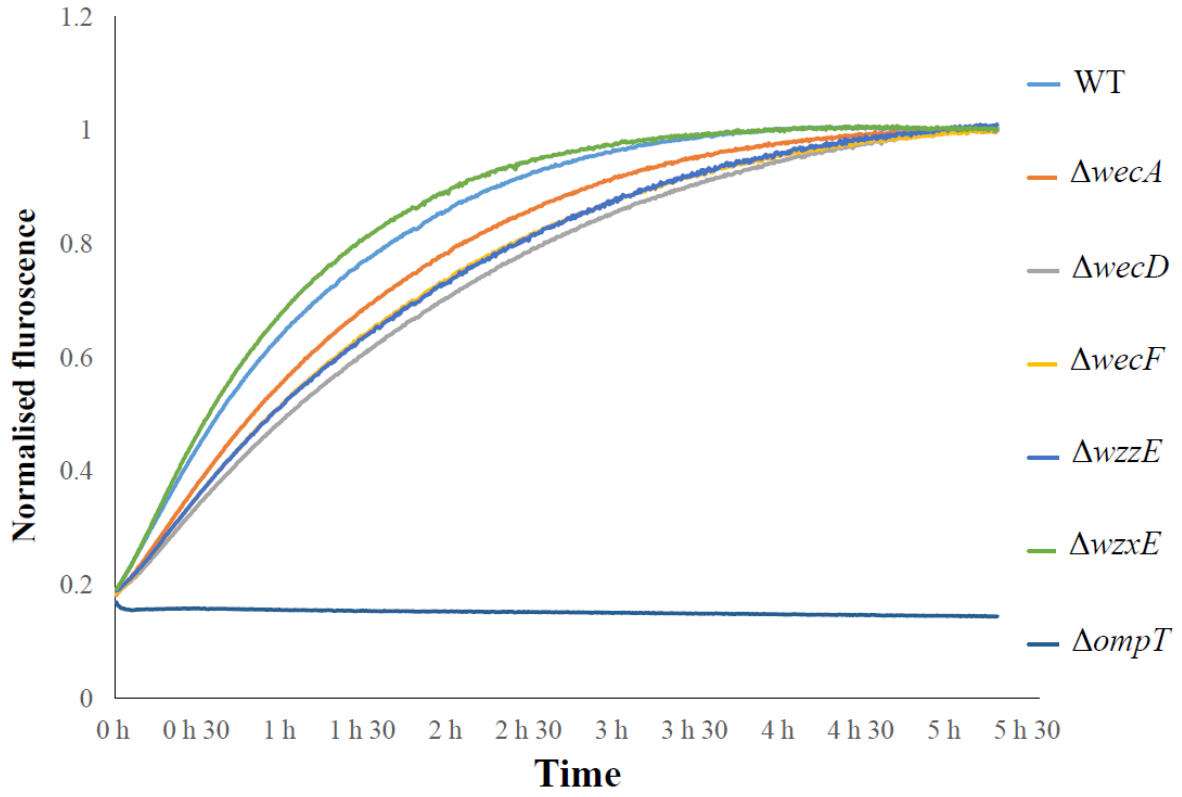


Fig. 5.11 OmpT cleavage assay in mutants impaired in the synthesis of ECA. Successful folding and insertion of OmpT into the OM by the BAM complex was monitored via fluorescence on a plate reader. OmpT cleavage was measured in biological and technical triplicate in the parent (WT) and in the mutants Δ*wecA*; Δ*wecD*; Δ*wecF*; Δ*wzxE*; Δ*wzzE*; and Δ*ompT*. The fluorescence was calculated as a percentage of the maximum fluorescence achieved and the normalised fluorescence for the Δ*ompT* negative control was normalised to the maximum achieved for all experiments. No OmpT cleavage occurred in the Δ*ompT* mutant, while only minor differences in OmpT activity were noted between the parent and the other mutants tested.

In summary, ECA biosynthesis is conditionally essential in the Δ*surA* TraDIS library. Three forms of ECA exist. However, data from this study suggests that the Δ*surA* mutant only requires the cyclic form. The synthetic lethality between ECA and the Δ*surA* mutant is not due to downstream negative effects on peptidoglycan synthesis and is not a result of ECA_{CYC} chaperoning OmpT to the BAM complex. This result was specific to the Δ*surA* TraDIS library and did not occur in the Δ*degP* or Δ*skp* TraDIS libraries.

5.2.8. The coordination of lipopolysaccharide synthesis with OMP biogenesis

In the Δ*surA* and Δ*degP* TraDIS datasets, GO categories involved in the synthesis of LPS were significantly enriched. This result was specific to these mutants and did not occur in the Δ*skp* TraDIS dataset (fig. 5.12). In the Δ*degP* TraDIS library, all four genes involved in the synthesis of heptose were essential: *gmhA*; *gmhB*; *hldE*; and *waaD* (fig. 5.12). In the Δ*surA* TraDIS library, *gmhA*, *hldE* and *waaD* were essential, while there was a decrease in the number of mutants recovered with transposon insertions in the gene *gmhB*, compared to the parent (fig. 5.12). Both *gmhB* and *waaD* are not required for the production of heptose in the cell, but are essential/functionally important in these mutants due to their role in maximising the efficiency of heptose production.

In the Δ*degP* and Δ*surA* TraDIS libraries, the genes *waaC* and *waaF* were also conditionally essential (fig. 5.13). WaaC and WaaF transfer the first and second heptose sugars into the inner core of LPS, respectively. In addition, in the Δ*surA* and Δ*degP* TraDIS libraries, there was a slight decrease in the number of mutants recovered with transposon insertions in the genes *waaP* and *waaG*, compared to the parent TraDIS library (fig. 5.14). More specifically, the insertion index scores for the *waaP* and *waaG* genes were 7 and 4.7 times higher in the parent TraDIS library than in the Δ*surA* and Δ*degP* TraDIS libraries. WaaG adds a glucose to heptose II, while WaaP adds a phosphate to heptose I (Gronow and Brade, 2001). The remainder of the

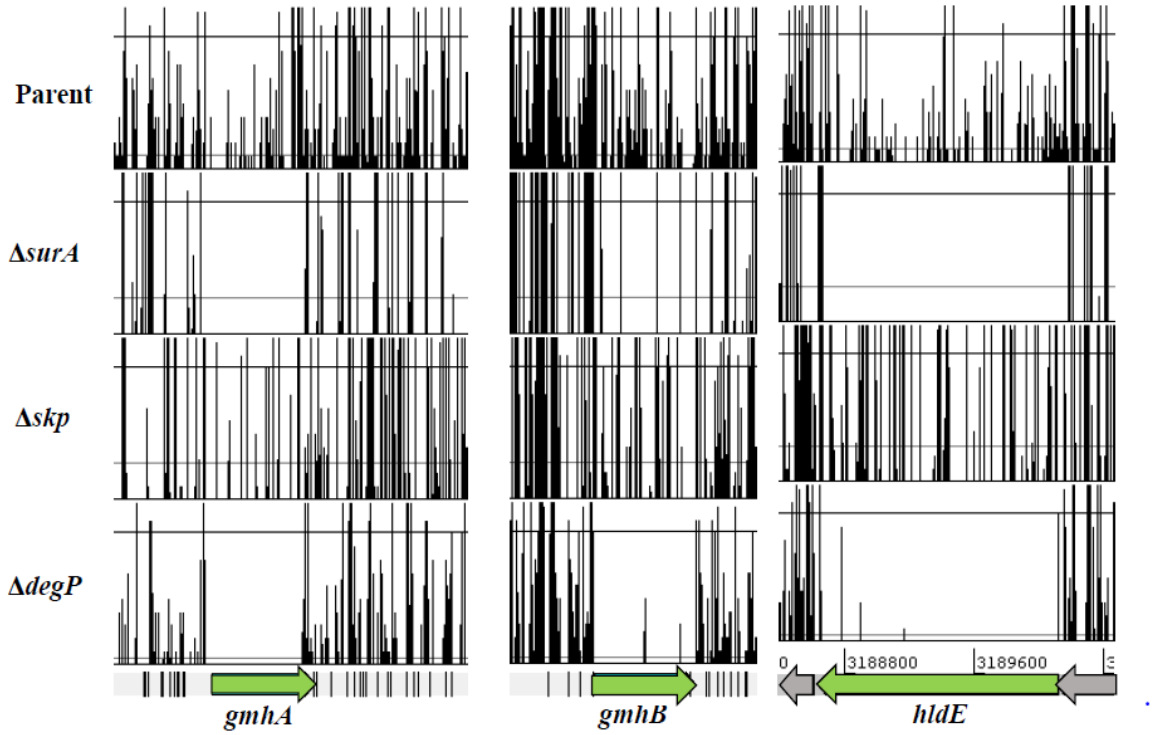


Fig. 5.12 The synthesis of heptose is conditionally essential in specific BAM mutants. Transposon insertions sites in the genes *gmhA*, *gmhB* and *hldE* in the parent (WT), Δ*surA*, Δ*skp* and Δ*degP* TraDIS libraries. In this figure, the transposon cut-off was set to ten. Essential genes are marked in green, while the non-essential genes are represented by grey. In the Δ*surA* and Δ*degP* TraDIS libraries, genes involved in heptose production were conditionally essential. Upon visual inspection of the Δ*surA* TraDIS library, the gene *gmhB* was also identified as functionally important. This result was specific to the Δ*surA* and Δ*degP* datasets and did not occur in the Δ*skp* dataset.

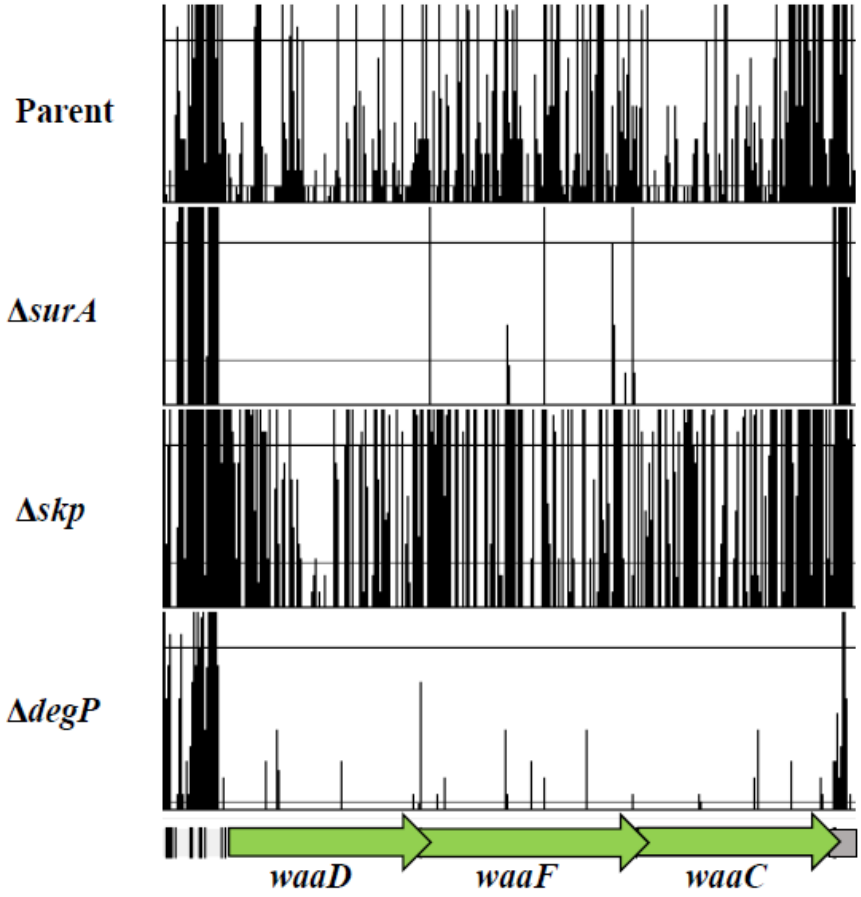


Fig. 5.13 Incorporation of heptose I and heptose II into LPS is conditionally essential in specific BAM mutants. Transposon insertion sites in the genes *waaD*, *waaF* and *waaC* in the parent, Δ*surA*, Δ*skp* and Δ*degP* TraDIS libraries. The transposon cut-off was set to ten. In the parent strain, the minimum LPS required for survival is lipid A and the Kdo backbone. In the Δ*surA* and Δ*degP* TraDIS libraries, the genes *waaD* and *waaC* were conditionally essential. In the Δ*degP* TraDIS library, the gene *waaF* was identified as conditionally essential by the statistical analysis. In contrast, in the Δ*surA* TraDIS library, the gene *waaF* was identified as conditionally essential via visual inspection. In the Δ*skp* TraDIS library, the genes *waaD*, *waaF* and *waaC* were non-essential.

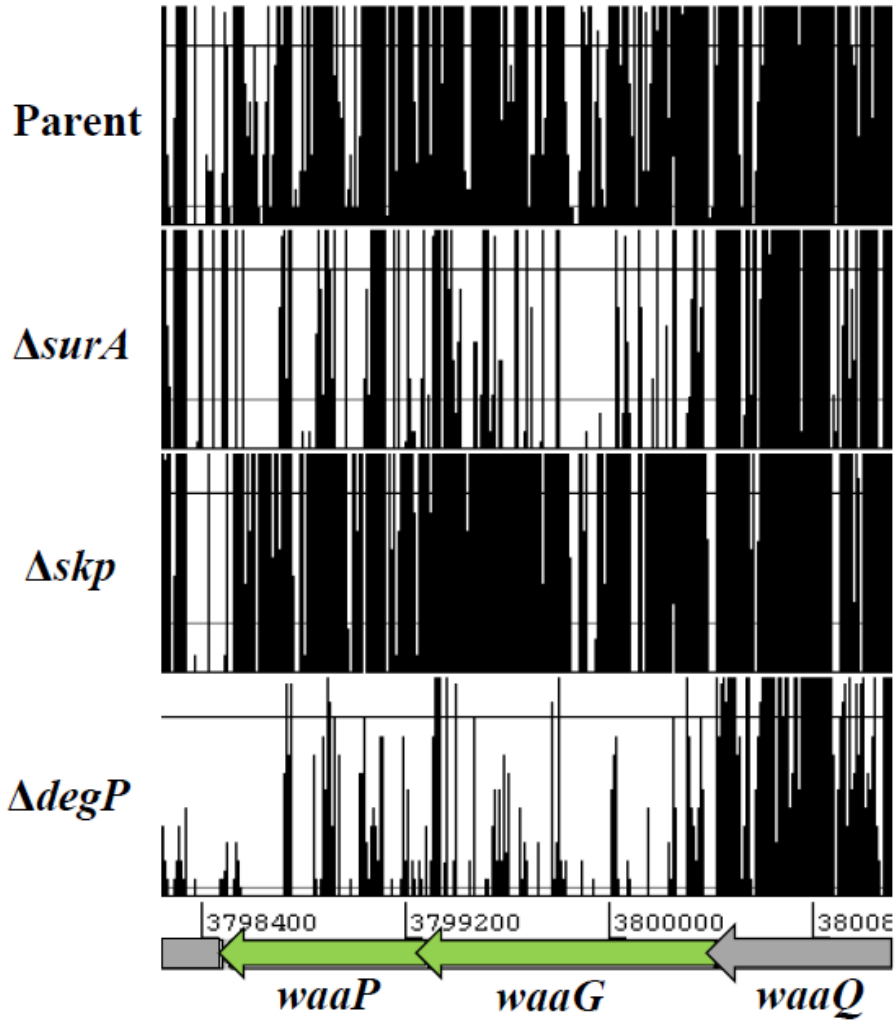


Fig. 5.14 The importance of additional genes involved in LPS synthesis in the Δ*surA* and Δ*degP* TraDIS libraries. In the Δ*surA* and Δ*degP* TraDIS libraries, fewer mutants were recovered with transposon insertions in the genes *waaP* and *waaG* than in the parent TraDIS library.

LPS genes, including *waaY,* which adds a phosphate to heptose II, were not functionally important in the Δ*surA* and Δ*degP* mutants. In summary, no genes involved in LPS assembly were conditionally essential in the Δ*skp* mutant, while the Δ*degP* and Δ*surA* mutants required a number of genes involved in heptose production and incorporation into LPS. These genes were further investigated in chapter six.

5.3. Conclusion

In an attempt to understand the synthetic lethality between *surA*, *skp* and *degP*, TraDIS libraries were constructed in Δ*surA*, Δ*skp* and Δ*degP* mutants and their respective synthetic lethal partners were identified. In the Δ*surA*, Δ*skp* and Δ*degP* TraDIS libraries, 54, 9 and 44 conditionally essential genes were identified, respectively. There were clear differences between the Δ*surA* and Δ*skp* TraDIS datasets, which suggests that SurA and Skp fulfil different functional roles. In contrast, there was an overlap of 17 conditionally essential genes between the Δ*surA* and Δ*degP* datasets, which suggests that a functional overlap exists between these two proteins.

