An investigation into the factors that affect membrane vesicle composition in bacteria

Sarah Amy Blackburn

2020

Thesis for the degree of PhD in Microbiology

School of Biosciences, Faculty of Sciences, University of Kent

Abstract

Outer membrane vesicles (OMVs) are nano-sized structures that are formed when portions of the bacterial membrane bud and pinch off from the cell in a process called vesiculation. This process entraps a diverse range of bacterial products within the vesicles (including virulence factors) which are later released into the environment. The main project aims were to gain a fundamental understanding of vesiculation in a range of bacterial species and to enable targeted expression of recombinant proteins and other molecules for delivery and inclusion in OMVs. OMVs were isolated and characterised from various strains of *Escherichia coli* and *Pseudomonas aeruginosa*. It was found that OMVs from different bacterial strains are similar in appearance but have very different compositions and cargo. The proteins FimA and Flagellin were found to be heavily enriched within *E. coli* K-12 OMVs in a mutually exclusive way. They are known virulence factors that have been shown to be reciprocally regulated in *E. coli* cells but not in OMVs. FimA has previously been found to have an anti-inflammatory effect on human immune cells whereas Flagellin has a pro-inflammatory effect, which may be the reason that the two proteins are not packaged within OMVs together. This was further explored by purification of OMVs from a series of *E. coli* gene knockouts and clinical isolates to compare the protein profiles of the OMVs. Lastly, an *E. coli* strain containing GFP fused to FimA was trialled as a method of targeted delivery within OMVs. This method was successful as the GFP-FimA protein fusion was detected in the OMVs purified from this strain. Recombinant protein fusions such as this could allow use of *E. coli* OMVs for therapeutic applications such as drug delivery and vaccines. Furthermore, the packaging of FimA and Flagellin into *E. coli* OMVs may play a significant role in its pathogenicity and ability to modulate the host response to infection. These findings could highlight potential new drug targets against OMV-producing pathogens such as *E. coli* as well as providing further insight into using OMVs for drug delivery and vaccines.

Thesis page count: 242

Declaration

No part of the thesis has been submitted in the support of an application for any degree or other qualification of the University of Kent, or any other University or Institution of learning.

Acknowledgments

Firstly, I would like to thank my supervisor Dr Gary Robinson for believing in me and giving me the opportunity to do this PhD. I am grateful for his continued support and guidance throughout all my time at the University of Kent. Thank you also to my co-supervisor Professor Simon Carding (University of East Anglia) and to Eastern Arc for the funding.

I would also like to thank Dr Mark Shepherd and Dr Ian Blomfield for their interesting discussions and suggestions for my project at our weekly lab meetings. I am also grateful for the friendship of all members of the Shepherd-Blomfield-Robinson ('Shepblomson') lab past and present, particularly Büke Yücel, Taylor Monaghan and Cláudia Ribeiro, who have been there for me since my first day in the lab.

During my PhD, I have needed to use the expertise and advice of many other labs within the School of Biosciences and I am grateful that I have been working in a community where so many people are willing and able to help others. I would particularly like to thank Ian Brown for his help and support with electron microscopy, Matt Lee for his help with confocal microscopy and Kevin Howland for his mass spectrometry expertise. I also appreciate all the help and guidance I received from Alex Moores and Joe Baker, which enabled me to do the molecular cloning work.

Throughout my PhD, I have received so much support from my family and friends, which are too numerous to mention individually! Special thanks to my Mum, Dad, brother Michael and grandparents for their continual encouragement and support in all I do and thank you to Paul Morris-Hill for always being there for me through my highs and lows. Last, but by no means least, to Marta Skryzypiec for your never-ending confidence in me.

Contents

Abstract

Outer membrane vesicles (OMVs) are nano-sized structures that are formed when portions of the bacterial membrane bud and pinch off from the cell in a process called vesiculation. This process entraps a diverse range of bacterial products within the vesicles (including virulence factors) which are later released into the environment. The main project aims were to gain a fundamental understanding of vesiculation in a range of bacterial species and to enable targeted expression of recombinant proteins and other molecules for delivery and inclusion in OMVs. OMVs were isolated and characterised from various strains of *Escherichia coli* and *Pseudomonas aeruginosa*. It was found that OMVs from different bacterial strains are similar in appearance but have very different compositions and cargo.

The proteins FimA and Flagellin were found to be heavily enriched within *E. coli* K-12 OMVs in a mutually exclusive way. They are known virulence factors that have been shown to be reciprocally regulated in *E. coli* cells but not in OMVs. FimA has previously been found to have an anti-inflammatory effect on human immune cells whereas Flagellin has a pro-inflammatory effect, which may be the reason that the two proteins are not packaged within OMVs together. This was further explored by purification of OMVs from a series of *E. coli* gene knockouts and clinical isolates to compare the protein profiles of the OMVs. Lastly, an *E. coli* strain containing GFP fused to FimA was trialled as a method of targeted delivery within OMVs. This method was successful as the GFP-FimA protein fusion was detected in the OMVs purified from this strain. Recombinant protein fusions such as this could allow use of *E. coli* OMVs for therapeutic applications such as drug delivery and vaccines. These findings were also relevant to the study of *P. aeruginosa* OMVs which were also found to be enriched in Flagellin.

E. coli and *P. aeruginosa* are Gram-negative bacterial strains which produce OMVs derived from the outer membrane of the bacterial cell envelope. As a comparison, membrane vesicles (MVs) were also purified from *Streptomyces* S4, which is a Gram-positive bacterium. Gram-positive bacteria do not have an outer membrane so MVs are derived from the cytoplasmic membrane instead. It had previously been found that *Streptomyces* S4 produces the antifungal agents candicidin and antimycin but it was not known how they were secreted. This study showed for the first time that *Streptomyces* S4 packages candicidin within membrane vesicles for release into the environment. Understanding of the natural packaging of cargo into the MVs of each bacterial strain is essential to maximise the selectivity and yield of cargo for therapeutic purposes. Furthermore, the packaging of FimA and Flagellin into *E. coli* OMVs may play a significant role in its pathogenicity and ability to modulate the host response to infection. These findings could highlight potential new drug targets against MV-producing pathogens such as *E. coli* as well as providing further insight into using MVs for drug delivery and vaccines.

List of Abbreviations

AMR	Antimicrobial resistance
CAB	Sodium cacodylate buffer
CGSC	Coli Genetic Stock Center
DLS	Dynamic light scattering
HEPES	Hydroxyethylpiperazine ethane sulfonic acid
IM	Inner membrane
KO	Knockout
lacZYA	Lactose operon
LB	Lysogeny broth
LPS	Lipopolysaccharide
MES	2-(N-morpholino)ethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
MQ	Milli-Q (water)
OD	Optical density
OM	Outer membrane
OMP(s)	Outer membrane protein(s)
OMV(s)	Outer membrane vesicle(s)
ORF	Opening reading frame
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RSB	Reducing sample buffer
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
T1F	Type 1 fimbriae
TAE	Tris-acetate-EDTA buffer
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
TCA	Trichloroacetic acid
(T)EM	(Transmission) electron microscopy
TSB	Tryptone soy broth
UEA	University of East Anglia, Norwich
UKC	University of Kent, Canterbury
UTI	Urinary tract infection
v/v	Volume/volume
w/v	Weight/volume
WGA-FITC	Wheat Germ Agglutinin (WGA) conjugated to the fluorescent conjugate fluorescein isothiocyanate (FITC)
WT	Wild type
YEME	Yeast extract-malt extract medium
YPD	Yeast extract peptone dextrose

List of Figures

List of Tables

Chapter 1: Introduction

1.1 Introduction to Outer Membrane Vesicles

Outer membrane vesicles (OMVs) are produced and secreted by Gram-negative bacteria. They are nano-sized, spherical vesicles, which are formed from the bacterial Outer Membrane (OM). OMVs can contain a wide range of cargo, which travel within the OMVs to reach their target in a concentrated and protected form. OMV cargo often includes virulence factors such as toxins or proteases which are beneficial to the bacteria that secrete them. Membrane vesicle release is conserved and mechanistically similar across a range of both prokaryotic and eukaryotic microorganisms (Prangishvili *et al*. 2000, Deatherage *et al.* 2012).

1.1.1 The discovery of OMVs

The formation of OMVs was first observed by electron microscopy in the 1960s (Chatterjee, Das. 1967). This was demonstrated using actively growing *Vibrio cholerae* cells that were in the logarithmic stage of growth. The electron microscopy images showed the bulging of certain parts of the bacterial cell wall. These portions of the membrane then pinched off to form vesicles that were released extracellularly (Figure 1.1). The release of these vesicles had no effect on the cell wall, which remained intact (Chatterjee, Das. 1966 and 1967). The authors proposed that this was a novel secretory system which allowed the secretion of non-diffusible materials from the bacterial periplasm (Chatterjee, Das. 1966 and 1967).

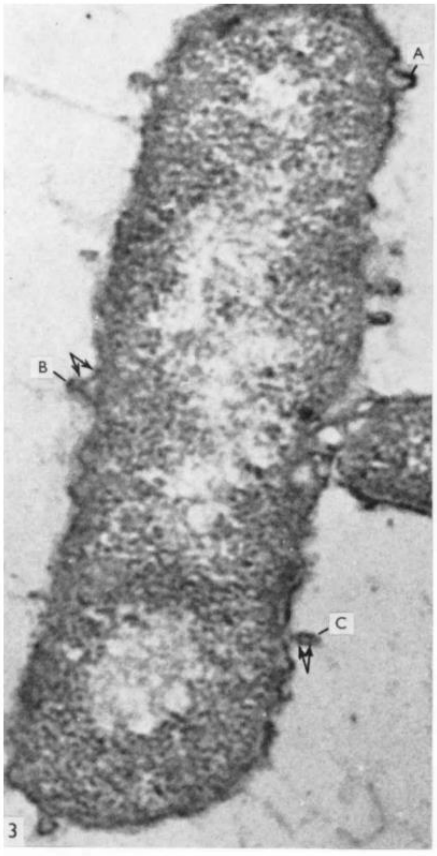


Figure 1.1 Electron microscopy images of the formation of membrane vesicles from *Vibrio cholerae* cells
V. cholerae cells were grown in peptone water to logarithmic growth phase. The cells were stained with lead and electron microscopy was used to visualise the bulging and budding from the cell membrane. The authors labelled the sequence of the budding process as A, B and C. The arrows represent granules associated with the cell wall. Magnification x 100,000. Image and experiment details sourced from Chatterjee *et al.* 1967.

These membrane sacs were later known as OMVs and were found to be produced by many Gram-negative bacteria during growth phase (McBroom *et al.* 2005). The production and secretion of OMVs was also established as a new bacterial secretory mechanism (Kuehn *et al.* 2005). Lastly, OMVs were found to contain a range of cargo which are advantageous to the parent cell (discussed in Section 1.3).

1.2 Composition of the cell envelope in Gram-negative bacteria

1.2.1 Summary of the main differences in the composition of the cell envelope in Gram-positive and Gram-negative bacteria

The role of the bacterial cell envelope is to protect bacteria from extreme environments and prevent lysis of the cell. The majority of bacterial cell envelopes fall into two categories: Gram-positive or Gram-negative. The Gram-positive bacterial cell envelope contains a plasma membrane and a thick layer of peptidoglycan cell wall. The Gram-negative bacterial cell envelope is composed of an inner (plasma) membrane, a thin peptidoglycan cell wall and an outer membrane (OM). The periplasm is the space between the inner and outer membrane in Gram-negative bacteria, which contains the peptidoglycan layer (Figure 1.2).