Following identification of synthetic lethal partners, GO categories were assigned and the TraDIS data demonstrated that loss of genes involved in the synthesis or incorporation of heptose into LPS combined with loss of *surA* or *degP* is lethal to the cell. In addition to LPS defects, ECA synthesis defects were synthetically lethal in a Δ*surA* mutant. In summary, the TraDIS data highlighted the importance of the synthesis and trafficking pathways for LPS and ECA in specific BAM mutants.

CHAPTER 6

Lipopolysaccharide and peptidoglycan synthesis in mutants of non-essential components of the BAM complex

6.1. Introduction

Bacteria must synchronize the synthesis, growth and division of the cell envelope components both spatially and temporally. An imbalance in these processes can compromise the permeability barrier and the structural integrity of the cell. How these processes are coordinated remains unresolved. This chapter focuses on how outer membrane protein biogenesis is coordinated with other cell envelope pathways. TraDIS libraries constructed in Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp* and *degP* mutants were compared to identify cell envelope pathways that become more important in more than one of the BAM mutants.

To build a stable and functional OM, the synthesis, transport and assembly of LPS and OMP biogenesis must be coordinated. One aim of this chapter was to determine the effect loss of a non-essential member of the BAM pathway has on LPS biogenesis. In the Δ*bamB* TraDIS library, fewer mutants were recovered with transposon insertions in genes involved in heptose production and incorporation into LPS than in the parent strain (fig. 3.11-3.12). Thus, genes involved in LPS assembly are functionally important to the Δ*bamB* mutant. In the Δ*surA* and Δ*degP* TraDIS libraries, genes involved in heptose production and incorporation into LPS were conditionally essential (fig. 5.12-5.13). Thus, genes involved in LPS biogenesis are crucial to the Δ*degP* and Δ*surA* mutants (fig. 6.1). In contrast to this, there was no significant difference in the recovery of transposon mutants in the Δ*bamC*, Δ*bamE* and Δ*skp* TraDIS libraries, compared to the parent TraDIS library.

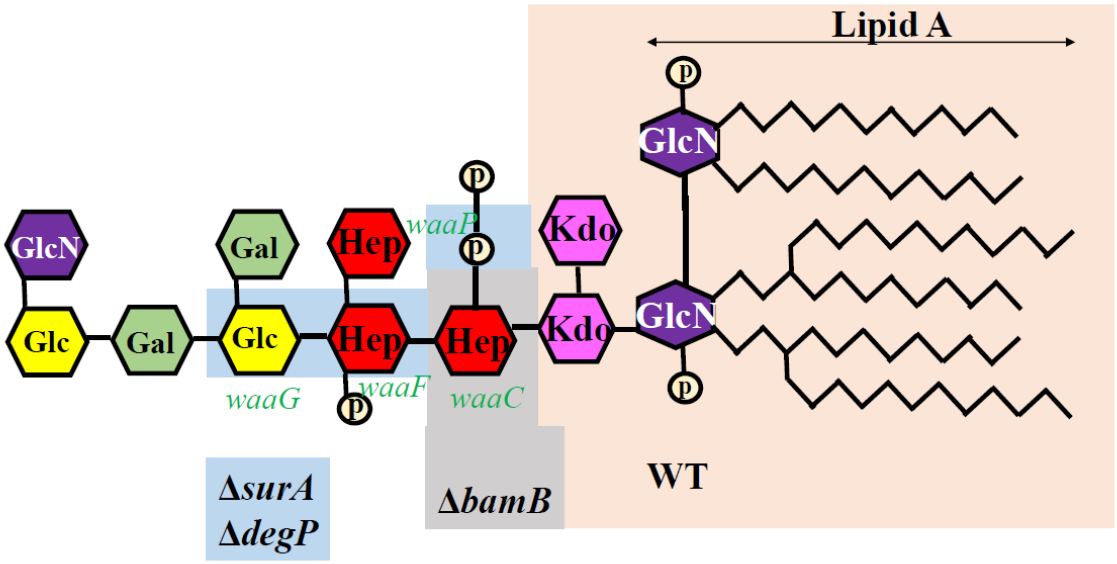


Fig. 6.1 The importance of genes involved in LPS synthesis differs between Δ*bamB*, Δ*surA* and Δ*degP* mutants. In the Δ*bamB* TraDIS library, fewer mutants were recovered with transposon insertions in the gene *waaC* than in the parent TraDIS library. In the Δ*surA* and Δ*degP* TraDIS libraries, the genes *waaC* and *waaF* were conditionally essential. In addition, in the Δ*surA* and Δ*degP* TraDIS libraries, fewer mutants were recovered with transposon insertions in the genes *waaG* and *waaP* than in the parent TraDIS library.

6.2. Results

6.2.1. The structure of LPS in non-essential mutants of the BAM complex

Declaration: Mutants were constructed by the author and all strains of interest were transferred to a laboratory at the University of Naples Federico II (UNINA). At this laboratory, Pilar Garcia del Vello Moreno extracted the LPS from the non-essential BAM mutants and the parent strain and subjected the LPS to a number of different techniques discussed in this section. The analysis of the LPS structural work was generated by Vello Moreno's supervisors Cristina De Castro and Antonio Molinaro. The following three sections are conclusions from the collobrators in UNINA, which form the basis for subsequent experiments by the author.

The TraDIS results demonstrated that a number of genes involved in LPS biogenesis are synethically lethal with specfic members of the BAM pathway. Upon depletion of Lpt proteins (LptA; LptB; LptC; LptD; LptE), modifications in LPS occur, where the LPS is ligated to repeating units of colanic acid in the outer leaflet of the OM (Sperandeo *et al.*, 2008). This suggests that cross-talk exists between LPS biogenesis and colonic acid production. Similarly, it was investigated whether failure in the BAM system due to loss of a member of the BAM pathway induces changes in the LPS structure. A possibility considered was that a modification in the structure of LPS combined with loss of a member of the LPS biogenesis pathway would impair membrane integrity and lead to cell death. This would provide a possible explanation behind the synthetic lethality between members of the LPS biogenesis pathway and members of the BAM pathway.

Using the phenol, chloroform and light petroleum extraction (PCP) procedure, the LPS was extracted from the parent and the non-essential BAM mutants Δ*bamB,* Δ*bamC,* Δ*bamE,* Δ*skp,* Δ*surA* and Δ*degP* (Galanos *et al.,* 1969). The structure of the LPS was determined using a

number of different techniques including: SDS-PAGE screening; monosaccharide composition by derivatization as fully acetylated methylated glycosides; profiling of the core region by proton nuclear magnetic resonance (^{1}H NMR) and lipid profiling as ester-derivatives by gas chromatography-mass spectrometry (GC-MS).

6.2.1.1. SDS-PAGE screening of LPS. The various LPS samples were screened by SDS-PAGE and visualised by silver staining (Kittelberg *et al.*, 1993). The pattern of mobility for the parent LPS sample (WT) was similar to the pattern of mobility for all of the mutant LPS samples (fig. 6.2). However, some tailing occurred in the Δ*skp* and the parent LPS samples due to overloading of the sample onto the SDS-PAGE gel. These minor differences were not considered to be due to significant structural variations.

6.2.1.2. Evaluation of the monosaccharide composition of LPS. The monosaccharide composition of each LPS sample was evaluated to determine whether the contents of the LPS core was different in the BAM mutants than in the parent. Following LPS extraction, the various LPS were converted into the corresponding acetylated methylglycosides by derivatization. This derivatization is required for detection of these sugars by GC-MS. Following GC-MS, the parent and the mutant LPS samples produced similar chromatograms. The monosaccharide composition of each LPS core region was compared and no significant differences were noted between the mutant and the parent LPS samples (fig. 6.3A). To confirm that the minor differences between the monosaccharide components were not relevant, the LPS core was also evaluated by ^{1}H NMR. Following LPS extraction, the core of the LPS was cleaved from lipid A by mild acid treatment. The oligosaccharides were separated from the lipid A by centrifugation and analysed by ^{1}H NMR. The composition of each glycan component from the mutant LPS samples resembled the parent LPS sample. Thus, mutations in the BAM members does not affect the glycan component of the LPS (fig. 6.3B).

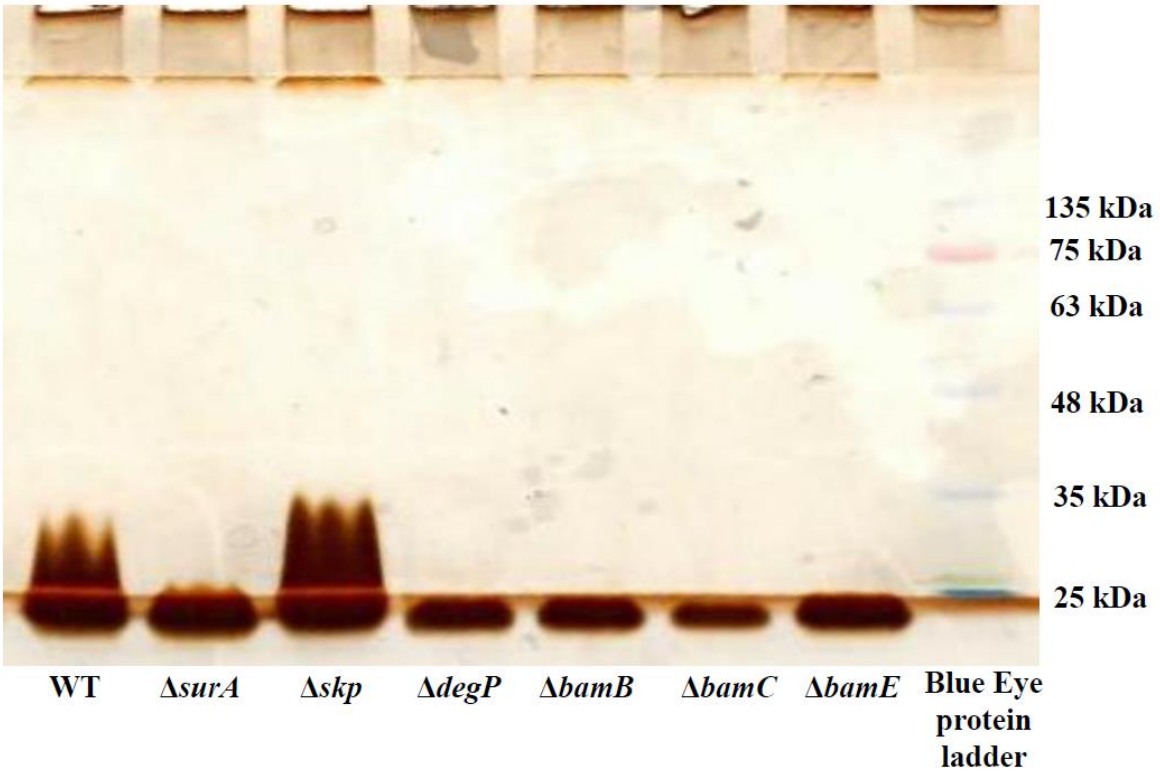


Fig. 6.2 SDS-PAGE screening of *E. coli* BAM mutants. LPS was extracted from the BAM mutants and the parent. Samples (~8 μg) were separated by SDS-PAGE and visualised by silver staining. The patterns of mobility were similar between each of the mutants and the parent strain.

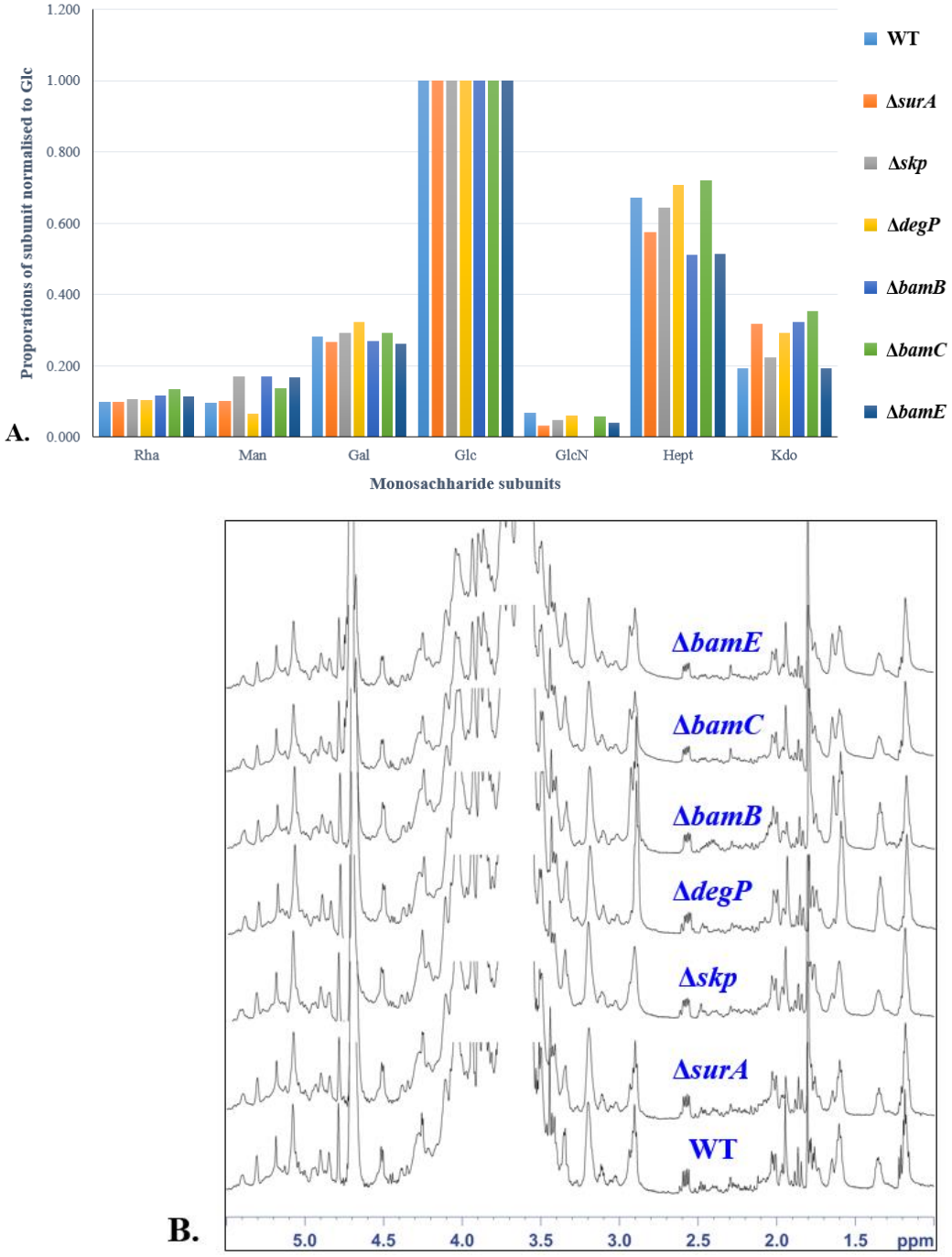


Fig. 6.3 The monosaccharide composition of the LPS extracted from the BAM mutants and the parent strain. (A) There were no significant differences in monosaccharide composition between the BAM mutants and the parent. The other monosaccharide components were normalised with respect to glucose (Glc), where Rha = rhamnose; Man = mannose; Gal = galactose; GlcN = N-Acetylglucosamine; Hept = heptose; and Kdo = 2-keto-3-deoxyoctulosonic acid. (B) In the core region profile of the oligosaccharides derived from mild acid hydrolysis of the LPS, there were no differences in the glycan profile between the mutants and the parent strain.

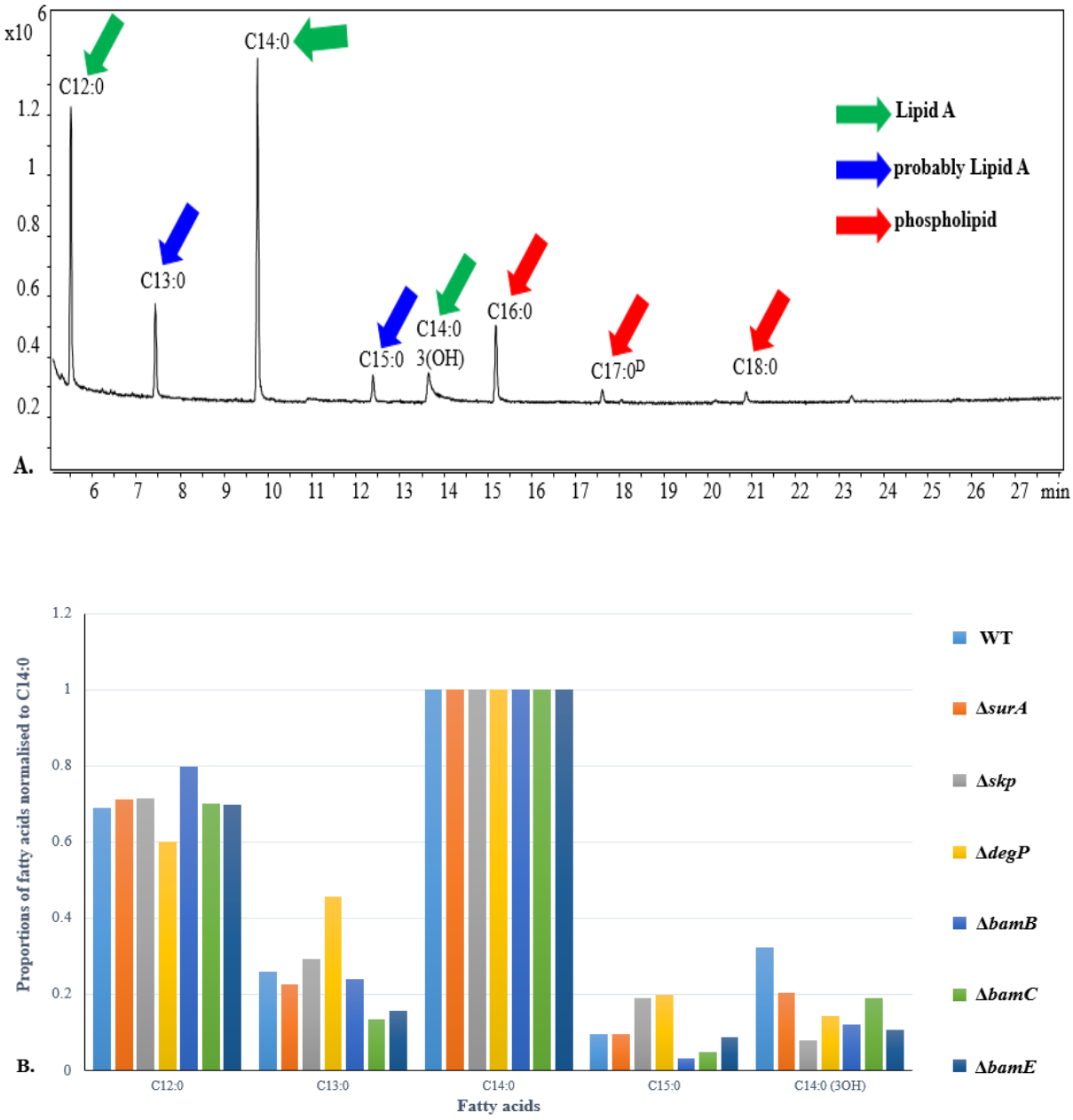


Fig. 6.4 The composition of Lipid A. (A) A GC-MS profile of fatty acid methylesters of the *E. coli* K12 parent strain. Lipid A species known in the literature were marked with a green arrow, while fatty acids belonging to phospholipids were marked with a red arrow. The blue arrow marks fatty acids that probably belong to lipid A but are not recorded in the literature. (B) Fatty acid composition of the samples were normalised relative to C14:0. There were no significant differences in the composition of fatty acids between the mutants and the parent strain.

6.2.1.3. Evaluation of the lipid content of LPS. The lipid content of each LPS sample was investigated to determine whether the lipid A profile was different in the BAM mutants than in the parent. The fatty acids were converted to ester derivates and these sugars were detected by GC-MS. The GC-MS chromatogram contained peaks that represented all lipids present in the samples, including phospholipids C16:0, C17:0 and C18:0, which co-extracted with the LPS. Each LPS contained the expected lipids C12:0, C14:0, C14:0 (3OH) and two additional fatty acids that contained an odd number of carbon atoms C13:0 and C15:0. The two additional fatty acids also appeared in the parent strain. Consequently, the presence of these fatty acids was not due to any of the mutations in the BAM pathway. Thus, there were no significant differences in the fatty acid composition of Lipid A between the mutants and the parent strain (fig. 6.4).

In summary, three methods were utilised to compare the LPS extracted from the parent and the mutants defective in the non-essential genes of the BAM pathway. There were no significant differences in LPS composition between the parent and the BAM mutants. Thus, no explanation was found for the synthetic lethality between specific BAM mutants and genes involved in LPS biogenesis.

6.2.2. The coordination of LPS biogenesis and membrane fluidity

This study demonstrated that loss of a non-essential member of the BAM pathway does not induce a significant difference in the LPS structure. However, this did not explain the synthetic lethality between specific BAM mutants and genes involved in heptose production and heptose incorporation into LPS. An aim of this study was to understand this synthetic lethality. Recently, Storek *et al.* (2019) demonstrated that a severe modification in the LPS structure lead to a change in membrane fluidity, which was detrimental to the activity of the BAM complex. These results imply that the BAM function and the OM environment are interrelated. Based on this study, the possibility was investigated whether the synthetic lethality between genes

involved in LPS biogenesis and specific members of the BAM pathway was due to fluctuations in membrane fluidity, which would therefore affect BAM activity.

Membrane fluidity was measured using a membrane fluidity kit (abcam), which contains a lipophilic pyrene probe that is incorporated into bacterial membranes. Once membrane incorporation occurs, the probes can move around the membrane and form excited dimers (excimers), which dramatically shift the emission spectrum of the pyrene probe to a longer red wavelength. The membrane fluidity was quantitatively determined by measuring the ratio of excimer (470 nm emission) to monomer (405 nm emission) fluorescence.

The membrane fluidity of the Δ*gmhA*, Δ*gmhB*, Δ*hldE* and Δ*waaD* mutants was measured and compared to the parent strain (fig. 6.5). The membrane fluidity of all four mutants was higher than that of the parent. Of the mutants tested, the membrane fluidity of the Δ*gmhB* mutant was the lowest. An independent sample T-test demonstrated that the differences between the parent and the mutants were statistically significant, where $p \leq 0.01$.

The membrane fluidity of the Δ*waaC*, Δ*waaF*, Δ*waaP*, Δ*waaG* and Δ*waaY* mutants was measured and compared to the parent strain (fig. 6.6). In the parent and mutant TraDIS libraries, similar levels of mutants were recovered with transposon insertions in the gene *waaY*, which encodes the enzyme responsible for the addition of the first phosphate group to heptose II. Therefore, the Δ*waaY* mutant was utilised as a control in this study. The membrane fluidity of the Δ*waaC*, Δ*waaF*, Δ*waaG* and Δ*waaP* mutants was higher than that of the parent strain. An independent sample T-test confirmed that the differences between the parent and the mutants Δ*waaC*, Δ*waaF* and Δ*waaG* were statistically significant, where $p < 0.05$. Of all the mutants tested, the difference between the Δ*waaY* mutant and the parent was the smallest. This difference was not statistically different. This is consistent with the TraDIS data that

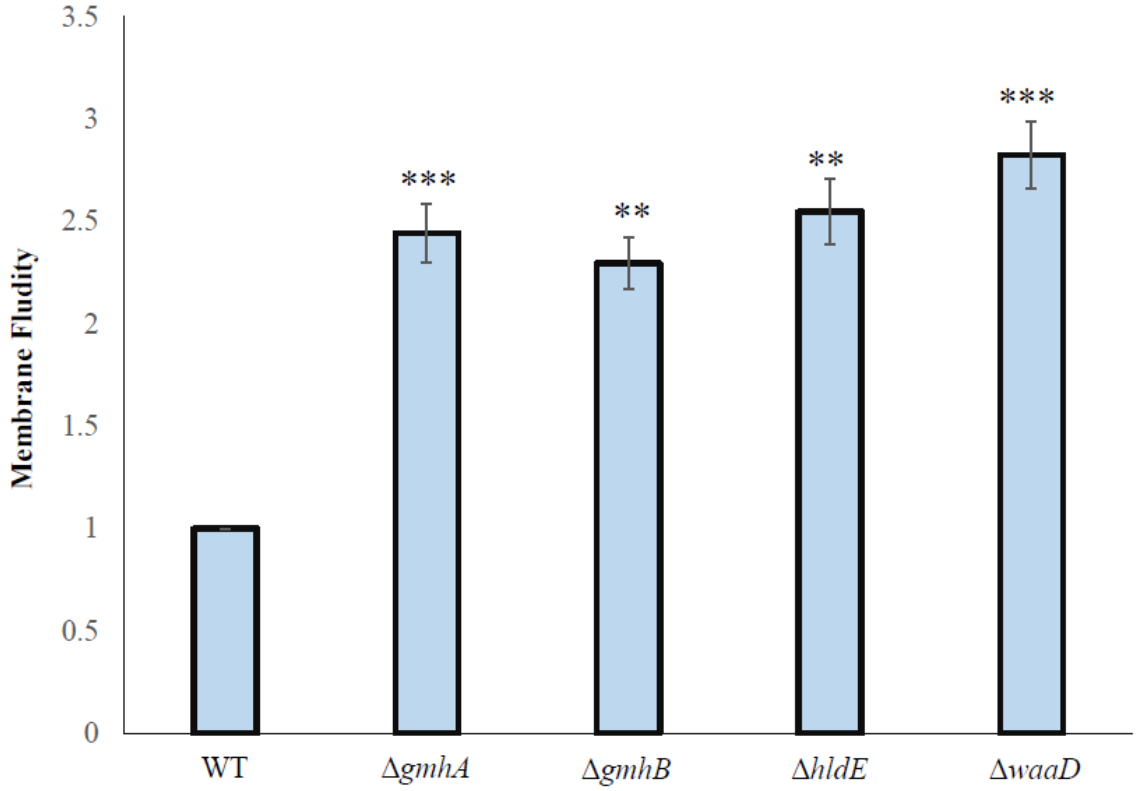


Fig. 6.5 Membrane fluidity of mutants impaired in the synthesis of heptose compared to the parent strain. Membrane fluidity of the mutants and the parent was measured in technical and biological triplicate. The membranes of the Δ*gmhA*, Δ*gmhB*, Δ*hldE* and Δ*waaD* mutants were more fluid than the parent strain. An independent sample T-test confirmed that the differences between the parent and these mutants were statistically significant, where $p \leq 0.01$.

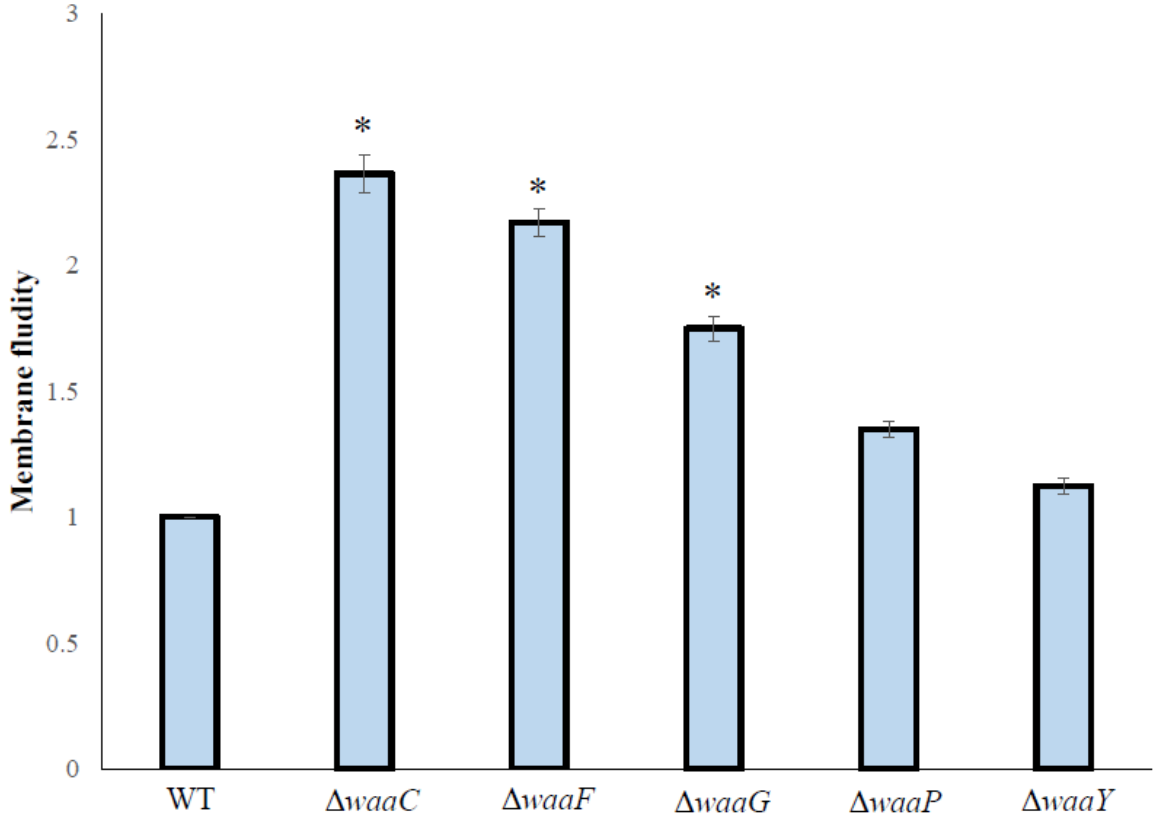


Fig. 6.6 Membrane fluidity of Δ*waaC*, Δ*waaF*, Δ*waaG*, Δ*waaP* and Δ*waaY* mutants compared to the parent strain. Membrane fluidity of the mutants and the parent was measured in technical and biological triplicate. The membranes of the Δ*waaC*, Δ*waaF*, Δ*waaG*, Δ*waaP* mutants were more fluid than the parent and the Δ*waaY* mutant. An independent sample T-test identified statistically significant differences. The membrane of the Δ*waaC* mutant was more fluid than any of the other mutants tested.

demonstrated that this gene was not functionally important in any of the BAM mutants tested.

In summary, membrane fluidity of the parent and the LPS biogenesis mutants was measured. The TraDIS data demonstrated that genes involved in LPS biogenesis were functionally important to the Δ*bamB*, Δ*surA* and Δ*degP* mutants. Upon loss of any of these genes, the membrane becomes more fluid. The more intact the inner core of the LPS molecules are in the cell, the lower the membrane fluidity. Thus, membrane fluidity is impacted to differing degrees depending on the length of the inner core of the LPS.

6.2.3. The link between membrane fluidity and BAM activity

Membrane fluidity was higher in mutants impaired in heptose production than in the parent strain. Next it was investigated whether impairment of the synthesis of heptose affected the activity of the BAM complex. Activity of the BAM complex was monitored by an in vivo OmpT fluorescence assay, which measured the insertion of OmpT into the OM. The inserted OmpT cleaved a fluorogenic peptide, which produced a fluorescence emission that was recorded on a plate reader.

OmpT was at least 50% less active in the Δ*gmhA*, Δ*gmhB*, Δ*hldE* and Δ*waaD* mutants than in the parent strain (fig. 6.7). The decrease in OmpT activity was the highest in the Δ*gmhA* mutant compared to other three *waa* mutants (fig. 6.7). However, there were only minor differences in OmpT activity between the Δ*gmhA*, Δ*hldE* and Δ*waaD* mutants. The TraDIS data demonstrated that loss of *gmhB* had the smallest effect on fitness compared to loss of the other heptose synthesis genes. Both the decrease in OmpT activity and the increase in membrane fluidity were lowest in the Δ*gmhB* mutant compared to the other mutants (fig. 6.7). These results suggest that mutations in the heptose production pathway impact membrane fluidity and BAM complex efficiency to differing degrees.