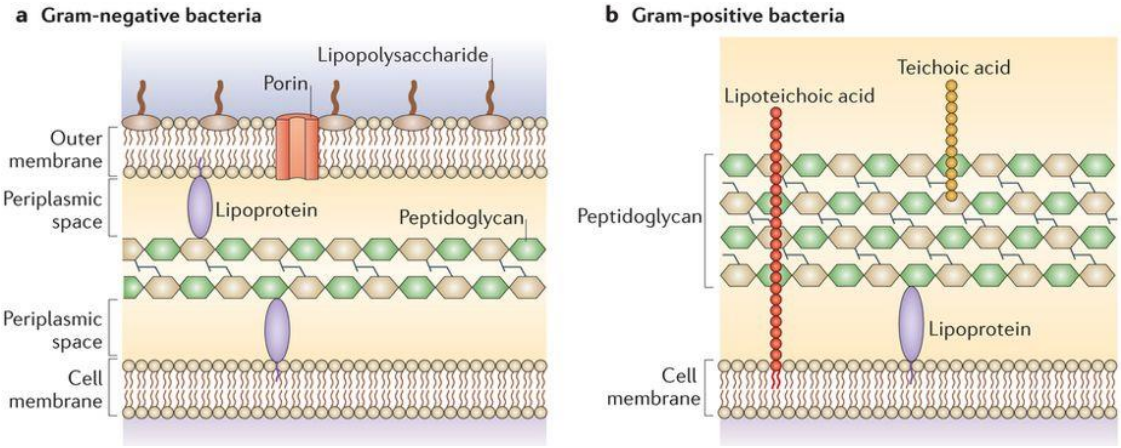


Figure 1.2 Comparison of the cell envelope in Gram-positive and Gram-negative cells
The Gram-negative bacterial cell envelope is composed of the inner (plasma) membrane, a thin peptidoglycan cell wall and an outer membrane (**a**). The Gram-positive bacterial cell envelope is composed of the plasma membrane and a thick peptidoglycan cell wall (**b**). Image sourced from Brown *et al.* 2015.

Lastly, it should be noted that there are some examples of bacteria that do not fit into either category. For example, the cell wall of *Mycobacterium tuberculosis* contains characteristics of both Gram-negative and Gram-positive bacteria (Fu *et al.* 2002).

1.2.2 Composition of the cell envelope in Gram-negative bacteria

The Gram-negative cell envelope is composed of the inner (cytoplasmic) membrane, the periplasm and the outer membrane which contains the lipopolysaccharide component (Figure 1.3).

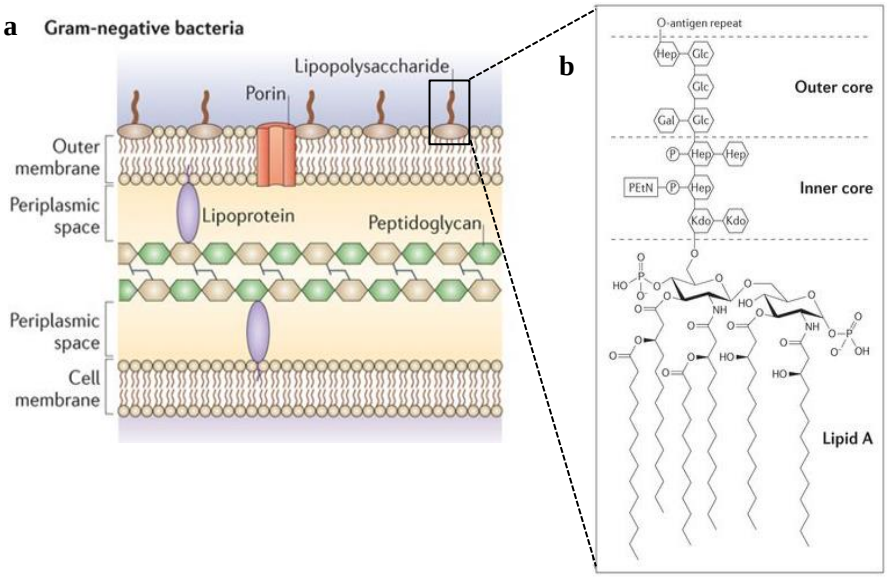


Figure 1.3 The composition of the cell envelope in Gram-negative bacteria
There are three layers to Gram-negative cell envelope: 1. The outer membrane: an asymmetrical bilayer composed of an inner leaflet formed of phospholipids and an outer leaflet composed of lipopolysaccharide which projects outwards. **2.** The peptidoglycan cell wall: a polymer that provides structure to the bacterial cell envelope, located within the periplasm. **3.** The inner membrane: composed of phospholipids, also known as the cytoplasmic membrane (**a)**. **The lipopolysaccharide is formed of: 1.** Lipid-A **2.** Core polysaccharide which split into the inner and outer cores **3.** O antigen polysaccharide which projects outwards from the cell wall (**b**). Image sourced from Brown *et al.* 2015 and Okuda *et al.* 2016.

1.2.2.1 The Outer Membrane (OM)

OMVs are formed from the OM of Gram-negative bacteria. The OM serves as a barrier to protect the cell from the environment. The OM is composed of the inner and outer leaflet. The inner leaflet contains phospholipids and lipoproteins and the outer leaflet is composed of lipopolysaccharide (LPS).

Lipopolysaccharide (LPS)

LPS is unique to Gram-negative bacteria and is exposed on the bacterial cell surface. The LPS molecules are tightly bound and packed together making an effective barrier against the entry of hydrophobic molecules. The LPS component of Gram-negative bacteria is pro-inflammatory and can cause the endotoxic shock observed in septicaemia (Raetz, Whitfield. 2002). LPS has three different components: lipid A, a core polysaccharide and an O antigen polysaccharide which projects outwards from the cell wall (Figure 1.3).

Lipid A

Lipid A is located next to the phospholipid layer of the OM and anchors the LPS to the bacterial membrane. It is composed of a β (1→6)-linked glucosamine disaccharide backbone (Steimle *et al.* 2016). It is usually hexa-acylated meaning that there are six acyl chains associated with the backbone (Steimle *et al.* 2016). However, the number of acyl groups can vary as well as the length of the chains. Lipid A is responsible for much of the toxicity of Gram-negative bacteria. When Gram-negative bacteria are lysed by the hosts' immune system, LPS (and Lipid A) can be released into the hosts circulation. It is a highly potent activator of immune cells and during bacterial infection can cause the body to go into septic shock which can be fatal.

Core polysaccharide

The core polysaccharide links Lipid A to the O antigen which projects outwards from the cell. The outer core is composed of more common sugars including hexoses such as glucose and galactose (Erridge *et al.* 2002). The inner core contains many phosphorylated glycan residues which increase the negative charge of the outer membrane and help to stabilise the cell. It contains a 'high proportion of unusual sugars such as 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-manno heptose (Hep)' (Erridge *et al.* 2002).

O-antigen

The O-antigen is composed of repeating oligosaccharides and is highly variable in the number of repeating units and sugars. Due to its variability, bacteria are classed by serotype based on the O antigen found on each strain (Erridge *et al.* 2002). On some bacterial species, the O-antigen is truncated or absent altogether. As the O antigen is expressed on the outside of the cell, it is one of the main antigens targeted by the host immune system (Erridge *et al.* 2002).

Outer membrane proteins

The majority of proteins from the OM can be classed as lipoproteins or β-barrel proteins (Silhavy *et al.* 2010). Almost all of the integral, transmembrane proteins found in the OM are in a β-barrel conformation (Silhavy *et al.* 2010). This includes the Outer Membrane Proteins (OMPs) such as the porins which form channels in the membrane and allow the passage of small ions and molecules across the OM. Example of porins are OmpC and OmpF in *E. coli* (Figure 1.4). The porins present in the OM also prevent the entry of hydrophilic molecules above 700 Daltons to limit diffusion into the cell (Silhavy *et al.* 2010). For these reasons, the OM gives Gram-negative bacteria resistance to environmental stress and antibiotics.

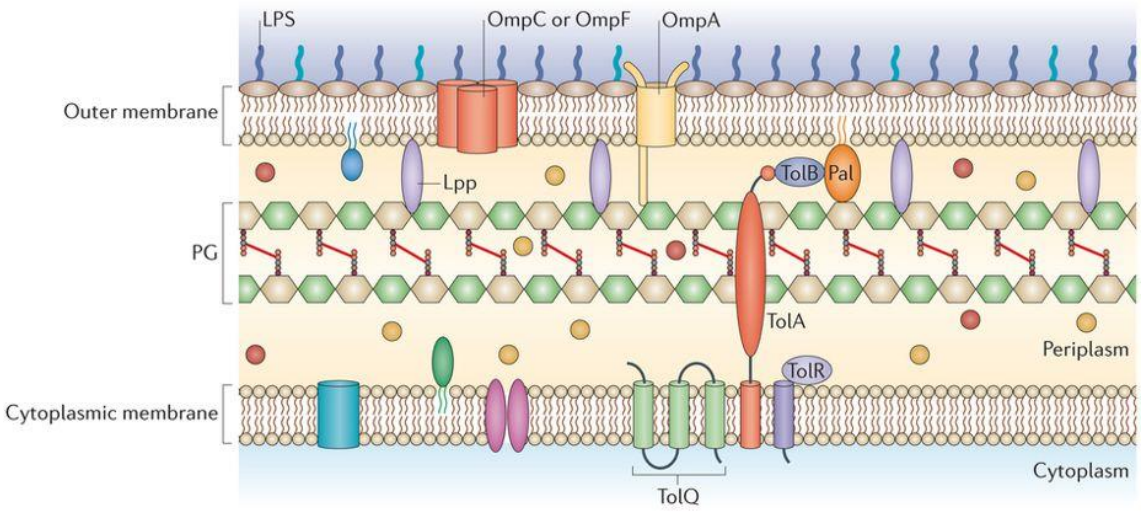


Figure 1.4 Structure of the Gram-negative bacterial cell envelope highlighting the location of OmpC, OmpF, OmpA and Lpp
The cell envelope is composed of the inner (cytoplasmic) membrane, the outer membrane and the periplasmic space which contains the peptidoglycan layer (PG). The stability of the cell envelope is due to the protein links between the IM, peptidoglycan and OM. The lipoprotein Lpp forms crosslinks between the OM and the layer of peptidoglycan (purple ovals). OmpA is situated in the OM and also forms links to the peptidoglycan (yellow). Lastly, the Tol-Pal complex spans the whole cell envelope from the IM to the OM for stability (includes proteins TolQ, TolR, TolA, TolB and Pal). Image sourced from Schwechheimer, Kuehn (2015).

The stability of the cell envelope is due to the protein links between the IM, peptidoglycan and OM. The OM is attached to the peptidoglycan layer by an outer membrane lipoprotein called Lpp. If there are places in the membrane where the Lpp link is absent, this is often the location for OMV biogenesis (discussed further in Section 1.4). OmpA is a protein in *E. coli*, which binds and links the OM to the peptidoglycan layer (Figure 1.4). OmpA is known to be present in *E. coli* OMVs, which is discussed further in Section 3.1.2.

Outer membrane-associated enzymes

OMVs contain proteins from the OM from which they are formed. The OM of Gram-negative bacteria (such as *E. coli*) contain a family of outer membrane proteases called Omptins (Vandeputte-Rutten *et al.* 2001). Omptins appear to be virulence factors in pathogenic Gram-negative bacteria as they cleave a range of substrates at the host-pathogen interface such as antimicrobial peptides and plasminogen (Brannon *et al.* 2015). An example of this is the finding that the *ompT* gene is present in *E. coli* clinical isolates from patients with complicated urinary tract infections (Webb *et al.* 1996). OmpT cleaves and inactivates the antimicrobial peptide protamine which is produced by epithelial cells in the urinary tract (Stumpe *et al.* 1998). This degradation in the OM prevents protamine from reaching the inner (cytoplasmic) membrane which is its site of action (Stumpe *et al.* 1998).