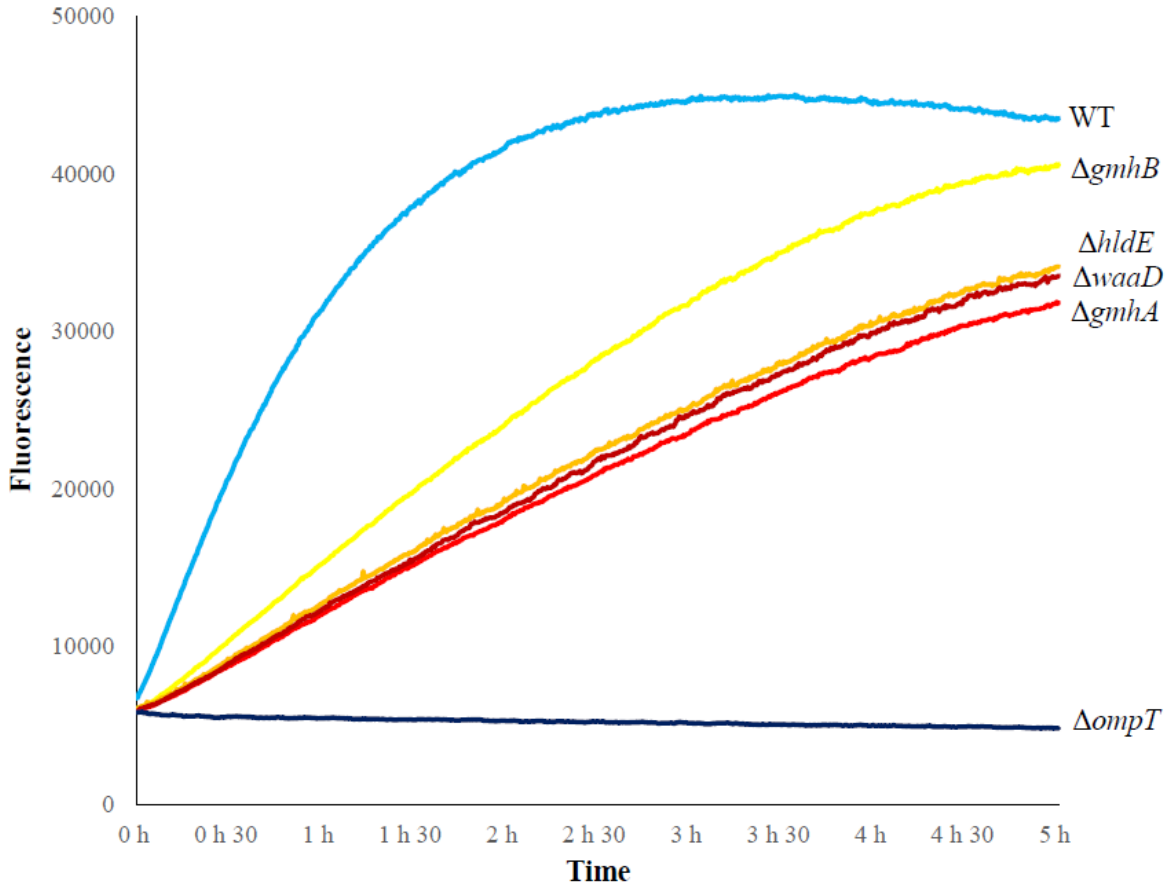


Fig. 6.7 BAM activity in the Δ*gmhA*, Δ*gmhB,* Δ*hldE* and Δ*waaD* mutants compared to the parent strain. To determine the rate of BAM activity, OmpT insertion into the OM was measured in biological and technical triplicate in the Δ*gmhA*, Δ*gmhB,* Δ*hldE* and Δ*waaD* mutants and in the parent strain. OmpT was not active in the control Δ*ompT* mutant. OmpT was less active in the Δ*gmhA*, Δ*gmhB,* Δ*hldE* and Δ*waaD* mutants than in the parent.

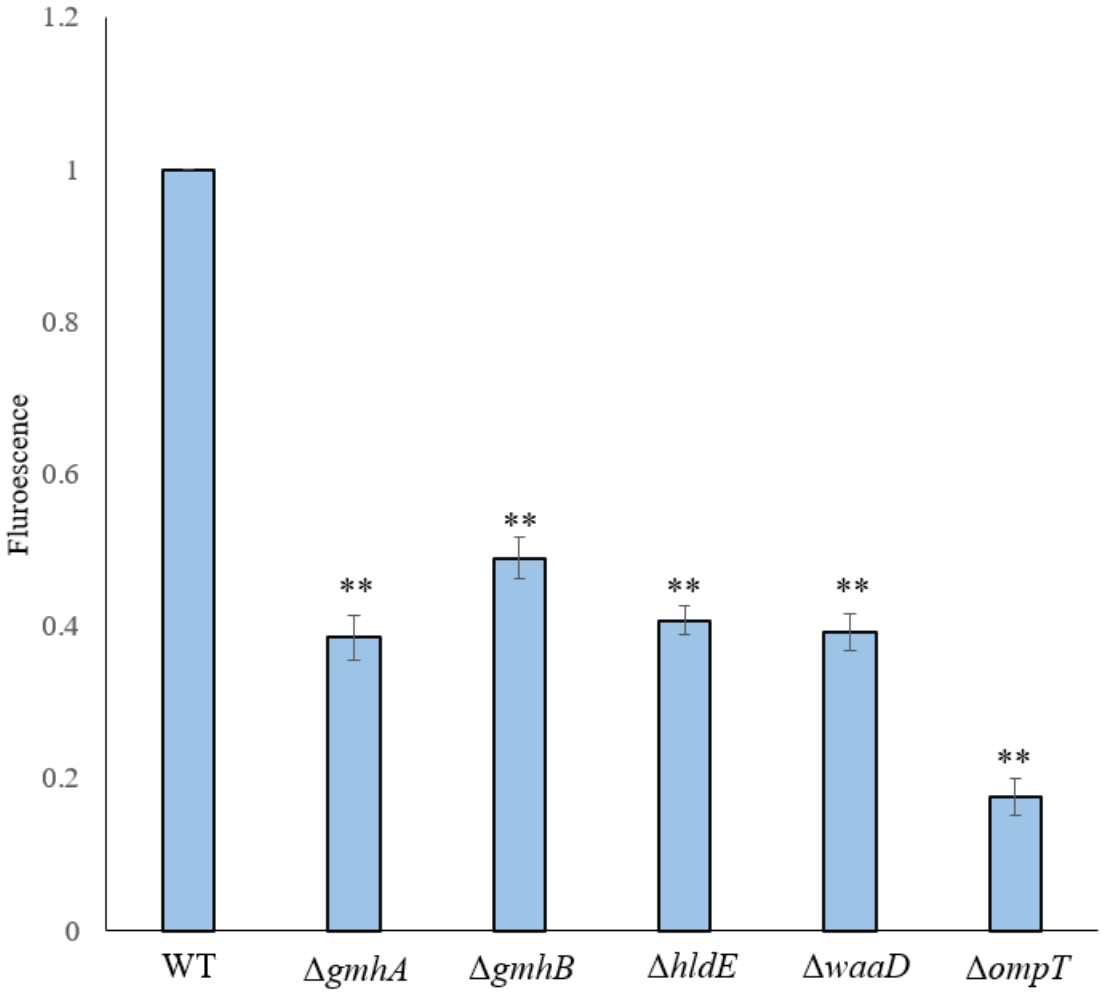


Fig. 6.8 OmpT insertion and cleavage monitored by fluorescence emission. A bar-chart representation of fluorescence emission of the OmpT cleavage assay at hour one. OmpT was at least 50% less active in the mutants tested than in the parent strain. An independent sample T-test confirmed that these differences were statistically significant, where $p \leq 0.01$.

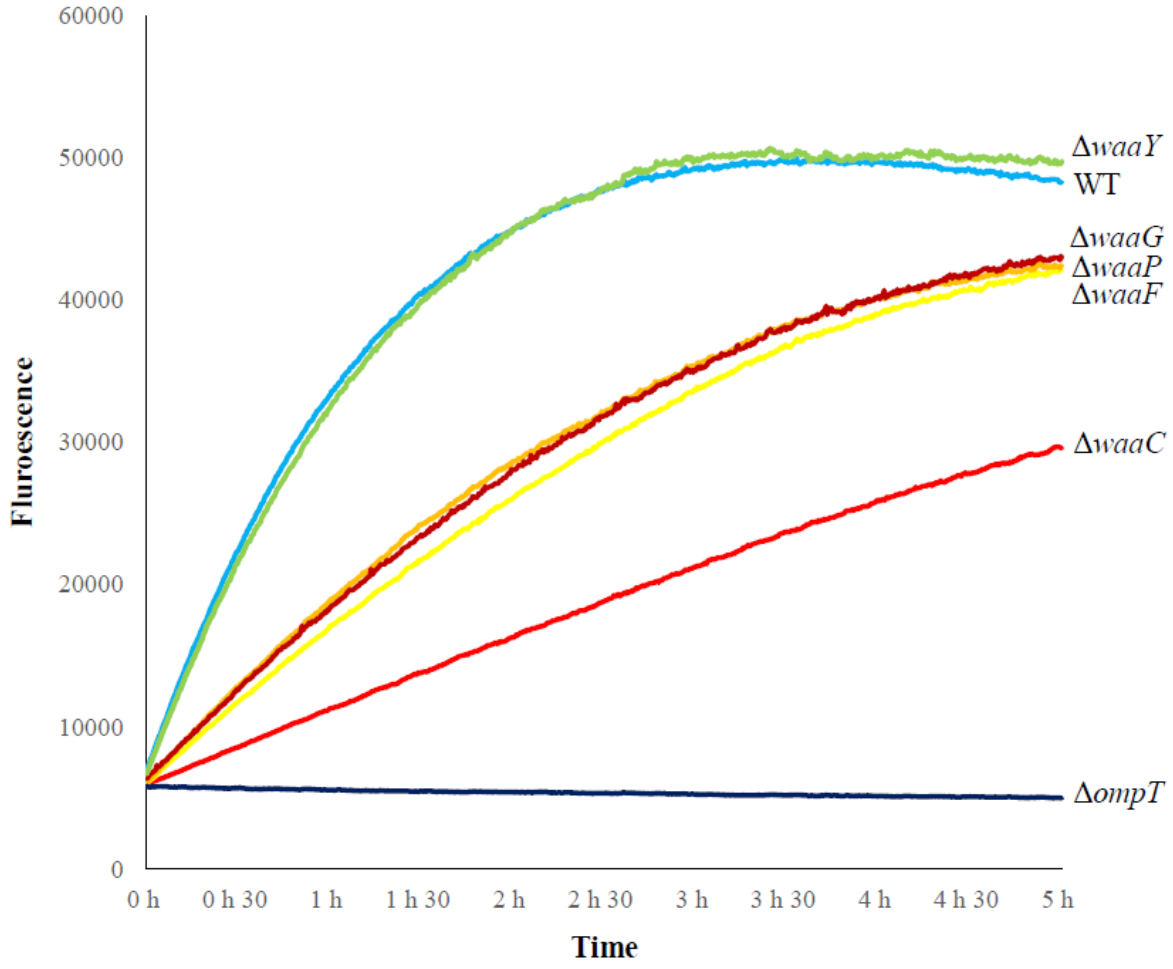


Fig. 6.9 OmpT cleavage assay monitored in LPS mutants. Successful folding and insertion of OmpT into the OM was measured in biological and technical triplicate in Δ*waaC*, Δ*waaF*, Δ*waaP*, Δ*waaG* and Δ*waaY* mutants and in the parent strain. OmpT was not active in the control Δ*ompT* mutant. OmpT was less active in the Δ*waaC*, Δ*waaF*, Δ*waaP* and Δ*waaG* mutants than in the parent and the Δ*waaY* mutant.

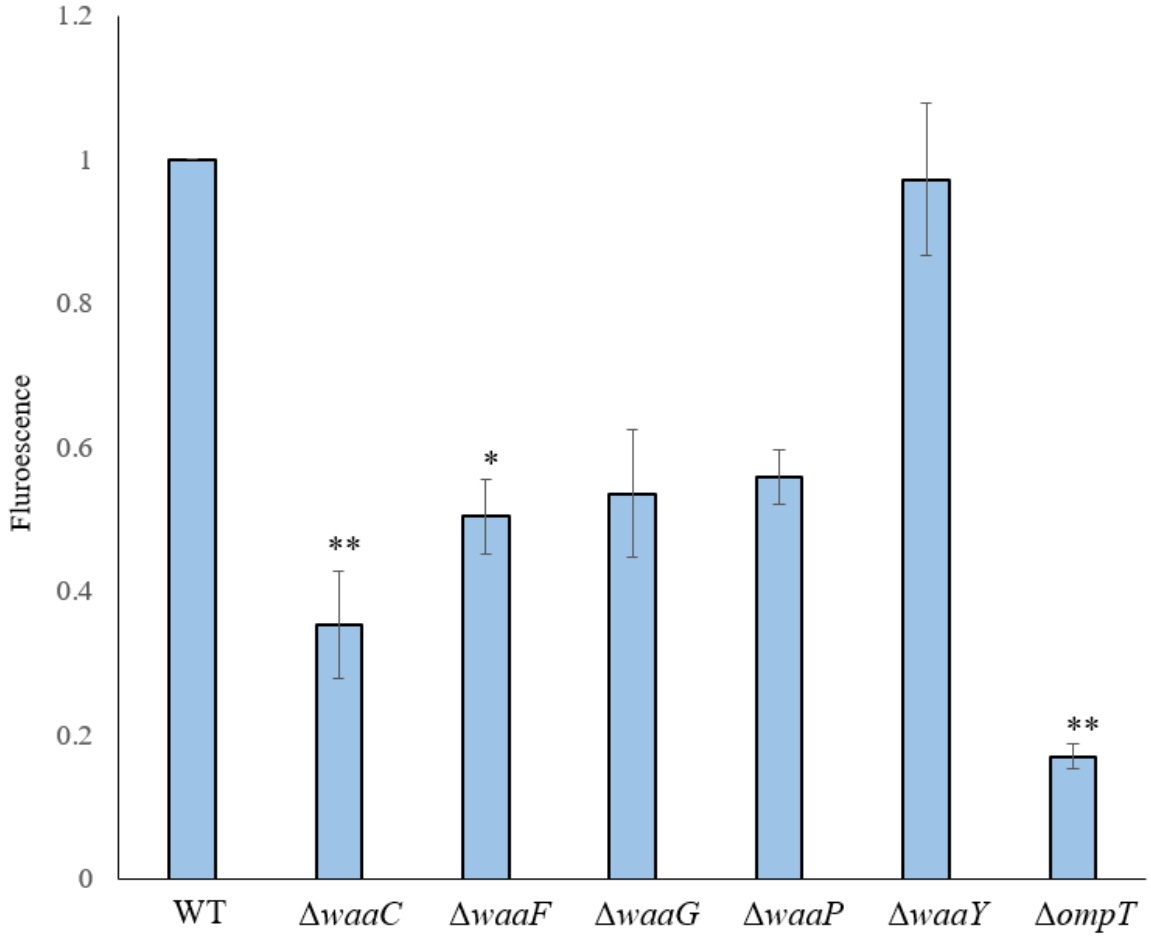


Fig. 6.10 Fluorescence emission at hour 1 in the OmpT in vivo assay. A bar-chart representation of fluorescence emission of the OmpT cleavage assay at hour one. OmpT was at least 40% less active in the Δ*waaC*, Δ*waaF*, Δ*waaG* and Δ*waaP* mutants than in the parent. No difference was noted in the rate of OmpT insertion into the OM between the Δ*waaY* mutant and the parent.

OmpT insertion into the OM of the Δ*waaC*, Δ*waaF*, Δ*waaP*, Δ*waaG* and Δ*waaY* mutants was measured and compared to the parent strain. OmpT was at least 40% less active in the Δ*waaC*, Δ*waaF*, Δ*waaP* and Δ*waaG* mutants than in the parent strain (fig. 6.9). The defects were larger in the Δ*waaC* mutant than in the other three *waa* mutants (fig. 6.10). In addition, loss of *waaY* did not compromise BAM activity. Thus, the length of the LPS inner core affects the activity of the BAM complex.

In summary, the BAM complex was less active in the Δ*gmhA*, Δ*gmhB*, Δ*hldE*, Δ*waaD*, Δ*waaC*, Δ*waaF*, Δ*waaG* and Δ*waaP* LPS mutants than in the parent and the Δ*waaY* mutant. Thus, BAM activity is impacted to differing degrees depending on the efficiency of the heptose production pathway and the length of the LPS inner core.

6.2.4. The coordination of peptidoglycan synthesis with OMP biogenesis

This study identified two cell envelope processes, LPS and ECA biogenesis, which become more important upon impairment of OMP biogenesis. To help understand the coordination between OMP biogenesis and other cell envelope processes, the TraDIS data were searched for additional synthetic lethal partners that are involved in cell envelope pathways. The synthetic lethality between members of the BAM pathway and genes involved in peptidoglycan synthesis is of interest to this study as the coordination between peptidoglycan synthesis and OMP biogenesis remains poorly understood.

The gene *dapF* was identified as conditionally essential in the Δ*bamB*, Δ*bamC* and the Δ*surA* TraDIS libraries (fig. 6.11A). DapF converts LL-diaminopimelate (LL-DAP) to *meso*-diaminopimelate (*meso*-DAP), which is either decarboxylated by LysA to produce L-lysine or utilised in the biosynthesis of peptidoglycan (Dewey and Work, 1952; Richaud *et al.*,

1987). The gene *lysA* was non-essential in the Δ*bamB*, Δ*bamC* and the Δ*surA* TraDIS libraries (fig. 6.11B). Thus, the gene *dapF* is required for the survival of these mutants due to its role in the synthesis of peptidoglycan.

6.2.5. The importance of *dapF* in the non-essential BAM mutants

The gene *dapF* was simultaneously inactivated alongside the genes encoding BamB, BamC, BamE, SurA, Skp and DegP, in the presence of *meso*-DAP supplied externally, which alleviates the loss of *dapF*. Double mutants were constructed to determine the fitness defects that occur upon deletion of *dapF* alongside components of the BAM pathway. The single Δ*dapF*, Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp* and Δ*degP* mutants and double mutants Δ*bamB*Δ*dapF*, Δ*bamC*Δ*dapF*, Δ*bamE*Δ*dapF*, Δ*surA*Δ*dapF*, Δ*skp*Δ*dapF* and Δ*degP*Δ*dapF* were screened on LB agar with and without *meso*-DAP.

The presence and absence of *meso*-DAP did not affect the growth of the parent or any of the single mutants: Δ*dapF*; Δ*bamB*; Δ*bamC*; Δ*bamE*; Δ*surA*; Δ*skp* and Δ*degP* (fig. 6.12). However, in the absence of *meso*-DAP, the growth of all of the double mutants were severely impaired. In contrast, the growth of the Δ*bamC*Δ*dapF*, Δ*bamE*Δ*dapF*, Δ*skp*Δ*dapF* and Δ*degP*Δ*dapF* double mutants were restored on LB agar supplemented with 1 mM *meso*-DAP (fig. 6.12). However, the growth of the Δ*surA*Δ*dapF* and Δ*bamB*Δ*dapF* double mutants were only partially restored in the presence of 1 mM *meso*-DAP, which suggests that the most severe fitness defect occurs in these mutants (fig. 6.12A). Thus, a negative genetic interaction occurs when *dapF* is simultaneously deleted alongside *bam*C, *bamB, bamE, surA, degP* or *skp*.

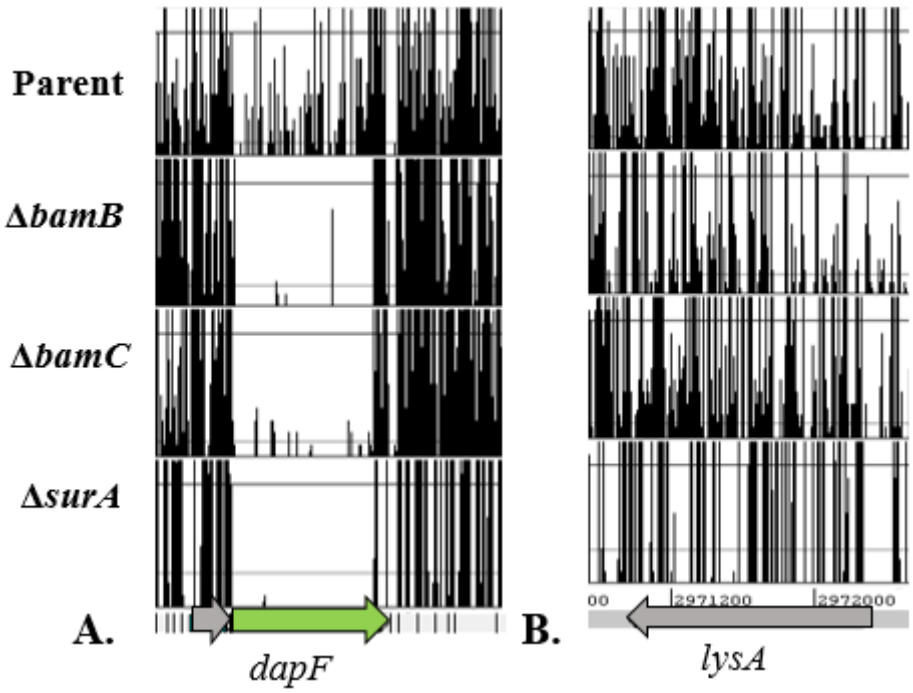


Fig. 6.11 Transposon insertions in the genes *dapF* and *lysA* in the parent and mutant TraDIS libraries. (A) In the Δ*bamB*, Δ*bamC* and Δ*surA* TraDIS libraries, the gene *dapF* was identified as conditionally essential. (B) The gene *lysA* was dispensable in all of the libraries tested.

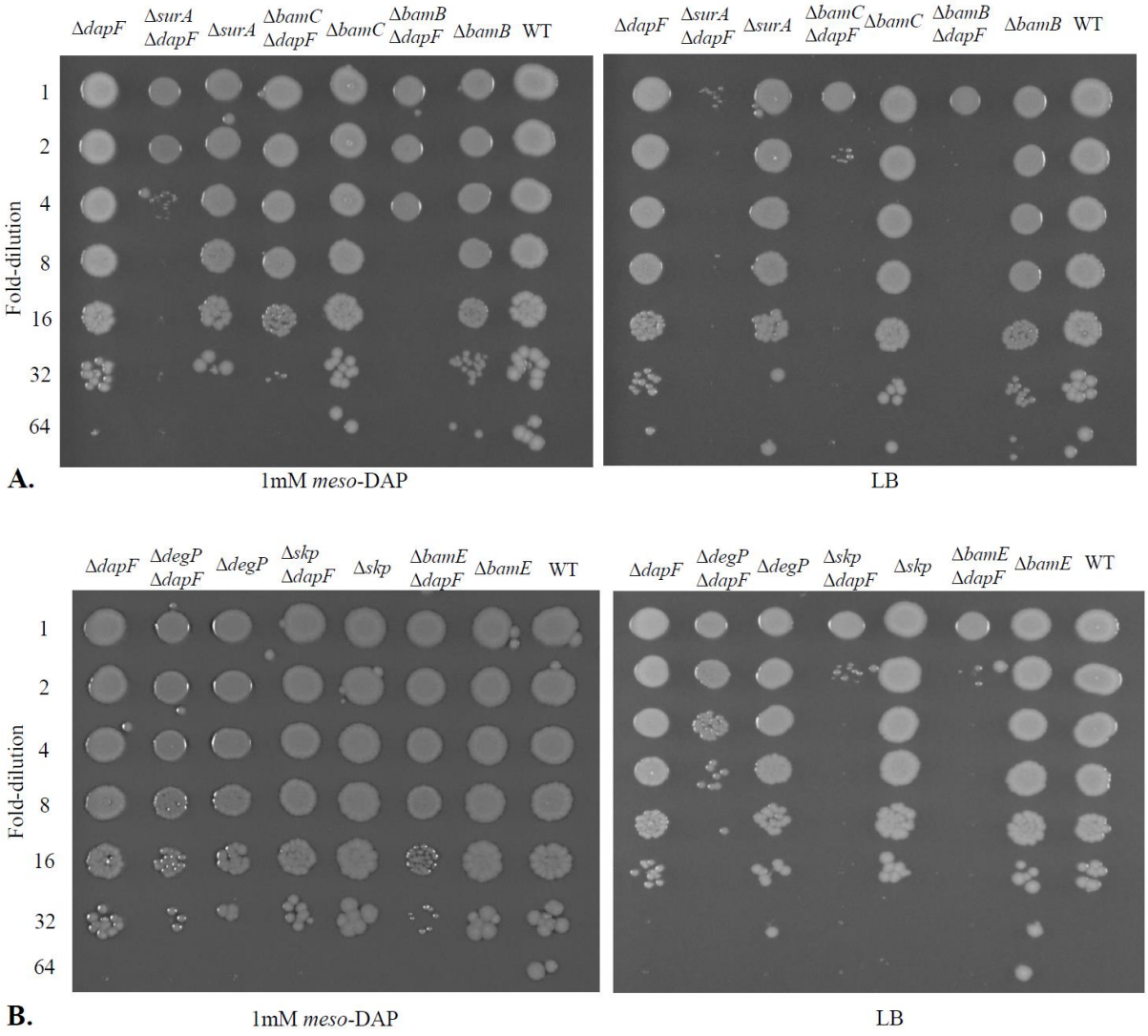


Fig. 6.12 The importance of the gene *dapF* to non-essential BAM mutants. (A) Strains (from left to right) were Δ*dapF*, Δ*surA*Δ*dapF*, Δ*surA*, Δ*bamC*Δ*dapF*, Δ*bamC*, Δ*bamB*Δ*dapF*, Δ*bamB* and the parent (WT). Strains were grown overnight, diluted to an OD_{600} of 1 and 2 µl of 10-fold serially diluted strains were inoculated onto agar plates with and without 1 mM *meso*-DAP. (B) Strains (from left to right) were Δ*dapF*, Δ*degP*Δ*dapF*, Δ*degP*, Δ*skp*Δ*dapF*, Δ*skp*, Δ*bamE*Δ*dapF*, Δ*bamE* and the parent (WT). Strains were grown overnight and diluted to an OD_{600} of 1. Serially diluted strains were inoculated onto agar plates in the presence and absence of 1 mM *meso*-DAP.

6.2.6. The effect of the combined loss of *dapF* and members of the BAM pathway on growth kinetics

Growth kinetics of the Δ*bamB*Δ*dapF*, Δ*bamE*Δ*dapF*, Δ*bamC*Δ*dapF* and Δ*surA*Δ*dapF* double mutants and the respective single Δ*bamB,* Δ*bamE,* Δ*surA*, Δ*dapF* mutants were monitored for 9 hours (fig. 6.13-6.14). All mutants were grown in the presence and absence of various concentrations of *meso*-DAP: 1 mM *meso*-DAP, 0.1 mM *meso*-DAP or 0.01 mM *meso*-DAP. In the absence of *meso*-DAP, the Δ*dapF* mutant had a decreased growth rate (fig. 6.13B). However, the absence of *meso*-DAP severely impaired or inhibited the growth of the double mutants. In contrast, the presence of *meso*-DAP partially restored the growth of the double mutants in a concentration dependent manner (fig. 6.13-6.14). The higher the concentration of *meso*-DAP, the more the growth of the double mutants were restored to that of the single mutants. Consequently, the availability of *meso*-DAP is important to OMP biogenesis.

6.2.7. Lysis of the Δ*bamB*Δ*dapF*, Δ*bamC*Δ*dapF* and Δ*bamE*Δ*dapF* double mutants

Compromising the integrity of the OM elicits different modes and rates of lysis. The mode and rate of lysis can be utilised to understand how conditions induce lysis or how a mutation in an essential gene induces lysis. For example, beta-lactams inhibit cell wall synthesis and induce lysis through a bulge-mediated mechanism (Yao *et al.,* 2012). Time-lapse microscopy was utilised to monitor lysis of the Δ*bamC*Δ*dapF*, Δ*bamB*Δ*dapF* and Δ*bamE*Δ*dapF* double mutants in the absence of *meso*-DAP. The rate and manner of lysis of these double mutants can help determine why *dapF* is functionally important in the BAM mutants.

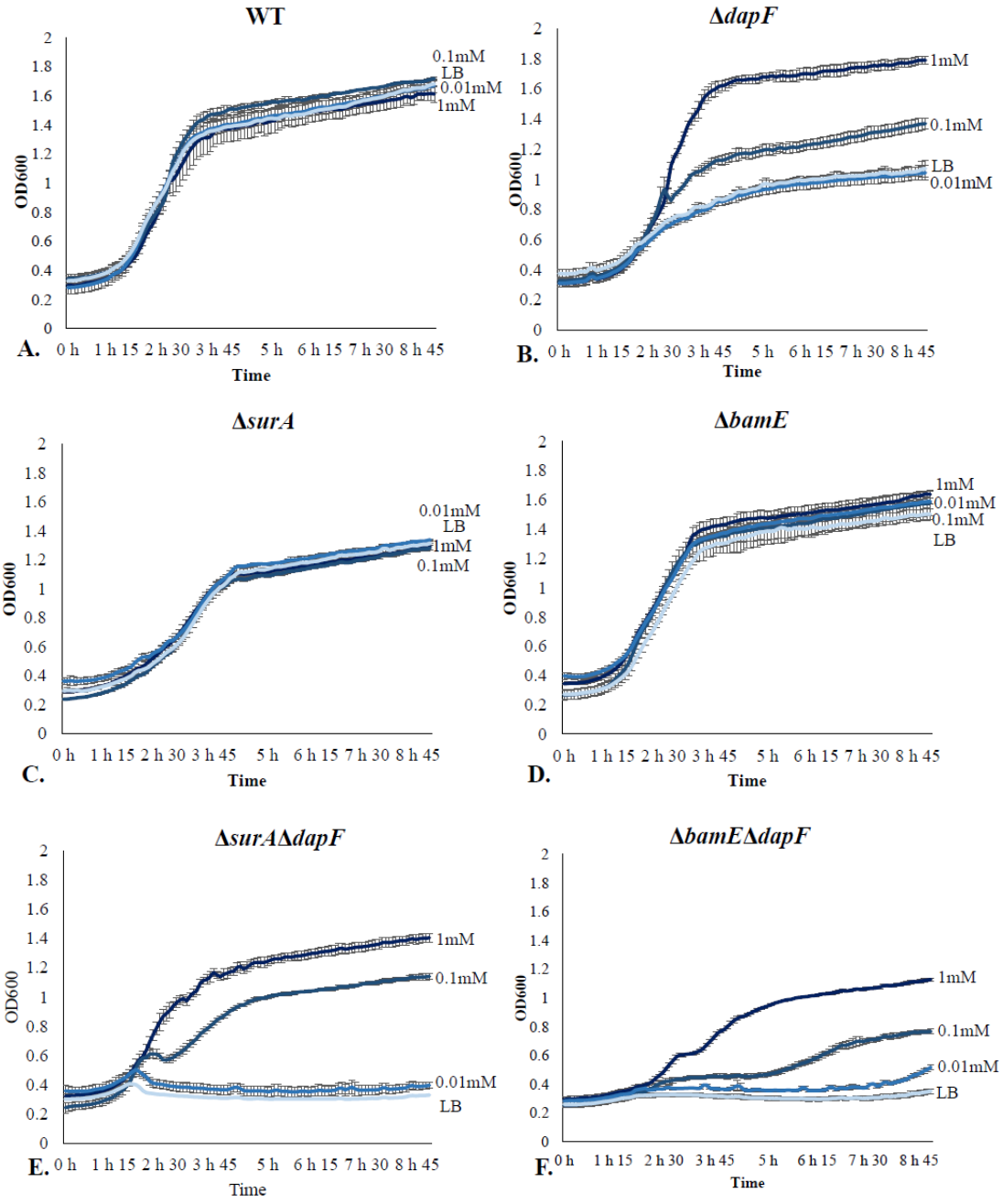


Fig. 6.13 The effect of *meso*-DAP on OMP biogenesis. The growth kinetics of the Δ*bamE*Δ*dapF* and Δ*surA*Δ*dapF* double mutants and the respective single Δ*bamE*, Δ*surA* and Δ*dapF* mutants were monitored for 9 hours, with and without *meso*-DAP. (A, C-D) The presence of *meso*-DAP did not affect the growth of the parent strain or the Δ*bamE* and Δ*surA* mutants. (B) The absence of *meso*-DAP reduced the growth rate of the Δ*dapF* mutant. However, this decrease in growth rate was significantly smaller in the Δ*dapF* mutant than in any of the double mutants tested. (E-F) In the absence of *meso*-DAP, growth of the Δ*bamE*Δ*dapF* and Δ*surA*Δ*dapF* mutants were inhibited. (E-F) The presence of *meso*-DAP partially restored the growth of the Δ*bamE*Δ*dapF* and Δ*surA*Δ*dapF* mutants, in a concentration dependent manner.