1.2.2.2 Peptidoglycan and the Periplasm

The periplasm is a viscous, aqueous compartment between the inner and outer membrane. It is densely packed with enzymes and proteins, particularly those associated with the secretory pathway. It is also a membrane bound compartment where potentially harmful toxins or degradative enzymes can be sequestered. Within the periplasmic space, there is a thin layer of peptidoglycan which is composed of the monosaccharides N-acetyl glucosamine (NAG) and N-acetyl muramic acid (NAM). The sugar component of the peptidoglycan contains strands of alternating residues of NAG and NAM, which are connected by the cross-linking of peptide chains present on the NAM residues (Figure 1.5). The result is an extremely strong 3D structure that gives the cell structure and prevents bacterial lysis (Silhavy *et al.* 2010)

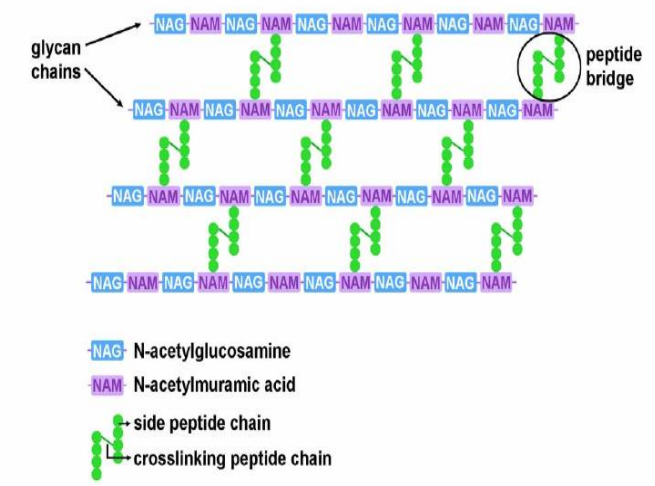


Figure 1.5 Schematic representation of the structure of peptidoglycan
Peptidoglycan is formed of the monosaccharides N-acetyl glucosamine (blue squares labelled NAG) and N-acetyl muramic acid (purple squares labelled NAM). The sugar component of the peptidoglycan contains strands of alternating residues of NAG and NAM which are connected by the cross-linking of peptide chains present on the NAM residues (green links). Image sourced from Malinicova *et al.* 2010.

This layer is rigid and provides structure to the cell to prevent cell lysis (Silhavy *et al.* 2010). The peptidoglycan layer is connected to the outer and inner membrane by various proteins for strength and stability of the bacterial cell structure.

1.2.2.3 The Inner Membrane (IM)

The IM is a phospholipid bilayer that encloses the cytoplasm. The membrane proteins in the IM function in lipid biosynthesis, protein secretion and energy production. In Gram-negative bacteria, OMVs are formed from the outer membrane rather than the inner membrane.

1.3 OMV composition and cargo

1.3.1 OMVs are formed from the Outer Membrane of the Gram-negative cell envelope

OMVs range from 50 nm to 300 nm in diameter (Brandon *et al.* 2014) and are spherical in shape (Chatterjee, Chaudhuri. 2011). The formation of OMVs from the OM appears to be a controlled process

that does not affect the cellular structure or integrity. Evidence suggests that OMVs are formed at sites where the lipoprotein (Lpp) link between the peptidoglycan layer and OM are missing or broken. The formation of OMVs involves the budding and pinching off of a segment of the OM which entraps some components of the periplasm (Ellis *et al.* 2010). Therefore, OMVs contain phospholipids, periplasmic proteins, OMPs and LPS which projects outwards (Figure 1.6). It is also evident that OMVs include material from the periplasm, which appears to involve a specific sorting mechanism that is not yet fully understood.

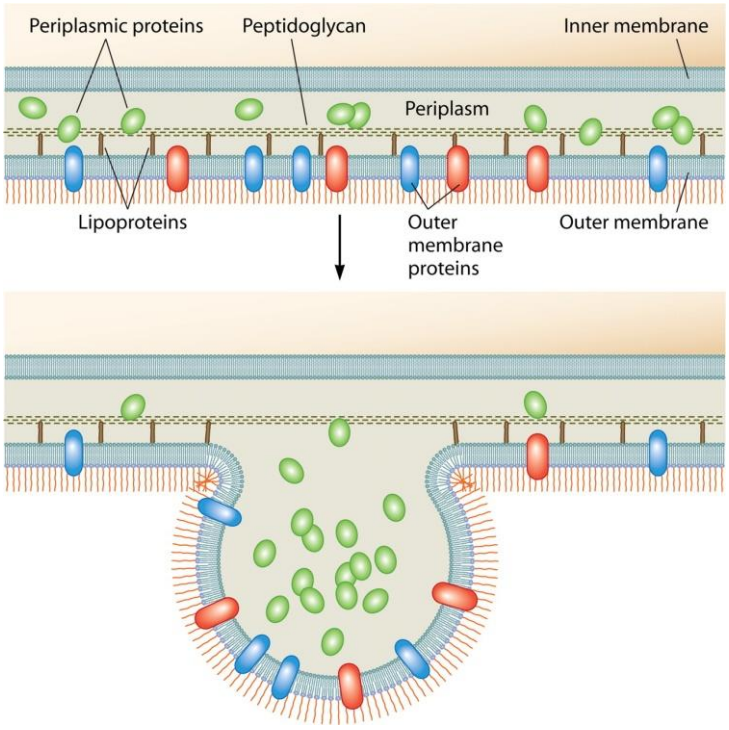


Figure 1.6 Model to show OMV production
In this model, OMVs bud from the Gram-negative bacterial envelope. OMVs contain proteins and lipids from the OM and material from the periplasm. It is thought that budding occurs at sites where the lipoprotein (Lpp) link between the peptidoglycan layer and OM are missing or broken. Image sourced from Ellis, Kuehn. 2010.

1.3.2 Chemical components of OMVs

Within OMVs, the ratios of each lipid type, fatty acid content and the ratio of phospholipid to protein (Brandon *et al.* 2014) resemble that of the bacterial outer membrane but not the inner membrane. As discussed in the Section 1.2, the sugar KDO is a marker of LPS from the OM of Gram-negative bacteria. KDO has also been detected on OMVs (Kato *et al.* 2002), which is further evidence that OMVs are formed from the OM. Analysis of OMV proteomes also found many OM proteins within OMVs such as OmpA and OmpF. OMVs also were found to selectively contain periplasmic proteins (Bauman *et al.* 2006). For example, proteins associated with peptidoglycan were found to be excluded from *E. coli* OMVs. Virulence factors (such as toxins) were found in high concentrations within the OMVs as if they were selectively targeted there (Horstman, Kuehn. 2002).

Material from the inner membrane (IM) and cytoplasm are generally thought to be excluded from OMVs (McBroom *et al.* 2006) although there have been exceptions. Marker proteins of the inner membrane such as succinate dehydrogenase and NADH oxidase are generally absent from purified OMVs (Brandon *et al.* 2014). This is strong evidence that OMV biogenesis is a controlled and deliberate process.

1.3.3 Summary of possible OMV cargo

There are two main advantages of packing and transporting molecules within OMVs. Firstly, certain proteins appear to be enriched in OMVs so that they become concentrated for release into the environment. Secondly, the packaged material can be transported while being protected from factors that may cause degradation in the environment (for example proteases). Possible cargo within OMVs include proteins, DNA and RNA. OMVs purified from pathogenic bacteria can contain toxins, multidrug efflux pumps and immunomodulatory compounds (Figure 1.7).

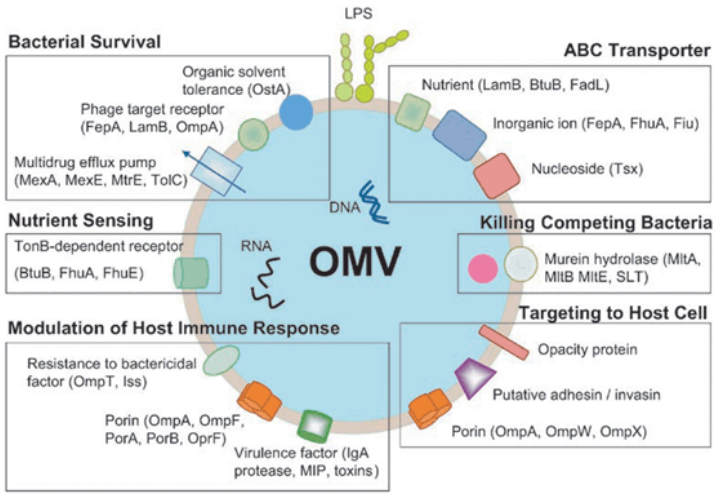


Figure 1.7 Cargo of a typical OMV
Findings from the proteomic, biochemical and biological studies of OMVs are summarised in this schematic diagram. Cargo types were split into the following categories: Bacterial survival, Nutrient sensing, Modulation of host immune response, ABC transporters, Killing competing bacteria and Targeting to the host cell. Image sourced from Brandon *et al.* 2014.

1.3.4 Why do bacteria secrete OMVs?

1.3.4.1 Aids Pathogenicity

Toxins

Many Gram-negative bacteria use OMVs to secrete virulence factors. OMVs are known to contain toxins that can cause disease in the host. Toxins known to be OMV-associated include the Shiga toxin from *Shigella dysenteria*e serotype 1 (Knockoutling *et al.* 1999), which is cytotoxic and causes host cell apoptosis (Dutta *et al.* 2004). Another example is Cytolysin (ClyA) found in OMVs from Enterohemorrhagic *E. coli* (EHEC) strains. ClyA monomers spontaneously form pore complexes at membranes, which cause cell membrane rupture and lysis (Roderer *et al.* 2016). ClyA in the periplasm exists in an oxidised form where disulphide bonds between cysteines keep ClyA in an inactive state (Wai *et al.* 2003). In the lumen of the OMVs, however, ClyA was found in a reduced form and was able

to form an active pore structure. Evidence suggests that ClyA within the OMV is more potent and cytotoxic to mammalian cells than ClyA purified from the periplasm (Wai *et al.* 2003).

Adhesins

OMVs contain proteins called adhesins in the outer membrane, which allow them to interact and adhere to host cells. Adhesins also allow OMVs to be internalised by host cells efficiently so that virulence factors can be released. An example of this is the Ail adhesin/invasin located in the OM of OMVs of some *E. coli* strains. OMVs from a laboratory *E. coli* strain that contained Ail were internalised by eukaryotic cells at a rate that was 10-fold higher than those that did not express Ail (Kesty *et al.* 2004).

Proteases

Proteases can also be associated with OMVs and when released they can act on either host cells or other competing bacteria. An example of this is the presence of murein hydrolases in *P. aeruginosa* OMVs. When released, these proteases cause degradation of the peptidoglycan layer in bacterial cell walls (Li *et al.* 1998). This is advantageous as this can cause lysis of competitor bacteria so that more resources are available in the environment for the OMV-producing bacterium as there is less competition. Another advantage may also be that lysis of other bacteria in the environment causes the release of organic compounds into the environment which can be used by the OMV-producing bacteria for growth.

1.3.4.2 Aids bacterial cell survival

Immunomodulatory functions

Many OMVs contain compounds that modulate the hosts' immune response. In *Helicobacter pylori*, the LPS of the OMVs are bound to Lewis antigens which induce a strong response from the host immune system (Hynes *et al.* 2005). This is beneficial for the OMV-producing cell as the host cells immune system functions to remove the OMVs rather than targeting the bacterial cell. Alternatively, OMV cargo can function to inhibit the hosts' immune response. For example, UspA1 and UspA2 associated with OMVs from *Moraxella catarrhalis* bind to a member of the complement cascade called C3 (Tan *et al.* 2007). This inhibits the complement cascade to protect the OMV-producing bacterial cell in its immediate environment (this is discussed further in Section 1.10).