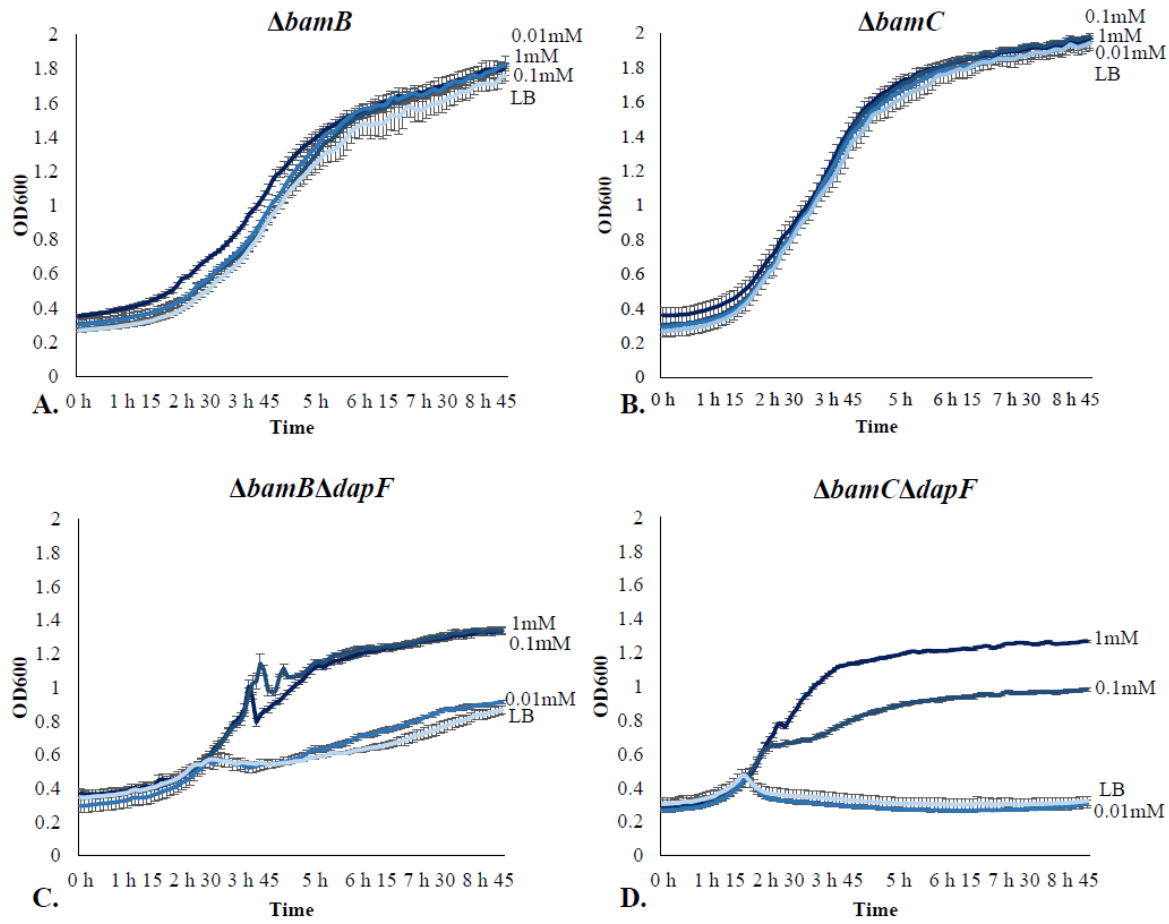


Fig. 6.14 The effect of the presence and absence of *meso*-DAP on the growth of the Δ*bamB*Δ*dapF* and Δ*bamC*Δ*dapF* mutants. The growth kinetics of the Δ*bamB*Δ*dapF* and Δ*bamC*Δ*dapF* double mutants and the respective single Δ*bamB* and Δ*bamC* mutants were monitored for 9 hours in the presence and absence of *meso*-DAP. (A-B) The presence of *meso*-DAP did not affect the growth of the Δ*bamB* and Δ*bamC* mutants. (C-D) The absence of *meso*-DAP severly impaired or inhibited the growth of the Δ*bamB*Δ*dapF* and Δ*bamC*Δ*dapF* mutants. The presence of *meso*-DAP partially restored the growth of the Δ*bamB*Δ*dapF* and Δ*bamC*Δ*dapF* mutants, in a concentration dependent manner.

6.2.7.1. Lysis of the Δ*bamB*Δ*dapF* mutant. Some of the Δ*bamB*Δ*dapF* cells divided once and in some rare cases twice before lysis. Lysis of the Δ*bamB*Δ*dapF* mutant can be divided into a number of steps (fig. 6.15A): filamentation (9 min); bulge formation (9 min); expansion of the bulge and length of the cell (9-11 min); cessation of growth, while the bulge continues to increase in size until lysis occurs (15-19 min). In the Δ*bamB*Δ*dapF* mutant, the bulge formed abruptly at the potential division site and lysis occurred before the two daughter cells divided. No membrane gaps were formed between the bulge and the dividing cells. Bulge formation (T_B) can be quantified ($T_B = t_3 – t_2$), where t_3 is the time required for bulge formation and t_2 is the time that bulge formation begins (Yao *et al.,* 2012). T_B of the Δ*bamB*Δ*dapF* mutant was 9 min, with ~86% of cells lysing in this manner (n=35). The remainder lysed prior to bulge formation.

6.2.7.2. Lysis of the Δ*bamE*Δ*dapF* mutant. The double mutant Δ*bamE*Δ*dapF* lysed in a similar manner to Δ*bamB*Δ*dapF* (fig. 6.15B). The cells filamented and formed a bulge (~7 min). This bulge rapidly increased in size and was shortly followed by lysis (11 min) (over 80% of cells where n=43). The bulge lifetime (T_{BL}), which refers to the length of time between onset of bulge formation (t_4) and lysis (t_2), was considerably shorter for the Δ*bamE*Δ*dapF* mutant (4 min) than the Δ*bamB*Δ*dapF* mutant (8 min). This is probably because the Δ*bamB* mutant grows more slowly than the Δ*bamE* mutant.

Yao *et al.* (2012) exposed *E. coli* cells to cephalexin, an antibiotic that disrupts PG synthesis. These cells underwent the same stages of lysis as the Δ*bamB*Δ*dapF* and the Δ*bamE*Δ*dapF* mutants, which suggests that the membrane protrusions that are formed at the potential division site in the Δ*bamB*Δ*dapF* and the Δ*bamE*Δ*dapF* mutants occur due to defects in PG.

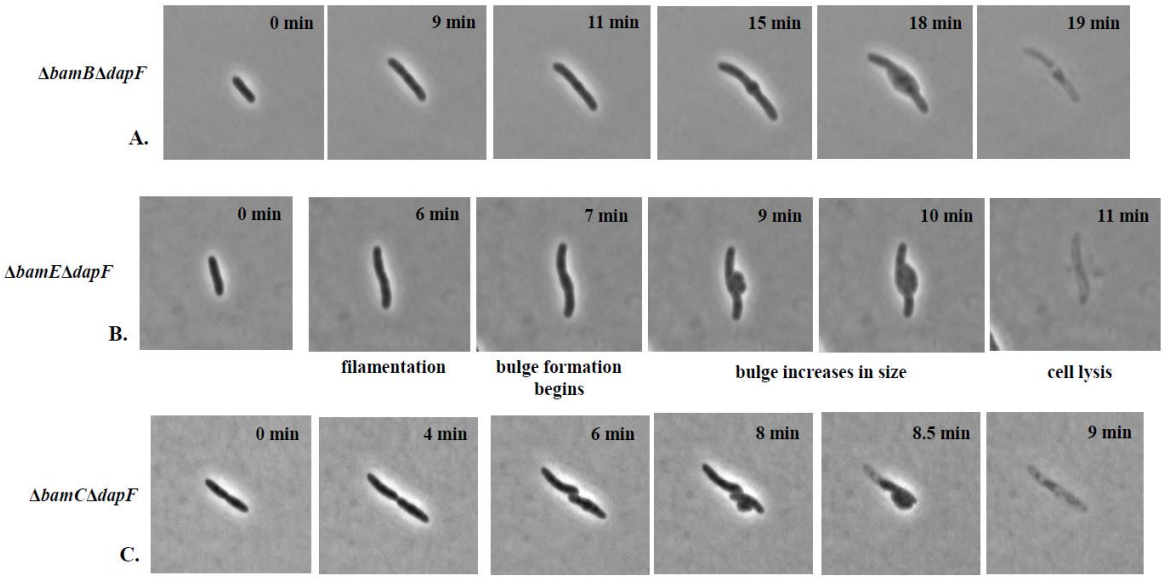


Fig. 6.15 Lysis of double mutants. (A) Lysis of Δ*bamB*Δ*dapF*, (B) Δ*bamE*Δ*dapF* and (C) Δ*bamC*Δ*dapF* double mutants visualised by time-lapse microscopy. Both the Δ*bamB*Δ*dapF* and the Δ*bamE*Δ*dapF* lysed via a five step process: filamentation; bulge formation; the cell and bulge increase in size; cessation of growth, while the bulge continues to increase in size; and cell lysis.

Possibilities of why *dapF* is synthetically lethal with *bamB* and *bamE* was discussed in chapter seven.

6.2.7.3. Lysis of the Δ*bamC*Δ*dapF* mutant. Lysis of the Δ*bamC*Δ*dapF* mutant was morphological distinct from the other double mutants (fig. 6.15C). It did lyse in a bulge-mediated method. However, septation was not blocked and the bulge did not form until after cell division was complete. The bulge did form at mid-cell, where the cytoplasm blebbed out of the cell wall, shifting the turgor pressure entirely onto the OM, which led to lysis of the cell. The morphological distinctions between lysis of the Δ*bamC*Δ*dapF* mutant and lysis of the Δ*bamB*Δ*dapF* and Δ*bamE*Δ*dapF* double mutants suggests that the synthetic lethality between *dapF* and *bamC* is not due to the same mechanism as the other BAM mutants.

In summary, the gene *dapF* is required for the survival of Δ*bamB*, Δ*bamC* and the Δ*surA* mutants due to its role in the synthesis of peptidoglycan. In addition, a negative genetic interaction occurs when *dapF* is simultaneously deleted alongside *bamE*, *degP* or *skp*. In the absence of *meso*-DAP, growth of the Δ*bamB*Δ*dapF*, Δ*bamC*Δ*dapF*, Δ*bamE*Δ*dapF* and Δ*surA*Δ*dapF* double mutants were inhibited. Lastly, lysis of the Δ*bamC*Δ*dapF* double mutant was morphologically distinct to lysis of Δ*bamB*Δ*dapF* and Δ*bamE*Δ*dapF* double mutants.

6.3. Conclusion

TraDIS libraries constructed in Δ*bamB*, Δ*bamC*, Δ*bamE*, Δ*surA*, Δ*skp* and Δ*degP* mutants were compared to increase understanding of how OMP biogenesis is coordinated with other cell envelope pathways. The TraDIS data demonstrated that loss of genes involved in the

synthesis and incorporation of heptose into LPS, combined with a sufficient defect in BAM function (loss of *bamB, surA* or *degP*) is lethal to the cell. The LPS extracted from the BAM mutants were analysed and compared to the parent strain. These results demonstrated that loss of a non-essential member of the BAM pathway did not induce a significant difference in the LPS structure. Next, it was investigated whether the synthetic lethality was due to fluctuations in membrane fluidity affecting the activity of the BAM complex. In the mutants Δ*gmhA*, Δ*gmhB*, Δ*hldE*, Δ*waaD*, Δ*waaC*, Δ*waaF*, Δ*waaG* and Δ*waaP*, the membranes were more fluid and the BAM complex was less active than in the parent and the Δ*waaY* mutant. Membrane fluidity and BAM activity was shown to be impacted to differing degrees depending on the efficiency of the heptose production pathway and the length of the inner core of LPS.

In addition, this study identified synthetic lethality between members of the BAM pathway and a gene involved in peptidoglycan synthesis. The gene *dapF* is conditionally essential in the Δ*bamB*, Δ*bamC* and the Δ*surA* mutants due to its role in peptidoglycan synthesis, not its role in lysine synthesis. This study validated the genetic interaction between *dapF* and components of the BAM pathway: *bam*C; *bamB*; *bamE*; *surA*; *degP*; and *skp*. In the absence of *meso*-DAP, growth of the Δ*bamB*Δ*dapF*, Δ*bamC*Δ*dapF*, Δ*bamE*Δ*dapF* and Δ*surA*Δ*dapF* double mutants were inhibited or severely impaired. In contrast, the growth of the double mutants were restored by the *meso*-DAP, in a concentration dependent manner. Lastly, the rate and manner of lysis of the Δ*bamC*Δ*dapF*, Δ*bamB*Δ*dapF* and Δ*bamE*Δ*dapF* double mutants were monitored in an attempt to understand why *dapF* is functionally important to the BAM mutants. The morphological distinctions between lysis of the Δ*bamC*Δ*dapF* mutant and lysis of the Δ*bamB*Δ*dapF* and Δ*bamE*Δ*dapF* double mutants suggests that the

synthetic lethality between *dapF* and *bamC* is not due to the same mechanism as the other BAM mutants.

CHAPTER 7

Discussion

7.1. Aims of this study

Overall, this Thesis examined the β-barrel assembly pathway. Chapter three centred on determining the role of BamB in outer membrane protein biogenesis, while in chapter four, the roles of BamC and BamE were considered. Critical to understanding OMP biogenesis is identifying the precise contributions of the three known BAM chaperones SurA, Skp and DegP. Consequently, an aim of this study was to determine whether these three putative chaperones are functionally redundant. This is largely resolved in chapter five. Lastly, chapter six explored the coordination between OMP biogenesis and other cell envelope pathways.

7.2. The non-essential BAM subunits

7.2.1. The role of BamB

In this study, TraDIS was utilised to help determine the roles of BamB, BamC and BamE in the cell. TraDIS libraries were constructed in mutants devoid of *bamB, bamC* or *bamE*. The data generated were compared to a parent TraDIS library to identify conditionally essential genes. This study hypothesized that the function of BamB was separate to BamC and BamE. Redundant proteins should contain similar synthetic lethal partners and as a result similar conditionally essential gene profiles. There were clear differences in regards to the number of conditionally essential genes identified and the contents of these essential gene lists between the Δ*bamB* and Δ*bamC* datasets and between the Δ*bamB* and Δ*bamE* datasets (fig. 4.8-4.9). Thus, BamB elicits a separate function to BamC and BamE. The results from the TraDIS data alone did not identify the function of BamB. However, it is possible that BamB coordinates OMP biogenesis with cell division. This hypothesis is based on the number of genes involved in cell division, recombination and repair that were identified as functionally

important in the Δ*bamB* mutant (fig. 3.8 and 3.10).

7.2.2. The role of BamC and BamE

Rigel *et al.* (2012) suggested that a functional overlap exists between BamC and BamE. However, there were clear differences between the Δ*bamC* and Δ*bamE* datasets in respect to the number of conditionally essential genes identified and the contents of these essential gene lists (fig. 4.8-4.9). Thus, the roles of BamC and BamE do not overlap. This study also proposes a potential new function for BamC in coordinating OMP biogenesis with peptidoglycan synthesis. This hypothesis is discussed in section 7.4.3.

7.3. The BAM chaperones

The three known BAM chaperones, SurA, Skp and DegP also elicit key roles in OMP biogenesis. Contrary to Rizzitello *et al.* (2001) and Sklar *et al.* (2007), there is no molecular evidence to suggest that the functions of SurA and Skp are redundant. To help understand this synthetic lethality, the number of conditionally essential genes and the contents of these essential gene lists were compared between the Δ*surA*, Δ*skp* and Δ*degP* TraDIS datasets. The highest number of conditionally essential genes were identified in the Δ*surA* TraDIS library (54). The number of conditionally essential genes identified in the Δ*degP* and Δ*skp* TraDIS libraries were 44 and 9, respectively (fig. 5.5C). A number of genes (17) were conditionally essential in both the Δ*surA* and Δ*degP* TraDIS libraries, which suggests the presence of an overlapping function between these two proteins.

In contrast, the number of conditionally essential genes identified in the Δ*surA* TraDIS library was quite different to the number identified in the Δ*skp* TraDIS library. The contents of these essential gene lists were also quite different (fig. 5.6). The Δ*surA* and Δ*skp* datasets

shared only three conditionally essential genes. Genes that were essential in both the Δ*surA* and Δ*degP* datasets were non-essential in the Δ*skp* dataset. Functionally redundant proteins or proteins that make up parallel pathways should contain similar synthetic lethal partners. Thus, the number of differences between the Δ*surA* and Δ*skp* datasets suggests that functional distinctions exist between these two proteins and that the role of these chaperones are not redundant.

7.3.1. The chaperone pathways to the BAM complex

The results from this study suggests that the functions of SurA and Skp are not redundant. Thus, the synthetic lethality between *surA* and *skp* might be due to reasons not previously reported. One possibility is that the synthetic lethality between *surA* and *skp* is not due to direct effects, but instead due to downstream effects. For example, loss of *surA* might negatively impact a pathway, pathway X, while loss of *skp* might negatively impact a different pathway, pathway Y. Loss of *surA* and *skp* in combination, impairs both pathway X and Y, which is lethal to the cell.

Another possibility is that both proteins act in sequence, where Skp delivers the OMPs to SurA and SurA docks the OMPs into the BAM complex. Losing one is kinetically unfavorable, while loss of both destabilizes OMP biogenesis, leading to a lack of OMP insertion into the OM, which results in cell death. Previous studies support this hypothesis, which demonstrated that Skp functions in early OMP biogenesis as Skp interacts with unfolded OMPs as they emerge from the SEC machinery and that no OMP depends on Skp for folding (Denoncin *et al.,* 2012; Harms *et al.,* 2001; Schafer *et al.,* 1999). In addition, SurA is the only periplasmic folding factor that can be cross-linked with BamA in vivo (Sklar

et al., 2007; Vuong *et al.*, 2008). In contrast, it is likely that proteins in the same pathway would share some synthetic lethal partners. Thus, this hypothesis is unlikely.

Similarly, it is possible that SurA functions as the main OMP chaperone, by transporting OMP intermediates from the IM to the BAM complex, while Skp acts as a holdase, disaggregating OMPs and protecting them from further aggregation (fig. 7.1). Thus, the functions of SurA and Skp would be distinct. As previously mentioned, Skp elicits holdase capabilities. Li *et al.* (2018) demonstrated that Skp is capable of converting aggregated OmpC into the monodisperse form, while SurA lacks this ability (Li *et al.*, 2018). Skp also prevents PagP aggregation in vitro and the shape of Skp is analogous to Prefoldin, a cytosolic chaperone that is a member of the holdase family (Goemans *et al.*, 2014; McMorran *et al.*, 2013). The lack of conditionally essential genes identified in the Δ*skp* TraDIS library suggests that loss of Skp has a minimum effect on fitness of the cell. However, under stress conditions, the holdase function of Skp might become crucial.

This study also demonstrated that an overlapping function exists between SurA and DegP. DegP is a dual acting protein with both protease and chaperone activity. In wild-type cells, DegP functions as a protease (≥28°C), degrading unfolded and misfolded OMPs. However, upon loss of SurA, DegP might partially fulfil the chaperone function to the BAM complex. Upon simultaneous deletion of SurA and DegP, there is no chaperone to deliver the OMPs to the BAM complex. This lack of OMP insertion results in demise of the cell. Upon deletion of SurA and Skp in combination, DegP is required to transport the OMPs to the BAM complex (fig. 7.1). However, the absence of SurA activates stress response systems, which upregulate the expression levels of OMPs. Therefore, OMPs are more prone to aggregation.

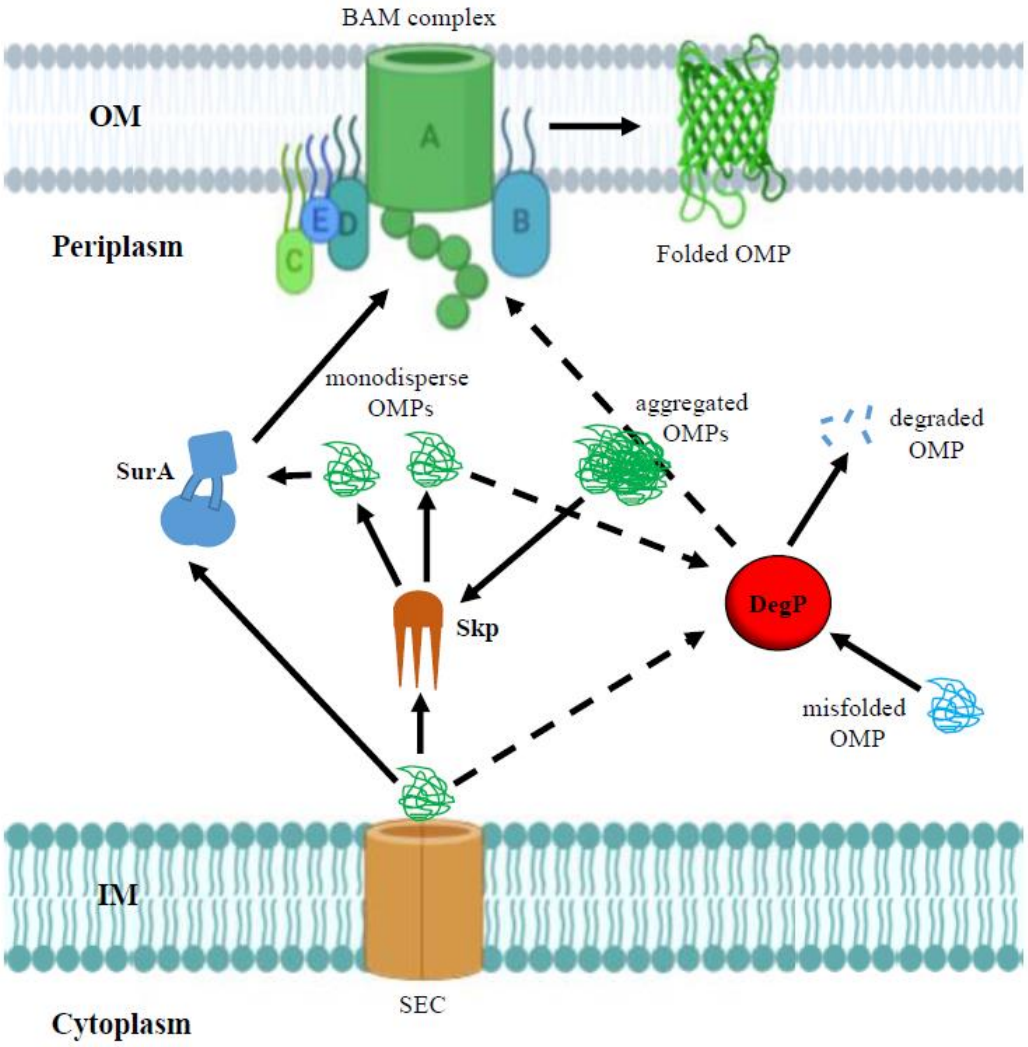


Fig. 7.1 Proposed mechanism for OMP transport to the BAM complex. In the periplasm, SurA functions as the main mechanism of OMP transport to the BAM complex. Skp acts as a holdase, disaggregating OMPs and protecting them from further aggregation, while DegP degrades misfolded and unfolded OMPs. An overlapping function exists between SurA and DegP. Thus, in the absence of SurA, DegP is required to transport the OMPs to the BAM complex (dashed arrows). Skp is required to disaggregate the OMPs. Skp converts the OMPs into the monodisperse form and reduces the toxicity of the aggregated proteins.

Skp is required to disaggregate the OMPs, which reduces the toxicity of the aggregated proteins. Skp converts the aggregated OMPs into the monodisperse form, which can then be transported to the BAM complex. Thus, in a Δ*surA*Δ*skp* double mutant, cell lysis occurs due to toxic OMP aggregation in the periplasm.

7.4. Cell envelope processes are highly coordinated

Multiple cell envelope processes including lipopolysaccharide synthesis, peptidoglycan synthesis and OMP biogenesis require coordination. However, these mechanisms of coordination are unknown. This study compared the conditionally essential genes identified in all of the mutant BAM TraDIS libraries in an attempt to unravel how OMP biogenesis coordinates with other cell envelope pathways.

7.4.1. The importance of ECA biosynthesis in OMP biogenesis

In the Δ*surA* TraDIS library, genes involved in the synthesis of enterobacterial common antigen **(**ECA) were conditionally essential. There are three forms of ECA: ECA covalently linked to LPS, ECA covalently linked to the phospholipid PG and the cyclic form. Data from this study suggests that the Δ*surA* mutant requires the cyclic form of ECA (fig. 5.9). A number of possibilities were considered for why the Δ*surA* mutant requires ECA. For example, disruption of ECA synthesis can cause downstream effects on other pathways including peptidoglycan synthesis (Mitchell *et al.,* 2018). Disruption of the first step in ECA synthesis increases the pool of precursors available for peptidoglycan synthesis. However, disruption of this step was not favourable in the Δ*surA* mutant (fig. 5.9B). Thus, the ECA pathway is not conditionally essential due to downstream effects on peptidoglycan synthesis.

In addition, this study demonstrated that ECA_{CYC} is unlikely to act as a chaperone to the BAM complex (fig. 5.11). However, it is possible that ECA_{CYC} might act as a holdase, preventing the aggregation of OMPs in the periplasm. Another possibility is that disruption of the ECA operon activates the Cpx, Rcs and σ^E stress responses (Danese *et al.*, 1998; Jorgenson *et al.*, 2016). In addition, loss of *surA* activates stress response pathways including the Rcs pathway (Castanie-Cornet *et al*., 2006). The combinational loss of *surA* and ECA might lead to excessive activation of stress responses, which might be lethal to the cell.

Lastly, it is possible that ECA_{CYC} helps stabilize the OM of a Δ*surA* mutant. Mitchell *et al.* (2018) demonstrated that ECA_{CYC} elicits a specific role in the maintenance of the OM permeability barrier. Loss of ECA_{CYC} suppresses the envelope permeability defect produced by an *yhdP* deletion, increases resistance to vancomycin and sensitivity to deoxycholate. This suggests that ECA_{CYC} catalyses changes to the permeability of the OM. Upon loss of SurA, these OM permeability changes might become crucial.

In summary, this study proposes a number of possibilities of why the Δ*surA* mutant requires ECA. Further experiments are required to determine if any of these possibilities are true.

7.4.1.1. Potential future experiments

7.4.1.1.1. The form of ECA that is essential in the Δ*surA* mutant. In order to confirm that the Δ*surA* mutant requires cyclic ECA, the *wzzE::aph* allele would be transferred from the Keio collection into a *surA* depletion strain, where the *surA* gene is under the control of the arabinose promoter. Thus, the mutant would be constructed in the presence of arabinose supplied externally, which induces the expression of the *surA* gene. Following strain construction, growth kinetics of the mutant would be monitored in the presence and absence of arabinose. The lack of arabinose would inhibit the expression of the *surA* gene. If the

Δ*surA* mutant requires the *wzzE* gene, the depletion of *surA* would be fatal to the mutant. In contrast, in the presence of arabinose, the growth of the mutant would be restored.

There is also the possibility that the Δ*surA* mutant does not require a specific form of ECA, where loss of ECA_{LPS}, ECA_{PG} or ECA_{CYC} individually is viable, even loss of any two forms is viable. However, the combinational loss of all three ECA forms is lethal to the Δ*surA* mutant. This is consistent with the hypothesis that the combinational loss of *surA* and ECA might lead to excessive activation of stress responses, which might be lethal to the cell.

7.4.1.1.2. The effect of stress response systems. A potential future experiment would be to determine if loss of the non-essential Cpx and Rcs stress response systems would suppress the requirement for ECA in the Δ*surA* mutant. Introduction of a resistance cassette in replace of the response regulators CpxR and RcsB removes the Cpx and Rcs stress responses, respectively (Dong *et al.*, 1993; Gottesman *et al.*, 1985). Following removal of *cpxR* and *rcsB* in the Δ*surA* mutant, loss-of-function mutations would be introduced into the ECA operon to produce Δ*surA*Δ*weccpxR::kan* and Δ*surA*Δ*wecrcsB::kan* mutants. If these mutants are viably isolated, it can be suggested that the synthetic lethality between *surA* and genes involved in ECA synthesis is due to over activation of the stress response systems.

7.4.1.1.3. Holdase activity of ECA. In order for ECA to elicit holdase activity, it must first interact with OMPs. Protein–protein interactions can be characterised through a number of different methods including immunoprecipitation (co-IP) and pull-down assays. Depending on whether ECA interacts with OMPs, it can then be determined whether ECA maintains OMPs in an unfolded state and/or contributes to the disaggregation of OMPs. For example,

single molecule FRET microscopy can be utilised to determine if ECA is capable of disaggregating OmpC (Li *et al.*, 2018).

7.4.2. Coordination of the synthesis of LPS and OMP biogenesis

The TraDIS data demonstrated that loss of genes involved in the synthesis and incorporation of heptose into LPS combined with a sufficient defect in BAM function is lethal to the cell. More specifically, in the Δ*bamB* TraDIS library, fewer mutants were recovered with transposon insertions in genes involved in heptose production and incorporation into LPS than in the parent TraDIS library (fig. 3.11-3.12). In the Δ*degP* and Δ*surA* TraDIS libraries, the genes *gmhA*, *hldE*, *waaD*, *waaC* and *waaF* were conditionally essential (fig. 5.12-5.13). Upon loss of these LPS synthesis genes, membrane fluidity increases, which decreases the activity of the BAM complex (fig. 6.5-6.10). However, the efficiency of the heptose production pathway and the length of the inner core of LPS affects membrane fluidity and BAM activity to differing degrees.

7.4.2.1. The importance of LPS synthesis in the Δ*bamB* mutant. A severe defect in OMP assembly occurs upon loss of BamB. More specifically, a reduction in OMP insertion into the OM occurs (Charlson *et al*., 2006; Wu *et al*., 2005). Loss of genes involved in the synthesis of LPS leads to an increase in membrane fluidity, which subsequently decreases the activity of the BAM complex (fig. 6.5-6.10). Thus, upon the combinational loss of *bamB* and LPS synthesis genes, not enough OMPs are inserted into the OM. This lack of OMP insertion results in a severe fitness defect, which ultimately might result in cell death. Consequently, genes involved in LPS synthesis are functionally important to a Δ*bamB* mutant.

The Δ*bamB* phenotype is the most pronounced of the three non-essential BAM subunits. Thus, genes involved in LPS biogenesis are more important to the Δ*bamB* mutant than to the Δ*bamC* or Δ*bamE* mutant. Storek *et al*. (2019) demonstrated that the BAM complex was less active and the membranes were more fluid in the Δ*bamC*Δ*waaD* and Δ*bamE*Δ*waaD* double mutants than in the single Δ*waaD* mutant. Therefore, defects in LPS synthesis affect membrane fluidity and BAM activity in Δ*bamC* and Δ*bamE* mutants, but not to a sufficient level that would make the cell inviable.

7.4.2.2. The importance of LPS synthesis in the Δ*surA* and Δ*degP* mutants. DegP acts as a protease and a chaperone, mopping up unfolded and misfolded OMPs in the periplasm. Loss of DegP decreases the number of OMPs in the OM and increases the level of unfolded OMPs in the periplasm (Krojer *et al.,* 2008). Loss of LPS synthesis genes decreases BAM activity, which results in the accumulation of additional OMPs in the periplasm. Consequently, the combined loss of *degP* and LPS synthesis genes leads to toxic OMP aggregation in the periplasm and eventually demise of the cell.

Similarly, loss of SurA decreases OMP insertion into the OM (Lazar and Kolter, 1996; Rouviere and Gross, 1996). Loss of LPS synthesis genes leads to a further decrease in BAM activity (fig. 6.7 and 6.9). Thus, loss of *surA* alongside an impairment in LPS synthesis leads to a significant reduction in OMP insertion into the OM. This lack of OMP insertion results in cell death. In the Δ*surA* and Δ*degP* mutants, genes involved in heptose production and incorporation into LPS were functionally more important than in the Δ*bamB* mutant. Therefore, these mutants are more sensitive to fluctuations in BAM activity.

In summary, specific LPS defects elicit an increase in membrane fluidity, which decreases BAM activity. A decrease in BAM activity combined with a compromised BAM system, leads to lack of OMP insertion into the OM and/or accumulation of OMPs in the periplasm, which results in demise of the cell. These results demonstrate that OMP biogenesis directly coordinates with the synthesis of LPS. Loss of members of the BAM pathway elicit different levels of defects in BAM, which coordinate with the amount of the LPS structure required for the survival of these mutants.

7.4.2.3. Potential future experiments. To confirm that OMP insertion is lower in the double mutants than in the single mutants, the number of OMPs in the OM in the Δ*bamE*, Δ*waaD* and Δ*bamE*Δ*waaD* mutants should be quantified. This would further validate that the synthetic lethality between members of the BAM pathway and genes involved in LPS synthesis is a result of a decrease in OMP insertion into the OM, due to a significant reduction in BAM activity.

7.4.3. Coordination of the synthesis of peptidoglycan and OMP biogenesis

This study identified synthetic lethality between the non-essential BAM mutants and the gene *dapF*, which is involved in peptidoglycan synthesis (fig. 6.11-6.13). This study proposes why *dapF* is functionally important in these mutants. Loss of BamB, BamE, SurA, Skp or DegP decreases OMP insertion into the OM and/or increases OMP accumulation in the periplasm, which negatively affects the stability of the OM, one of two load-bearing structures in the cell (Rojas *et al.*, 2018). A compromised OM elicits a higher mechanical pressure on the other load-bearing structure; the PG. Loss of DapF reduces cross-linking within the PG layer, which affects the stability of the PG (Richaud *et al.*, 1987). The

combinational loss of *dapF* and *bamB/bamE/surA/skp/degP* compromises both the OM and the PG layer, which results in cell lysis due to high internal osmotic pressure. This hypothesis is supported by a previous study, which monitored lysis of *E. coli* cells exposed to an antibiotic that targets PG synthesis (Yao *et al.*, 2012). Lysis of these cells were morphologically similar to lysis of the Δ*bamB*Δ*dapF* and Δ*bamE*Δ*dapF* mutants. This indicates a defect in PG synthesis is occurring in these mutants.