Antibiotic resistance

OMVs have also been found to contain multidrug efflux pumps which are used to move toxic compounds and antibiotics out of the OMVs (Gellatly, Hancock. 2013). Some OMVs contain proteases to catalyse degradation of antibiotics to remove them from the environment. For example, OMVs from *Pseudomonas aeruginosa* have been found to contain β-lactamase in the lumen of the vesicles (Bonnington, Kuehn. 2014), which aids resistance to β-lactam antibiotics such as penicillins (Chatterjee, Chaudhuri. 2012).

Antibacterial factors

Antibacterial factors that have been packaged in OMVs include autolysins and murein hydrolases (Li *et al.* 1996). In 1998, OMVs from Gram-negative bacteria were found to lyse both Gram-positive bacteria and other Gram-negative bacteria (Li *et al.* 1998). As well as reducing the competition in the environment, lysis of non-self bacterial cells releases nutrients into the environment which can be utilised by the OMV-secreting bacterium. For example, *Pseudomonas aeruginosa* eliminates neighbouring bacteria by secreting periplasmic peptidoglycan hydrolases through OMVs when nutrients are scarce (Kadurugamuwa, Beveridge. 1997).

Bacteriophage decoy

Bacteriophages are viruses that infect bacteria to reproduce, which ultimately results in bacterial cell lysis. In order to prevent this, evidence suggests that bacteria such a *Vibrio cholerae* secrete OMVs as a decoy for bacteriophages. It appears that bacteriophages bind to receptors on the surfaces of OMVs and sequesters them so that they cannot bind to the OMV-producing bacterial cell (Reyes *et al.* 2018).

Genetic diversity

Evidence suggests that a range of Gram-negative bacteria package DNA into their OMVs (Kolling *et al.* 1999). So far, chromosomal DNA, plasmid DNA and bacteriophage DNA have been detected within OMVs (Chatterjee, Chaudhuri. 2012). For example, the DNA within the lumen of *P. aeruginosa* OMVs remains present even after treatment with an extracellular DNase (Kadurugamuwa, Beveridge. 1995). This is evidence that the DNA is protected from degradation within the lumen of the OMV. It was hypothesised that DNA within OMVs could be transferred to another bacterial cell as a method of gene transformation (Yaron *et al.* 2000). To test this, OMVs were purified from an *E. coli* strain that expressed green fluorescent protein (GFP) on a plasmid with ampicillin resistance. These OMVs were able to transform this plasmid into a different *E. coli* strain that did not contain this plasmid or gene. The *E. coli* cells became ampicillin resistant and fluoresced green due to the GFP (Yaron *et al.* 2000). This evidence suggests that OMVs may play a role in gene transfer between bacteria.

1.3.4.3 Aids cell to cell communication

Biofilm formation

A biofilm is a community of microbial cells attached to a surface enclosed in a self-produced extracellular matrix. Biofilms create a protective and nutrient-rich environment that allow microbes to thrive. The extracellular matrix is composed of polysaccharides, proteins and extracellular nucleic acids such as DNA. The polysaccharide component of the extracellular matrix provides structure and protection and its adhesive properties allow bacteria to adhere to the surfaces and other cells. The biofilm protects the cells from antibiotics, cells of the hosts' immune system and extreme environments such as desiccation (Limoli *et al.* 2015). OMVs are used to release exopolysaccharides to form the matrix. They also allow bacteria in a biofilm to communicate via quorum sensing.

Quorum-sensing (QS)

Both Gram-negative and Gram-positive bacteria communicate using quorum sensing (Ramsey *et al.* 2009). QS involves the release of signalling molecules, which are used for both intraspecies and interspecies communication. Bacteria can detect when there is an accumulation of these molecules in the environment, which allows the cells to sense the number of bacteria (cell density). The molecules involved in QS can be secreted and delivered to neighbouring bacteria using OMVs (Bielig *et al.* 2001). QS allows bacteria in a population to coordinate gene expression according to cell density (Miller, Bassler. 2001). Bacteria use QS to work together to respond appropriately to a change in the environment. For example, QS is used to adapt to a change in nutrient availability and to form biofilms. Bacteria often use QS to co-ordinate their virulence (for example all secreting a toxin simultaneously) and to avoid the host immune response by forming biofilms.

Bacteria use different types of QS signalling molecules called autoinducers. The most common are the acyl-homoserine lactones (AHLs) (Cataldi *et al.* 2007). Some QS molecules are secreted into the environment without OMVs. However, some QS molecules are highly hydrophobic and unable to cross the LPS layer on Gram-negative cell envelopes or they are too large. In these cases, QS signalling molecules can be packaged into OMVs. An example of this is the quorum sensing molecule produced by *P. aeruginosa* called *Pseudomonas* Quinolone Signal (PQS). PQS is packaged into OMVs for transport from cell-to-cell as it is hydrophobic. PQS has been found to cause curvature in the OM of other *P. aeruginosa* cells and it has also been found to initiate and increase OMV production in other cells (Mashburn-Warren *et al.* 2008). This is discussed further in Section 1.4.1.

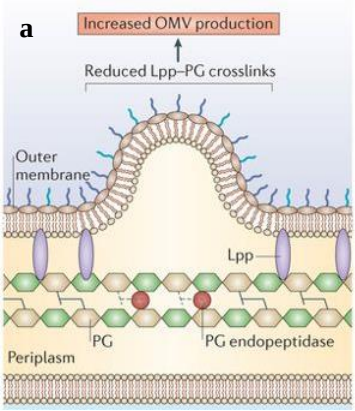
1.4 OMV biogenesis

1.4.1 Models of OMV biogenesis

Electron microscopy images suggest that OMVs are formed by bulging of the OM which pinches off and captures proteins from the periplasm. However, analysis of OMV composition suggests that the process is more regulated and complicated than this. For example, the composition of the OMV is similar but can differ from the bacterial OM it is derived from. Similarly, OMV-associated proteins can vary greatly from the periplasm as certain proteins appear to be enriched and others excluded from the OMVs (Schwechheimer, Kuehn. 2015). There are many models proposing different theories for the process of OMV biogenesis, which are discussed below. OMV biogenesis will be discussed in three parts: **1.** Bulging **2.** Cargo enrichment and exclusion **3.** Scission.

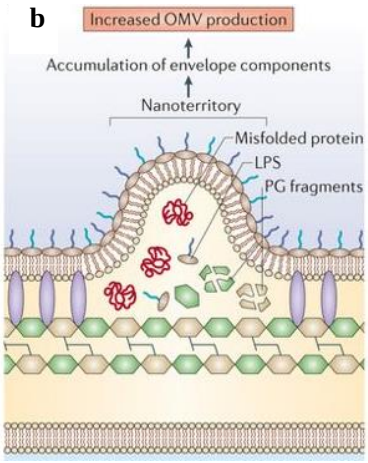
1.4.1.1 Outer Membrane Bulging

The first step of OMV biogenesis is the bulging of the OM, which can be explained by the four models below (Figure 1.8).



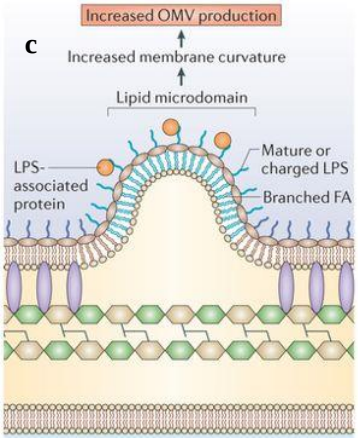
Model 1: Missing lipoprotein (Lpp link)

- For an OMV to form, the outer membrane must detach from the peptidoglycan layer
- Peptidoglycan (PG) endopeptidases are thought to form the Lpp link between the peptidoglycan and the outer membrane in the cell envelope
- At the position where the Lpp links are reduced or missing, localised membrane curvature occurs and OMVs are produced



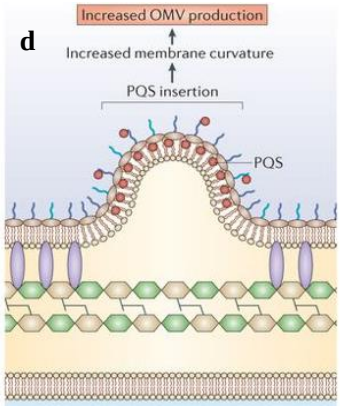
Model 2: Accumulation of envelope components or misfolded proteins

- OM bulging occurs where there is an accumulation of misfolded proteins or cell envelope components such as peptidoglycan fragments or LPS. These areas are known as Outer Membrane Nanoterritories and also appear to have reduced Lpp crosslinks between the OM and peptidoglycan
- This is thought to induce bulging of the OM and this leads to increased OMV production in these areas to remove these misfolded proteins from the cell in OMVs
- There is thought to be an imbalance in the production of peptidoglycan in certain regions of the periplasm. This leads to an excess of muramic acid which generates turgor pressure and causes OM bulging (Zhou *et al.* 1998)



Model 3: Lipid microdomains

- Some areas of the OM can become enriched with certain types of LPS, LPS-associated proteins and/or phospholipids. These areas are known as Lipid microdomains
- Lipid microdomains tend to bulge outwards due to the LPS charge or increased membrane fluidity. This leads to an increase in OMV formation in these areas



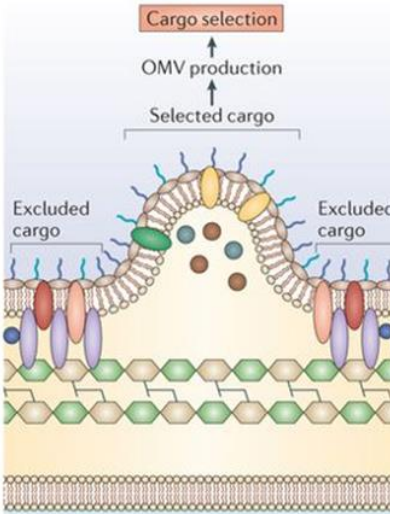
Model 4: Insertion of *Pseudomonas* quinolone signal (PQS) into the outer leaflet

- *P. aeruginosa* packages the quorum sensing molecule *Pseudomonas* Quinolone Signal (PQS) into OMVs for transport from cell-to-cell as it is hydrophobic
- PQS is bound to LPS in the bacterial outer membrane
- PQS has been found to cause curvature in the *P. aeruginosa* OM and it has also been found to initiate and increase OMV production (Mashburn-Warren *et al.* 2008)

Figure 1.8 Four models of how the outer membrane bulges to produce OMVs
The four models of OM bulging are: **1.** Missing lipoprotein link (**a**) **2.** Accumulation of envelope components or misfolded proteins (**b**) **3.** Lipid microdomains (**c**) **4.** Insertion of *Pseudomonas* quinolone signal (PQS) into the outer leaflet (**d**). Image sourced from Schwechheimer, Kuehn (2015).

1.4.1.2 Cargo enrichment and exclusion

The abundance of a particular protein in the OM and periplasm does not necessarily reflect the concentration of that protein within the OMV. A selective and regulated process of enriching and excluding certain proteins from the OMVs is thought to occur. It appears that cargo, which is destined to be incorporated into the OMVs, is targeted to the sites of OMV budding. Similarly, cargo that is not destined for the OMVs are located away from the sites of OM bulging (Figure 1.9). Currently, there is not a known signal sequence on proteins destined to be incorporated into OMVs (Kulp et al. 2010).



- Certain cargo from the periplasm appear to be selectively included or excluded from OMVs via a sorting mechanism
- Cargo to be included within the OMVs appear to interact with OM-associated proteins which are located in sites prone to budding
- Cargo to be excluded from OMVs are thought to interact with other cell envelope components which are not found at the sites of budding and OMV formation

Figure 1.9 Model for OMV cargo selection
Cargo to be included within the OMVs appear to interact with OM-associated proteins which are prone to budding. Cargo to be excluded from OMVs are thought to interact with other cell envelope components which are not found at the sites of budding and OMV formation
Image sourced from Schwechheimer, Kuehn (2015).

1.4.1.3 Scission

It has been proposed that OMVs are released from the cell when the bud grows to a size where the membrane curvature forces it to separate and causes scission. The source of energy for membrane scission remains unclear. One possibility could be the transfer of energy from the cytoplasm but the mechanism is still unknown and further research is needed (Kulp *et al.* 2010).

1.4.2 How are the contents of OMVs released at the target site?

1.4.2.1 Entry of OMVs into other Prokaryotic cells

Gram-negative bacteria

The fusion of OMVs with other Gram-negative cell membranes is the most likely route of entry as their membranes are so similar. Once the OMVs have fused to the bacterial cell, the OMV luminal contents are released into the periplasm (Kulp *et al.* 2018). OMVs may contain peptidoglycan hydrolase enzymes

which can degrade the peptidoglycan cell wall in the periplasm and cause bacterial cell lysis (Kadurugamuwa, Beveridge. 1996).

Gram-positive bacteria

Gram-positive bacteria have very different membrane compositions to OMVs produced from Gram-negative bacteria. Evidence suggests that OMVs adhere to the cell wall of Gram-positive bacterial membrane then lyse. The cargo is then released and enters the cell by diffusion through the membrane (Kulp *et al.* 2018). Some OMVs contain peptidoglycan hydrolase enzymes, which digest the Gram-positive peptidoglycan cell wall and cause lysis (Kadurugamuwa, Beveridge. 1996).

1.4.2.2 Entry of OMVs into Eukaryotic cells

There are four main methods that allow OMV entry into eukaryotic host cells which are summarised below (Figure 1.10).

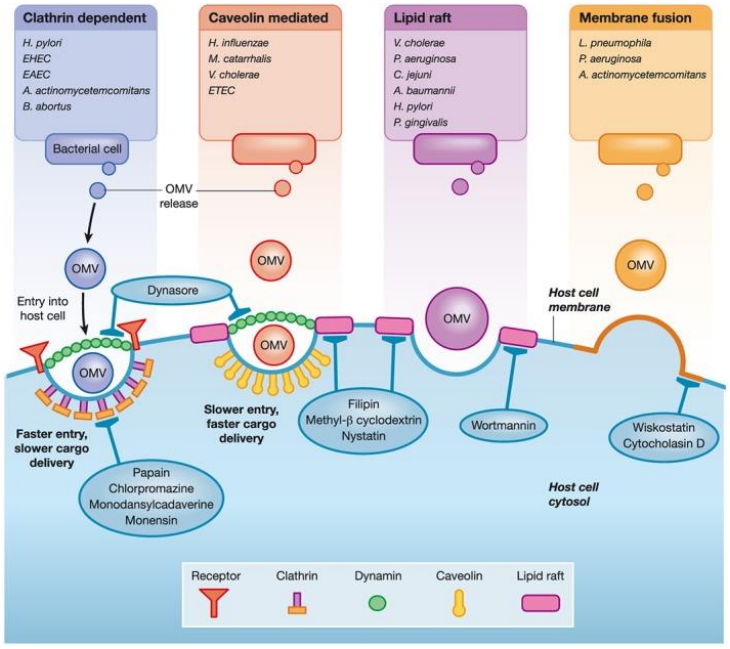


Figure 1.10 Entry of OMVs into eukaryotic cells
Diagrams of four routes of entry are shown above: 1. Clathrin dependent 2.Caveolin mediated 3. Lipid raft 4. Membrane fusion. Inhibitors of OMV entry are also listed above next to the relevant method. Image sourced from O'Donoghue, Krachler. 2016.

The routes of entry into host cells can be split into the following categories:

1. **Clathrin dependent:** Clathrin is a protein that plays a major role in clathrin-mediated endocytosis. Clathrin is the major scaffold protein that can assemble into a cagelike structure on cell membranes. When ligands bind to receptors on the cell surface, the clathrin structure forms around the desired cargo to become the inside surface of the vesicles formed during endocytosis. Clathrin coated pits are formed on the membrane of the host cell. OMVs contain ligands on their surface, which bind to the host cell receptors. The OMVs are internalised by the cell through clathrin-mediated endocytosis and enter the endosomal trafficking pathway of the host cells where the OMV cargo is released.

2. **Caveolin mediated:** Lipid rafts are microdomains of the plasma membrane with increased concentrations of cholesterol and sphingolipids (Mulcahy *et al.* 2014). These domains are

sometimes enriched with the membrane protein caveolin. Caveolins form caveolae which are cave-shaped invaginations of the host cells plasma membrane. The internalisation of the OMVs using caveolin-mediated endocytosis is thought to avoid fusion with lysosomes. This means that the cargo of the OMVs is delivered faster with less chance of degradation compared to clathrin-dependent endocytosis (Lim *et al.* 2014). OMVs contain ligands which bind to host cell receptors to trigger endocytosis.

3. **Lipid raft mediated:** Lipid raft domains are more compact than other areas of the plasma membrane and can cause an inwards curvature of the membrane (invaginations). Viruses are known to use lipid rafts to enter host cells and it is possible that OMVs use lipid rafts as points of entry too (Kulp *et al.* 2010). This route of OMV uptake is not reliant on clathrin or caveolin coated membrane invaginations.

4. **Membrane fusion:** OMVs have been found to enter eukaryotic host cells despite the differences in membrane architecture. Model membranes representing OMVs and host membranes have confirmed that fusion between the two membrane types can occur (Jager *et al.* 2014). However, the exact mechanism remains unclear.

5. **Macropinocytosis:** Macropinocytosis is a type of endocytosis that involves the internalisation of extracellular material from the environment. It is characterised by polymerisation of actin to form actin filaments at the cell membrane. The cell membrane ruffles and closes in a way that engulfs extracellular material from the surroundings (Weiner *et al.* 2016). It is possible that OMVs enter host cells via micropinocytosis, however, it is not believed to be an event induced by the OMVs themselves (O'Donoghue, Krachler. 2016).

1.5 Is OMV secretion a novel secretion system?

Gram-negative bacteria have six major secretory mechanisms, which are summarised later on in Section 1.5.2. In order for a molecule to be secreted from bacteria, there are two membranes to cross (the inner and outer membranes).

1.5.1 Summary of the Secretory pathway (Sec) and the Twin-arginine translocation pathway (Tat)

There are two different pathways to transport proteins across the inner membrane in Gram-negative bacteria: the Secretory pathway (Sec) and the Twin-arginine translocation pathway (Tat). In the Sec pathway, proteins are transported in an unfolded state. They are folded at the trans-side of the membrane. The Tat-pathway on the other hand transports proteins in their folded (native state).

Sec pathway: transport of unfolded proteins

Secreted proteins will either be released into the periplasm in an unfolded form or may be embedded in the inner membrane (Figures 1.11-1.13). Proteins destined for the Sec pathway have a hydrophobic signal sequence which is approximately twenty amino acids long. This signal sequence found at the N terminus of the protein is recognised by either SecB or a signal recognition particle (discussed below).

Sec pathway: Proteins destined for the periplasm or extracellular secretion

Proteins destined for the periplasm or secretion outside of the cell contain a removable signal sequence specific for this Sec pathway. SecB recognises the signal sequence and binds to it to keep the protein unfolded. SecB delivers the protein to SecA which guides the protein to a channel called SecYEG. The protein is transported in an unfolded state using the ATPase activity of SecA for energy (Figure 1.11).

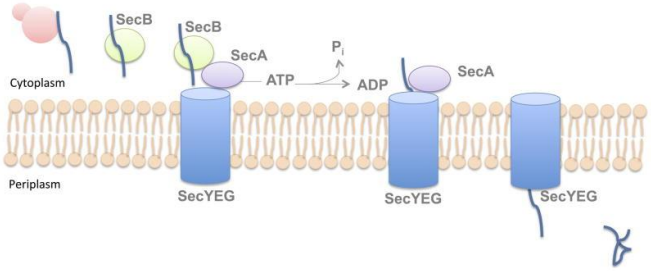


Figure 1.11 Secretory pathway for proteins destined for the periplasm
Image sourced from Green *et al.* 2016.

Sec pathway: Proteins destined for the inner membrane

Proteins that are destined for the inner membrane contain a different signal sequence recognised by SRP. SRP binds to the protein during translation at the ribosome. The docking protein FtsY then guides the protein-ribosome complex to the SecYEG channel. However, in this case, the protein passes out of the side of the SecYEG channel and remains embedded in the membrane (Figure 1.12).

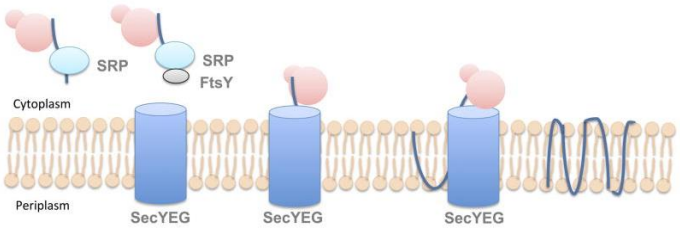


Figure 1.12 Secretory pathway for proteins destined to be embedded in the inner membrane
Image sourced from Green *et al.* 2016.

Tat pathway: transport of folded proteins

The Tat pathway is used for the secretion of folded proteins which are folded and/or post-translationally modified in the cytoplasm. In this pathway, TatB and TatC bind to a specific signal peptide on the N terminal of the protein. TatA is then recruited to the membrane to form a channel across the inner membrane to translocate the folded proteins (Figure 1.13).

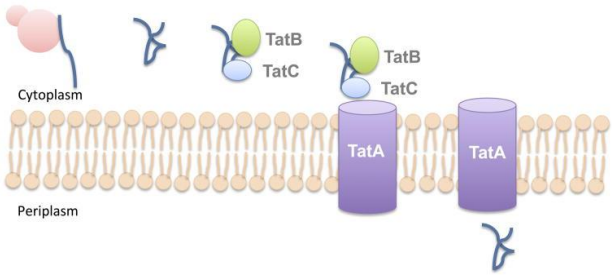


Figure 1.13 Twin-arginine translocation pathway for folded proteins
Image sourced from Green *et al.* 2016.

1.5.2 Overview of bacterial secretion systems

Bacterial secretion systems are complexes of proteins which are found on the bacterial cell membrane and are used for the extracellular secretion of proteins or other substances. They are used by pathogenic bacteria to secrete virulence factors which can cause damage to the host or other competing microorganisms in the environment. There are six major bacterial secretory systems in Gram-negative bacteria which are outlined in Table 1.1.