However, loss of *bamC* does not elicit any known phenotypic effects. The membrane is not compromised and there is no reduction in the number of OMPs in the OM (Wu *et al.*, 2005). Consequently, the synthetic lethality between *dapF* and *bamC* is not due to the same mechanism as the other BAM mutants. In addition, this study identified morphological distinctions between lysis of the Δ*bamC*Δ*dapF* mutant and lysis of the Δ*bamB*Δ*dapF* and Δ*bamE*Δ*dapF* mutants (fig. 6.14). This study proposes a new potential function for BamC. It was recently demonstrated that multiple components of the BAM complex interact with PG and that BamC interacts with PG crosslinks (Corona *et al.*, 2020 - data not yet published). However, loss of *meso*-DAP decreases crosslinking of PG (Richaud *et al.*, 1987). This study hypothesizes that BamC acts as a link between the two pathways, coordinating OMP biogenesis with PG synthesis. The cell cannot withstand loss of this mechanism of coordination alongside a compromised PG layer.

7.5. The clinical applications of this study

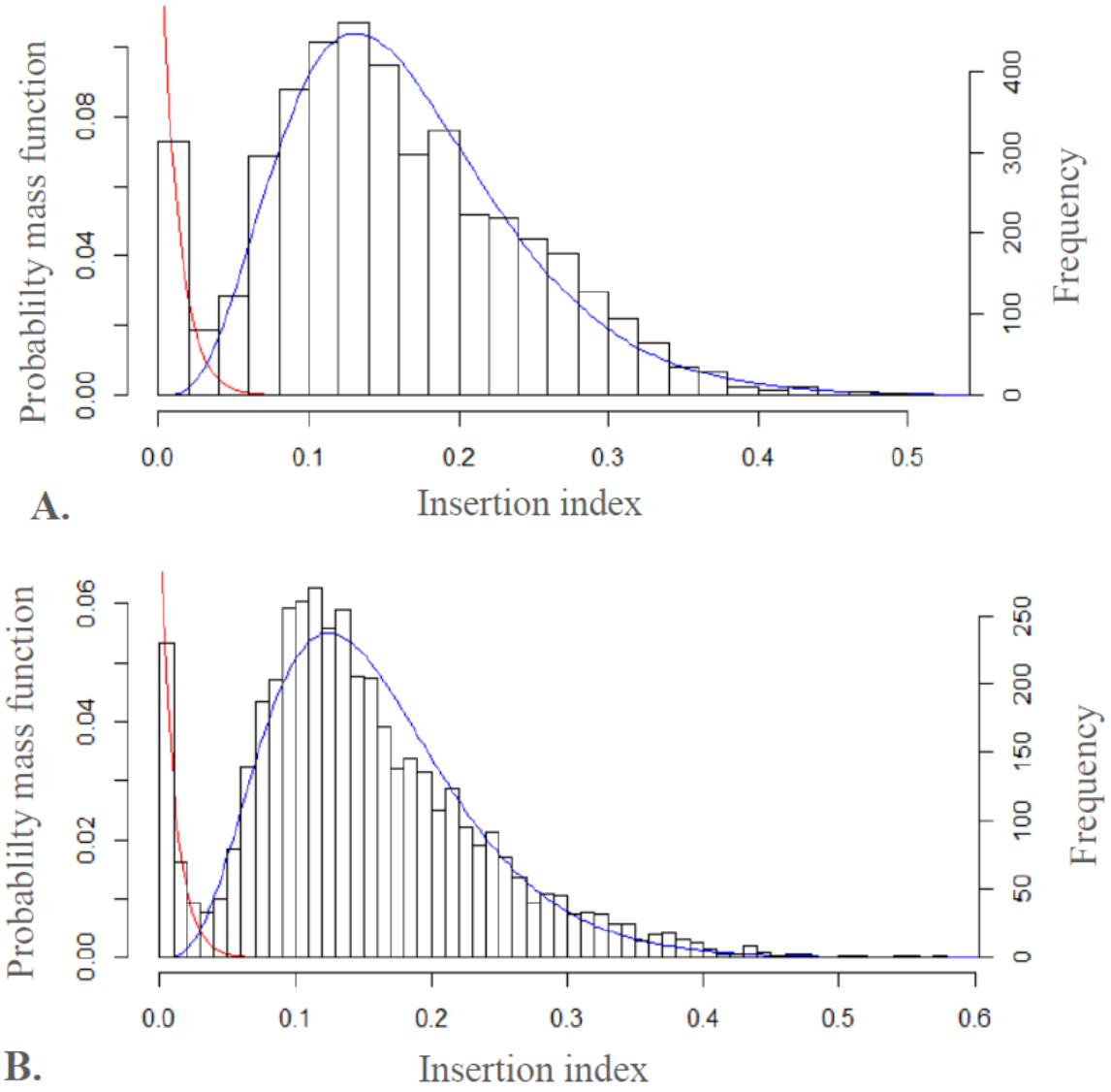
This study identified synthetic lethal partners that might act as drug targets in the development of antibacterials. Synthetic lethality offers the possibility of selectively targeting bacterial cells, while reducing the development of drug resistance. Synthetic

lethality can be utilised in the development of dual inhibitors, which elicit loss-of-function mutations in two genes. Loss of one gene is viable but loss of both genes leads to cell death. For example, the cancer therapy PARP inhibitors (PARPi) targets the combination of poly (adenosine diphosphate [ADP]-ribose) polymerase (*PARP*) and breast-cancer susceptibility genes 1 and 2 (*BRCA1* and *BRCA2*) (Lord and Ashworth, 2017).

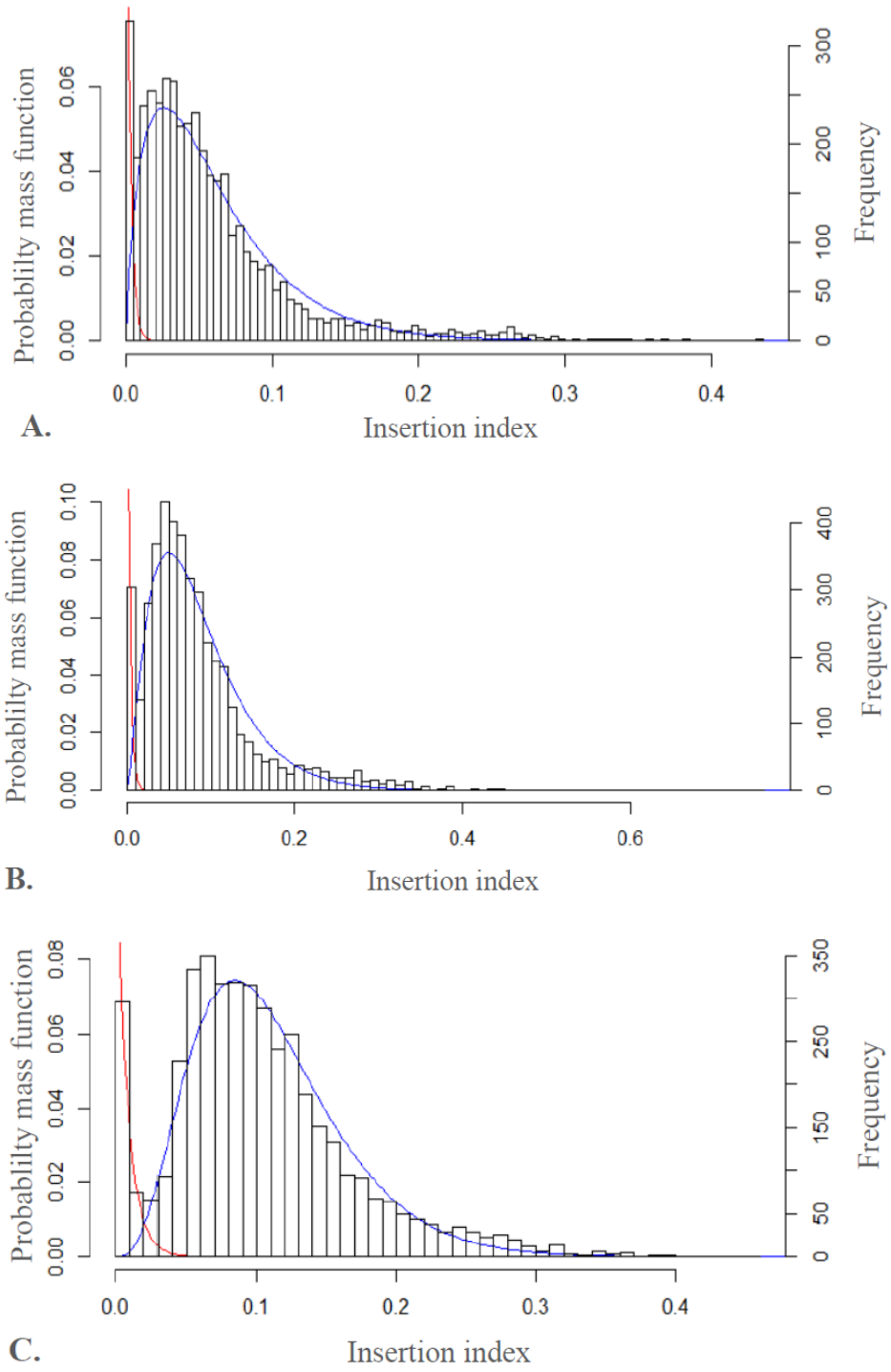
Synthetic lethality can also be utilised in combinational therapy, where the gene inhibitor is administered as a sensitizer alongside classical antibiotics. Combination therapy might provide the opportunity to reinstate antibiotics in therapy that were previously removed due to high levels of bacterial resistance. Thus, synthetic lethality provides a framework for the development of potential effective treatments for complex diseases including the treatment of resistant bacterial infections.

7.6. Concluding remarks

This study utilised TraDIS to identify synthetic lethal partners of *bamB*, *bamC*, *bamE*, *surA*, *skp* and *degP*. These synthetic lethal partners might act as drug targets in the development of antibacterials. This study demonstrated that there is no functional overlap between BamB, BamC and BamE and that the functions of SurA and Skp are not redundant. This study also explored the coordination between OMP biogenesis and other cell envelope processes such as the synthesis of LPS, PG or ECA. These results demonstrate that OMP biogenesis requires a network of components and that secondary negative consequences occur upon impairment of OMP biogenesis.



Appendix 4.1. Histogram illustrating the binomial frequency distribution of the genome-wide insertion sites of the Δ*bamC* and Δ*bamE* TraDIS libraries. The frequency of insertion index scores were plotted for the (A) Δ*bamC* and (B) Δ*bamE* TraDIS libraries. Both histograms followed a bimodal distribution. An exponential distribution model was fitted to the left (red). This mode contains essential genes i.e. genes with low or no transposon insertions. A gamma distribution model was fitted to the right (blue), which is the non-essential mode. This mode contains genes with numerous transposon insertions.



Appendix 5.1. Histogram illustrating the binomial frequency distribution of the genome-wide insertion sites of the Δ*surA*, Δ*skp* and Δ*degP* TraDIS libraries. The frequency of insertion index scores were plotted for the (A) Δ*surA*, (B) Δ*skp* and (C) Δ*degP* TraDIS libraries. All of the histograms followed a bimodal distribution. An exponential distribution model was fitted to the left (red), which includes essential genes. A gamma distribution model was fitted to the right (blue), which contains non-essential genes.

Supplementary tables

Supplementary table 3.1 Genes classified as conditionally non-essential in the Δ*bamB* TraDIS library

Conditionally non-essential genes				
birA	*hemL*	*mreD*	*rpsR*	*ubiE*
coaA	*hicA*	*pheM*	*rpsT*	*ubiF*
coaE	*hscA*	*ptsH*	*secD*	*ubiH*
crp	*iscS*	*ptsI*	*secF*	*ybeY*
fmt	*iscU*	*ratA*	*suhB*	*ygfZ*
folB	*ldrB*	*rluD*	*tadA*	*yihA*
folP	*lepB*	*rpmJ*	*tilS*	*ymjD*
grpE	*lipA*	*rpoC*	*tonB*	*ynfN*
hemC	*lpd*	*rpsG*	*tsaC*	*yobI*
hemD	*lptA*	*rpsI*	*ubiA*	*yqgF*
hemG	*mreC*	*rpsQ*	*ubiD*	

Supplementary table 4.1 Genes classified as conditionally non-essential in the Δ*bamE* TraDIS library

Conditionally non-essential genes		
crp	*ldrB*	*trpL*
cspI	*lpd*	*tsaC*
dnaT	*ptsH*	*ubiE*
folP	*ptsI*	*ubiF*
hicA	*ratA*	*ybeY*
hscA	*rpsG*	*ygfZ*
iscS	*rpsR*	*ymjD*
iscU	*tonB*	*ynfN*

Supplementary table 4.2 Genes classified as conditionally non-essential in the Δ*bamC* TraDIS library

Conditionally non-essential genes		
coaE	*ldrB*	*trpL*
crp	*ptsH*	*ubiE*
dcd	*ptsI*	*ubiF*
fmt	*ratA*	*ubiH*
folP	*rluD*	*ybeY*
gpsA	*rpsG*	*ygfZ*
hicA	*rpsI*	*ymjD*
hscA	*rpsR*	*ynfN*
iscS	*suhB*	*yqgF*
iscU	*tonB*	

Supplementary 5.1. Genes classified as conditionally non-essential in the Δ*skp* TraDIS library

Conditionally non-essential genes				
appY	*fmt*	*iscU*	*secD*	*ydcD*
bamD	*folB*	*lexA*	*secF*	*yddK*
birA	*folP*	*lpd*	*suhB*	*yddL*
coaE	*ftsL*	*lpxC*	*tadA*	*yedN*
crp	*gnsB*	*map*	*thiL*	*yffS*
cspI	*grpE*	*mtn*	*tilS*	*ygfZ*
cydA	*guaA*	*pth*	*tonB*	*ykfM*
cydB	*hemC*	*ptsH*	*trpL*	*ymfE*
cydC	*hemD*	*ptsI*	*tsaC*	*ymjD*
cydD	*hicA*	*ratA*	*ubiA*	*ynfN*
cysB	*holA*	*rluD*	*ubiD*	*yqgF*
dcd	*hscA*	*rplS*	*ubiE*	
dnaT	*infA*	*rpsG*	*ubiF*	
dut	*iraM*	*rpsR*	*ubiH*	
fabA	*iscS*	*secA*	*ybeY*	

Supplementary 5.2. Genes classified as conditionally non-essential in the Δ*degP* TraDIS library

Conditionally non-essential genes		
appY	*iscU*	*ubiH*
crp	*ptsH*	*ybeY*
cspI	*ratA*	*yddK*
dcd	*rpsG*	*ygfZ*
folP	*trpL*	*ymjD*
hicA	*tsaC*	
hscA	*ubiE*	

Supplementary 5.3. Genes classified as conditionally non-essential in the Δ*surA* TraDIS library

Conditionally non-essential genes				
appY	*ftsQ*	*lptD*	*ratA*	*ubiA*
bamA	*gnsB*	*lptF*	*ribA*	*ubiD*
birA	*gpsA*	*lpxB*	*rluD*	*ubiE*
can	*groL*	*lpxC*	*rpmI*	*ubiF*
cdsA	*groS*	*map*	*rpoC*	*ubiH*
coaA	*grpE*	*metG*	*rpsB*	*yddK*
coaE	*hemC*	*mraY*	*rpsG*	*yedN*
crp	*hemD*	*mtn*	*rpsP*	*yffS*
cspI	*hemE*	*murB*	*rpsR*	*ygfZ*
dapD	*hemG*	*murD*	*rpsU*	*yihA*
dcd	*hemL*	*nadD*	*rseP*	*ymfE*
dnaB	*holB*	*orn*	*ssb*	*ynfN*
dnaC	*hscA*	*pgsA*	*suhB*	*yqcG*
dnaT	*ileS*	*pheM*	*thiL*	*yqgF*
dut	*infA*	*plsB*	*tilS*	
fabZ	*iscS*	*proS*	*tonB*	
folB	*iscU*	*ptsH*	*topA*	
folD	*ispD*	*ptsI*	*trmD*	
folP	*lexA*	*pyrH*	*tsaC*	
ftsA	*lipA*	*ratA*	*tsaD*	
ftsL	*lpd*	*ribA*	*tsaE*	

Supplementary table 5.4 Genes that were conditionally essential in both the Δ*surA* and Δ*degP* TraDIS libraries

Genes common to both the Δ*degP* and Δ*surA* TraDIS libraries	
cmk	*waaC*
degS	*yajC*
gmhA	*yciM*
hldD	*yciS*
hldE	*yciU*
pbl	*yddM*
pgm	*ygeF*
prc	*zwf*
secB	

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