Table 1.1 – Comparison of the six secretion systems in Gram-negative bacteria

Name of Secretion System	Number of steps in Secretion	Uses Sec or Tat?	Summary of Mechanism	Molecules transported
Type I Section System (T1SS)	1	No	Protein contains a signal sequence specific for the ABC transporter. The protein is excreted outside of the OM through a tunnel-like protein channel	Proteins, ions, drugs, polysaccharides
Type II Section System (T2SS)	2	Yes	Proteins are initially transported to the periplasm through the Sec or Tat systems. Once in the periplasm, the molecule passes through the outer membrane using a multimeric protein complex	Proteins
Type III Section System (T3SS)	1-2	No	Similar to a syringe, proteins are injected into eukaryotic cell cytoplasm from the bacterial cytoplasm through the complex known as an injectosome	Toxic proteins
Type IV Section System (T4SS)	1	Yes	Uses an envelope spanning complex of proteins that forms a channel from the cytoplasm of the bacterial cell to the cytoplasm of a recipient cell.	DNA and protein macromolecules
Type V Section System (T5SS)	2	Yes	Proteins are transported through the inner membrane using the Sec pathway. These proteins can form β-barrel structures in their C-terminus which allows insertion into the OM (this is known as the autotransporter). The rest of the peptide can reach outside the cell which is known as the passenger domain. The autotransporters are often cleaved which releases the passenger domain while leaving the β-barrel domain in the OM	Proteins (such as adhesins)
Type VI Section System (T6SS)	1	No	Current models of this secretion system suggest that there is dynamic structure which closely resembles a bacteriophage. This is anchored to the cell by a complex which spans the cell envelope. Proteins can be transported to from the cytoplasm of the bacterial cell to a target cell using this machinery	Proteins

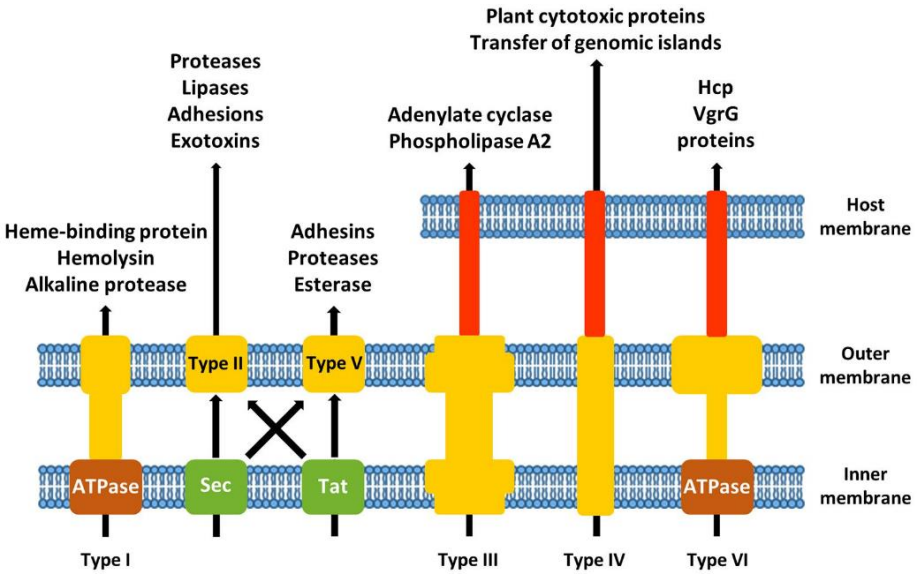


Figure 1.14 Schematic diagram of the bacterial secretory systems
Secretory systems Type I to VI are represented here along with the Sec and Tat systems. Image sourced from Depluverez *et al.* 2016.

1.5.3 Evidence that OMVs are a novel secretory system in Gram-negative bacteria

The formation and secretion of OMVs containing cargo was described as a seventh bacterial secretion system (McBroom, Kuehn. 2007). It can be labelled as a secretion system as cargo is packaged into OMVs in a deliberate and selective way for later release into the environment. It is also very different from the existing secretion systems outlined in Table 1.1 and Figure 1.14 above. The unique characteristics of OMVs as a secretion system were outlined in a review in 2010 (Kulp *et al.* 2010) and were as follows:

1. OMVs can be used to secrete bacterial lipids and other insoluble compounds that cannot be secreted via the other six secretory systems.
2. Cargo is protected within the lumen of the OMVs. This means that they are protected from extracellular proteases in the environment until they have reached their target destination.
3. Proteins (for example virulence factors) can be delivered to the target in high concentrations within the OMVs. OMVs allow these molecules to reach the target cells in a concentrated form where they are fully folded and biologically active.
4. OMVs can contain adhesins on their surface to target them to specific target cells bearing the correct receptors.
5. Lastly, multiple virulence factors can be packaged together for maximum damage to the target cell (Demuth *et al.* 2003).

1.6 Using *Escherichia coli* (*E. coli*) as a model system to study OMVs

1.6.1 Introduction to *E. coli*

E. coli is a commensal organism in humans and is part of the intestinal flora. It can also be found in every day food such as raw meat, raw egg, vegetable salads and unpasteurised milk. Some strains of *E. coli* can acquire virulence factors and become pathogenic. These strains can cause a range of diseases such as urinary tract infections, kidney infections, cystitis, cholangitis, food poisoning and bacteraemia. Treatment for infections caused by *E. coli* are becoming more difficult as they have developed resistance mechanisms to most first-line antibiotics (Sabaté *et al* 2008). Antibiotic resistance in *E. coli* is a major concern as it is the most common Gram-negative bacterial pathogen in humans (Rasheed *et al.* 2014). Virulence factors of pathogenic *E. coli* include adhesins, flagella, fimbriae and hemolysin. They are also easily able to acquire and accumulate antibiotic resistance genes through horizontal gene transfer making many strains multidrug resistant (Poirel *et al.* 2018).

1.6.2 *E. coli* pathogenicity

Urinary tract infections (UTIs)

Approximately 50 to 60% of women will have UTIs in their lifetimes, the majority of which are caused by *E. coli* (Al-Badr, Al-Shaikh. 2013). Some of these will develop recurrent UTIs where the same pathogen re-infects multiple times. UTIs can infect the bladder, urethra or kidneys and can currently be treated by antibiotics. In the worst cases, UTIs can cause bacteraemia, which can be fatal (Figure 1.15).

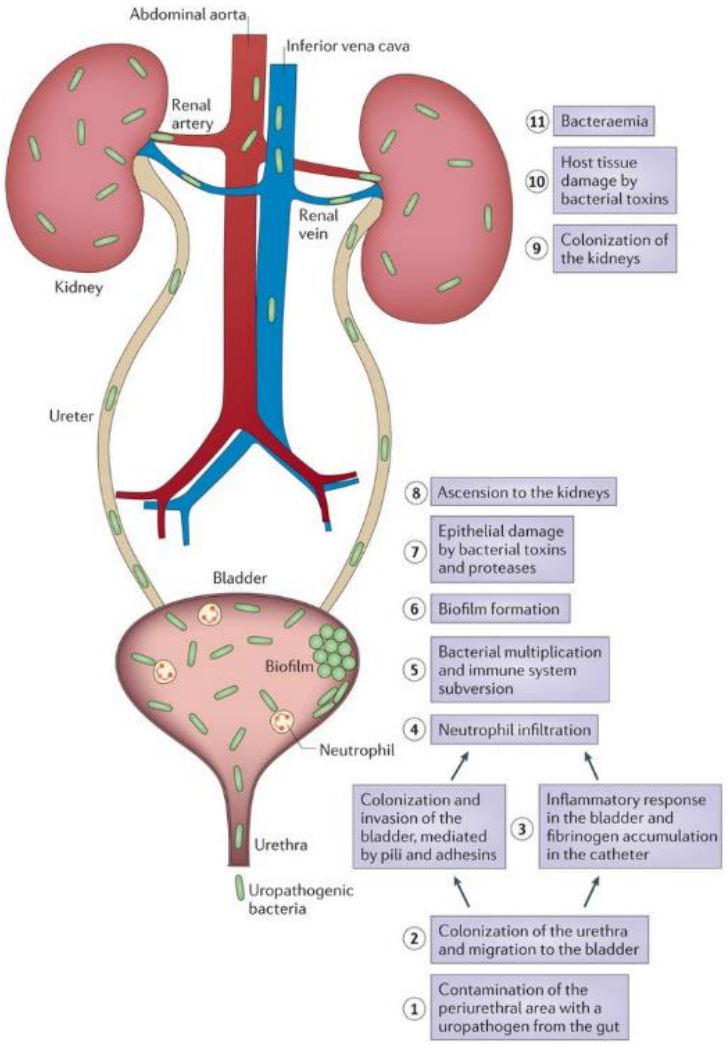


Figure 1.15 The stages of urinary tract infections (UTIs) The eleven stages of UTIs are described in detail below. In the case of infection due to a catheter, the immune response causes the accumulation of fibrinogen on the catheter. Uropathogens that express fibrinogen-binding proteins can form biofilms on the catheter (see step 3). Image sourced from Flores-Mireles *et al.* 2015.

Urinary tract infections begin when uropathogens, such as *E. coli*, contaminate the periurethral area. These bacteria can then colonise the urethra (step 1). After entering the urethra, *E. coli* can then migrate upwards towards the bladder (step 2). The bacteria can adhere to uroepthelial cells by expressing Type 1 fimbriae and adhesins (step 3). The immune response of the host causes neutrophils to arrive to the site of invasion (step 4). However, bacteria can evade the immune system due to morphological changes or invasion of host cells. These successful bacteria multiply (step 5) then form biofilms (step 6) for protection. Bacteria within the biofilm can secrete toxins and proteases to damage host epithelial cells (step 7). Bacteria can now migrate towards the kidneys (step 8) and begin colonisation of renal tubular epithelial cells by expressing Type 1 fimbriae (step 9). The tissues of the kidney are also damaged by release of toxins such a haemolysin (step 10). Cytokines are also induced, which causes an inflammatory immune response. This is known as pyelonephritis (a kidney infection). If the bacteria cross the kidney tubular epithelial barrier, the uropathogens can enter the blood and cause bacteraemia which can be fatal (step 11).

1.6.3 Type 1 fimbriae biosynthesis and introduction to FimA

Type 1 fimbriae are extracellular appendages that are synthesised by *E. coli* cells (Figure 1.16 **a**). Fimbriae allow bacteria to adhere to host cells and allow colonisation of host tissues. Type 1 fimbriae in *E. coli* bind to mannose receptors on the surface of urinary epithelial cells (Nishiyama *et al.* 2005) to initiate the colonisation of a certain area. They are 2-7 nm in diameter but can be up to 2 µm in length (Costello *et al.* 2012). Fimbriae are anchored in the outer membrane of Gram-negative bacteria by FimD (see Figure 1.16 **b**). The main structural component of Type 1 fimbriae is FimA. FimA monomer proteins polymerise to form the rod portion of the Type 1 fimbriae, which contains approximately 300-5000 FimA monomer subunits (Nishiyama *et al.* 2005). The tip fibrillum is synthesised first and consists of FimH, FimG and FimF. FimA monomers are then added and polymerise to form the main structure of the Type 1 fimbriae.

To assemble the Type 1 fimbriae, the chaperone protein FimC forms complexes with new proteins entering the periplasm. Fimbriae-associated proteins FimA, FimF, FimG and FimH are transported across the inner membrane (via the Sec pathway) into the periplasm. Type 1 fimbriae components are assembled by FimC (the periplasmic chaperone) and an usher protein called FimD. FimD is located in the outer membrane and is also known as the assembly platform as it is the location where Type 1 fimbriae are synthesised (Figure 1.16 **b**).

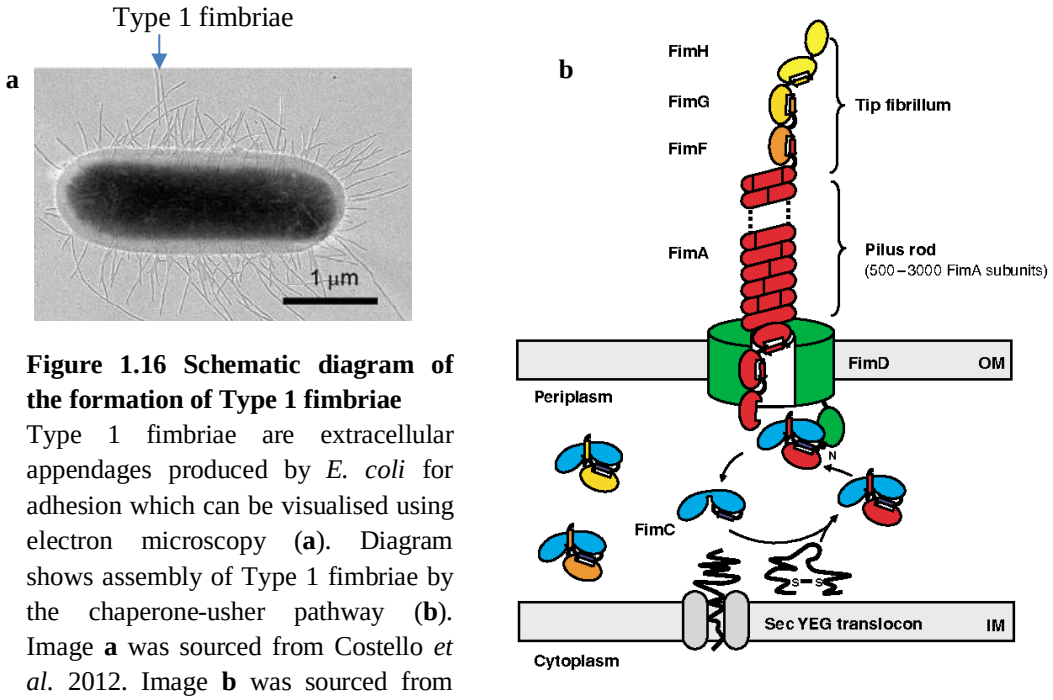


Figure 1.16 Schematic diagram of the formation of Type 1 fimbriae
Type 1 fimbriae are extracellular appendages produced by *E. coli* for adhesion which can be visualised using electron microscopy (**a**). Diagram shows assembly of Type 1 fimbriae by the chaperone-usher pathway (**b**). Image **a** was sourced from Costello *et al.* 2012. Image **b** was sourced from Nishiyama *et al.* 2005.

The fimbrial subunits have an immunoglobulin-like fold but are missing the seventh C-terminal β strand (known as the 'pilin fold'). This means that the Ig-like folds are incomplete and that the subunit folding is dependent on a periplasmic chaperone for the correct folding. The chaperone protein FimC forms complexes with each of the fimbrial subunits in the periplasm. FimC donates the missing β-strand to

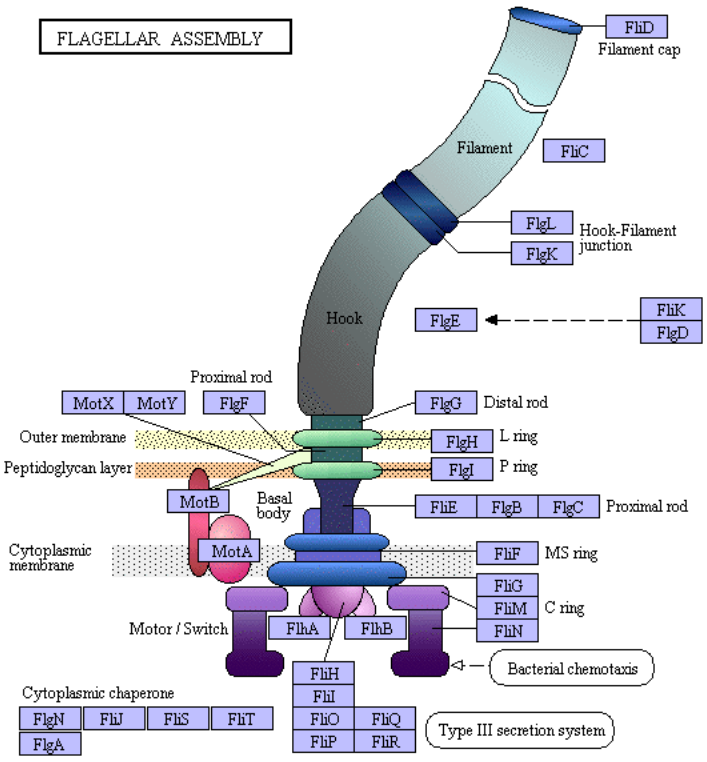
complete the protein so it is ready for incorporation into the Type 1fimbriae. The FimC-protein complexes are recognised by FimD in the outer membrane. The folded subunits are released from FimC are guided by FimD through the outer membrane and on to the growing Type 1 fimbriae.

In the assembled fimbriae, there is an N-terminal extension of approximately 15 residues preceding the 'pilin fold'. This acts as a donor strand to the subunit before it. Each fimbrial subunit gives its donor strand to the subunit before it and accepts a donor strand from the next subunit in the chain. It is thought that a conformation change occurs during subunit assembly, which makes Type 1 fimbriae so stable. The quaternary structure of the Type 1 fimbriae produced extracellularly is helical. These fimbrial proteins are important as FimA monomer was found to be heavily enriched in some *E. coli* OMVs.

1.6.4 Flagella biosynthesis and introduction to Flagellin

Flagella are extracellular appendages used by bacteria for motility which are used for host invasion. Flagella are approximately 10-30 nm in diameter and 5-20 µm in length (Atlas of Oral Microbiology, 2015). Bacterial flagellum are complex structures and typically over 50 genes are involved in flagellar synthesis and function (Figure 1.17).

a



b

FlgA	flagella basal body P-ring formation protein FlgA
FlgB	flagellar basal-body rod protein FlgB
FlgC	flagellar basal-body rod protein FlgC
FlgD	flagellar basal-body rod modification protein FlgD
FlgE	flagellar hook protein Flg
FlgF	flagellar basal-body rod protein FlgF
FlgG	flagellar basal-body rod protein FlgG
FlgH	flagellar L-ring protein precursor FlgH
FlgI	flagellar P-ring protein precursor FlgI
FlgK	flagellar hook-associated protein 1 FlgK
FlgL	flagellar hook-associated protein 3 FlgL
FlgN	flagella synthesis protein FlgN
FlhA	flagellar biosynthesis protein FlhA
FlhB	flagellar biosynthetic protein FlhB
FliC	flagellin
FliD	flagellar hook-associated protein 2
FliE	flagellar hook-basal body complex protein FliE
FliF	flagellar M-ring protein FliF
FliG	flagellar motor switch protein FliG
FliH	flagellar assembly protein FliH
FliI	flagellum-specific ATP synthase
FliJ	flagellar FliJ protein
FliK	flagellar hook-length control protein FliK
FliM	flagellar motor switch protein FliM
FliN	flagellar motor switch protein FliN/FliY
FliO	flagellar protein FliO/FliZ
FliP	flagellar biosynthetic protein FliP
FliQ	flagellar biosynthetic protein FliQ
FliR	flagellar biosynthetic protein FliR
FliS	flagellar protein FliS
FliT	flagellar protein FliT
MotA	chemotaxis protein MotA
MotB	chemotaxis protein MotB

Figure 1.17 Schematic diagram of flagellar assembly
A typical flagellum consists of the following components: 1. The basal body 2. A motor 3. A switch 4. A hook 5. A filament 6. Export apparatus (**a**). The long helical filament portion rotates as a propeller which allows motility. List of full protein names are found in (**b**). Image sourced from Kegg, 2017.

The main structural component of flagella is the protein Flagellin (FliC). Flagellin monomers are transported from the cytoplasm into a central channel through the basal body and hook structures to the filament. Flagellin monomer subunits polymerise to form the main structural subunit of the flagella and this occurs under the FliD filament cap. The final structure can contain 20,000-30,000 Flagellin subunits that form a helical structure.

Flagellin monomers have strong oligomerisation potential and polymerise into filaments *in vitro*. It is thought that the N and C terminals of FliC are responsible for Flagellin polymerisation. In the monomeric form of Flagellin, the N and C terminals are exposed and have no tertiary structure. However, these regions become folded and incorporated into the polymer. In the cytosol, spontaneous polymerisation is prevented by a cytosolic chaperone protein that is specific for binding to FliC called FliS. FliS binds to the C terminal of Flagellin and inhibits premature polymerisation (Auvray *et al.* 2008). Bacterial flagella are of interest as the Flagellin (FliC) monomer was found to be heavily enriched in some *E. coli* OMVs.

1.6.5 *E. coli* K-12 strains vs. B strains

E. coli K-12 strains and *E. coli* B strains are very commonly used as a model organism in the scientific community. *E. coli* is widely used in biotechnology due to its rapid doubling time, ease of genetic manipulation and our extensive knowledge of the genome, metabolomics and biochemistry. The origin of the *E. coli* K-12 strain can be traced to a stool sample in 1922 at Stanford University (Bachmann *et al.* 1972). Although the origins of the *E. coli* B strain are unclear, they are commonly used for bacterial transformations and expression of recombinant proteins. B strains are deficient in extracellular appendages such as flagella and fimbriae, which reduces their pathogenicity compared to K-12 strains (Marisch *et al.* 2013). *E. coli* B strains (such as BL21) are also deficient in certain proteases such as Lon and OmpT. They also have enhanced membrane permeability to allow uptake of plasmid DNA.

One of the ultimate project aims was to manipulate *E. coli* strains into producing OMVs with specific cargo for therapeutic use. For this study, OMVs were purified from the *E. coli* B strains: BL21 and BL21 (DE3). The DE3 designation means that the strain carries the gene for T7 RNA polymerase under control of the lacUV5 promoter. This allows inducible protein expression of any gene controlled by the T7 promoter when induced with IPTG. It is called DE3 as the T7 RNA polymerase gene is carried on a DE3 lysogen. OMVs were also purified from a range of K-12 strains and six clinical isolates for comparison (Chapter 4).

1.7 Using *Pseudomonas aeruginosa* OMVs as Gram-negative comparison

1.7.1 Introduction to *Pseudomonas aeruginosa* (*P. aeruginosa*)

P. aeruginosa are ubiquitous Gram-negative bacteria, which are commonly found in the environment in soil and water. They are classed as opportunistic pathogens as they rarely cause disease in healthy individuals, but are a major cause of infection in patients that are immunocompromised. *P. aeruginosa* is one of the most common causes of hospital-acquired infections and ventilator-associated pneumonia (Barbier *et al.* 2013). *P. aeruginosa* infection can be fatal in immunocompromised patients such as those with cystic fibrosis (CF). CF leads to the formation of a thick layer of mucus within the lungs of the patients. This prevents mucociliary clearance by the cilia, which line the lungs and function to clear any pathogens or inhaled particles from blocking the airways. This provides an ideal area for *P. aeruginosa* to colonise and form biofilms with other opportunistic pathogens such as *Bukholderia cenocepacia* (Eberl *et al.* 2004). Treating *P. aeruginosa* infections is becoming increasingly difficult as *P. aeruginosa* strains are versatile and able to adapt well to environmental changes. *P. aeruginosa* strains have now developed resistance to many of the current antibiotics available (Okamoto *et al.* 2001). The strains not only have intrinsic resistance to antibiotics, but are also easily able to acquire genes encoding resistance mechanisms making this strain a global threat to human health.

1.7.2 Roles of OMVs in the pathogenicity of *P. aeruginosa*

P. aeruginosa strains can form biofilms with other bacteria, such as *Burkholderia cenocepacia*, in the lungs of CF patients. Bacteria can communicate during the formation of biofilms by quorum sensing (Eberl *et al.* 2004). *P. aeruginosa* strains produce OMVs, which have been found to contain virulence factors, such as toxins and β-lactamases. The OMVs can diffuse across the mucus layer found within the lungs and are internalised by lung epithelial cells that cause the release of the cargo (Koeppen *et al.* 2016). Interestingly, the virulence factor Cif (CFTR inhibitory factor) has been found in OMVs which was found to reduce the host immune response (Koeppen *et al.* 2016). Cif causes lysosomal degradation of CFTR (CF transmembrane conductance regulator), which is essential for mucociliary clearance within the lungs. Also, Cif has been found to decrease MHC class 1 antigen presentation on lung epithelial cells (Koeppen *et al.* 2016). *P. aeruginosa* OMVs were studied as a Gram-negative bacterial comparison for *E. coli* OMVs with an aim to compare and contrast OMV cargo in another clinically relevant strain.

1.8 Membrane Vesicles from Gram-positive bacteria

1.8.1 Gram-positive cell envelope

As discussed in Section 1.2.1, the Gram-positive cell envelope is composed of a cytoplasmic membrane and a thick peptidoglycan cell wall, with no outer membrane present (Figure 1.18). Instead, there is a thick layer of peptidoglycan to protect the cell membrane from harsh environments and turgor pressure (Silhavy *et al.* 2010).

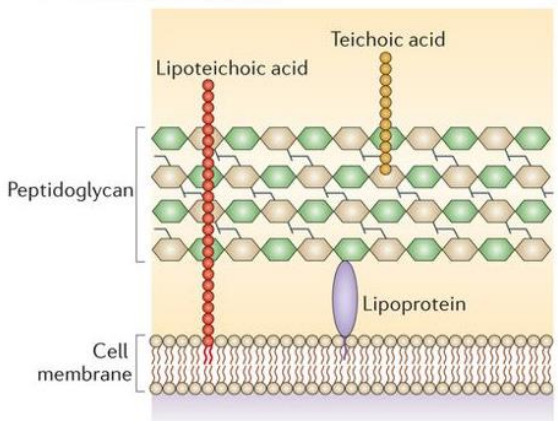


Figure 1.18 Gram-positive bacteria cell envelope
The Gram-positive bacterial cell envelope is composed of the plasma membrane and a thick peptidoglycan cell wall. Image sourced from Brown *et al.* 2015.

The composition of peptidoglycan was summarised in Section 1.2.2. The peptidoglycan layer in the Gram-positive cell envelope is approximately 20-80 nm in diameter with many layers compared with the peptidoglycan of Gram-negative bacteria which is less than 10 nm thick (Mai-Prochnow *et al.* 2016). Threading through the layers of peptidoglycan are teichoic acids. These are long anionic polymers that are covalently linked to the peptidoglycan layer. Lipoteichoic acids are also present, which are anchored to the cytoplasmic membrane and run through the peptidoglycan layer (Silhavy *et al.* 2010). There are also a range of proteins found within the cell envelope and some are associated with the cytoplasmic membrane and peptidoglycan layer. Lastly, surface proteins such as adhesins allow adhesion to host cells.

1.8.2 Comparison of Membrane Vesicle (MV) composition between Gram-negative and Gram-positive bacteria

Recent evidence in the literature suggests that Gram-positive bacteria produce membrane vesicles (MVs). These are different to OMVs in cargo and composition due to differences in the membrane structure (Brown *et al.* 2015, Bitto *et al.* 2017). OMVs derived from Gram-negative bacteria are composed of the outer membrane and contain cargo from the periplasm. Gram-positive membrane vesicles, however, are composed of the cytoplasmic membrane only and cargo from the cytosol (Figure 1.19). The term membrane vesicles (MVs) will be used to describe vesicles from Gram-positive bacteria as they are not *outer* membrane vesicles (OMVs). MVs may also be used as a term to describe both MVs from Gram-positive bacteria and OMVs from Gram-negative bacteria as it encompasses both.

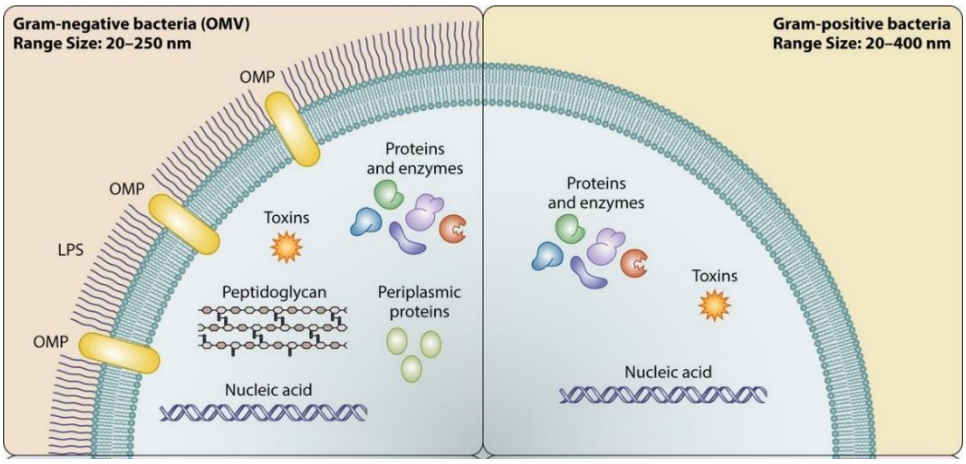


Figure 1.19 Comparison of membrane vesicles formed from Gram-negative and Gram-positive bacterial membranes.
There are significant differences in the composition of Gram-negative and Gram-positive bacterial membranes. This leads to variations in the composition of membrane vesicles produced and methods of MV biogenesis. This schematic summarises the OMV cargo and membrane composition of typical Gram-negative and Gram-positive OMVs. Image sourced from Joffe *et al.* 2016.

1.8.2.1 Gram-positive MV composition and cargo

In 2009, membrane vesicles were visualised on the surface of *Staphylococcus aureus* cells using TEM (Lee *et al.* 2009). The MVs were very similar in appearance to OMVs as they were nano-sized, spherical membranous structures of 20-100 nm in diameter (Lee *et al.* 2009). MVs were then purified from *S. aureus* using a method very similar to that used for OMV purification. The purified MVs were visualised by TEM and the size of the MVs was determined using dynamic light scattering. To determine the MV proteome, MV protein profiles were visualised by SDS-PAGE and proteins were characterised by microscopy (Lee *et al.* 2009).

MVs from Gram-positive organisms, (such as *Staphylococcus aureus*), have been found to contain cytoplasmic proteins, metabolic enzymes, DNA polymerases, ribosomal proteins and virulence factors (Lee *et al.* 2009). Many of the virulence factors found in Gram-negative OMVs have also been found in Gram-positive MVs. These include toxins, haemolysin, adhesins and β-lactamases (Joffe *et al.* 2016). As with OMVs, it appears that Gram-positive cells have a specific sorting mechanism for packaging cargo into the MVs. In 2010, it was found that MVs from *Bacillus anthracis* contained toxins (edema factor, lethal factor and protective antigen), which were not present freely in the supernatant (Rivera *et al.* 2010). The protein profile of the MVs were also confirmed to be different from that of the cytoplasmic membrane and cytosol making it unique.

1.8.3 Models for Gram-positive MV biogenesis

The method of MV biogenesis from Gram-positive bacteria is not fully understood. At first glance, it seems unclear how membrane vesicles can bud from the cytoplasmic membrane when the peptidoglycan layer is so thick. Three hypotheses of how membrane vesicles are formed from Gram-positive bacteria are outlined in Figure 1.20.

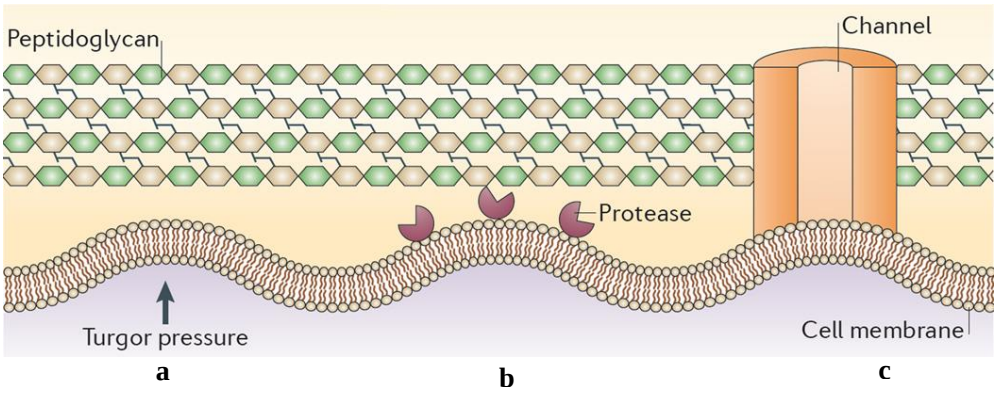


Figure 1.20 Hypotheses of MV formation in Gram-positive bacteria
Three main hypotheses exist of how Gram-positive bacteria secrete membrane vesicles through such a thick layer of peptidoglycan. The first is that MVs can be forced through by turgor pressure (**a**), the second is that enzymes degrade the peptidoglycan cell wall to allow MVs to bud (**b**) and the third is that protein channels are used to guide MVs extracellularly (**c**). These hypotheses are not mutually exclusive and may all be possible. Image sourced from Brown *et al.* 2015.

1.8.3.1 Turgor pressure

The first theory is that the turgor pressure produced after MVs are released from the cytoplasmic membrane is enough to force the MVs through the peptidoglycan cell wall. In this theory, there may be pore sizes within the cell wall which are large enough to allow MVs through or that there are certain parts of the cell wall which are thinner than others (Brown *et al.* 2015).

1.8.3.2 Proteases degrade peptidoglycan

MVs released by Gram-positive strains have been found to contain peptidoglycan-degrading enzymes. This may suggest that enzymes are secreted with the MVs which degrade the peptidoglycan layer, allowing the MVs to bud (Brown *et al.* 2015).

1.8.3.3 MVs are transported through a channel

One final hypothesis is that MVs are transported through the peptidoglycan layer using protein channels. This would allow the passage of MVs through the peptidoglycan layer without disrupting the cell wall (Brown *et al.* 2015).

1.9 Using *Streptomyces* S4 as a model system to study MVs

1.9.1 Introduction to *Streptomyces*

Antimicrobial resistance (AMR) hinders the effective treatment and prevention of infections caused by microbes such as bacteria, fungi, viruses and protozoa. AMR is a serious threat to health globally as the infections can no longer be treated and may then be spread to others (World Health Organization, Fact sheets on AMR, 2018). Bacteria from the genus *Streptomyces* produce the majority of antibiotics used for medicine, agriculture and veterinary practice (Chater, 2016). With a global emergence of multi-drug resistant pathogens, the study of *Streptomyces* bacteria for new antimicrobial compounds has never been more important.

Streptomyces are filamentous soil bacteria that play a key role in the decomposition and recycling of plants and fungi. Some have also developed symbiotic relationships with insects or plants and some have evolved pathogenic traits (Chater, 2016). *Streptomyces* are Gram-positive bacteria but are unusual as they grow as filamentous hyphae that resemble fungi. *Streptomyces* have a complex life cycle which includes the formation of spores (Figure 1.21).

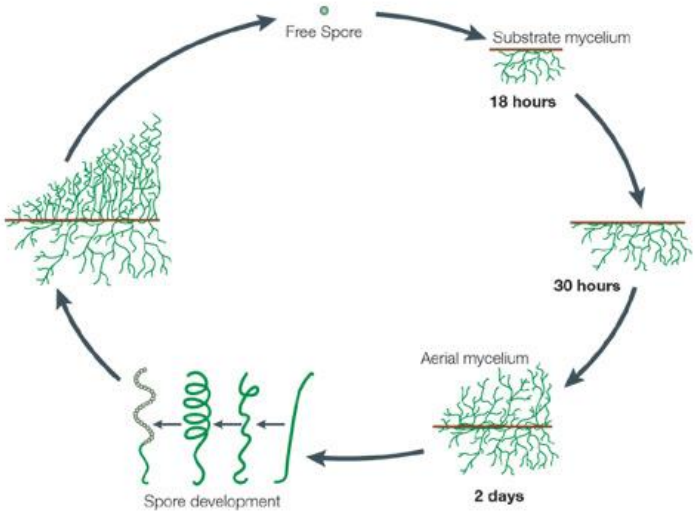


Figure 1.21 Life cycle of *Streptomyces coelicolor*
Diagram representing the stages of the *S. coelicolor* life cycle including the development of the substrate mycelium, aerial mycelium, spore development and dispersal. Image sourced from Angert, 2005.

In this life cycle, free spores germinate to form a substrate mycelium. These are networks of branching hyphae that grow by tip extension. When nutrients are depleted, these hyphae grow away from the substrate to reach nutrients. The hyphae can also grow upwards and outwards to form aerial mycelium. The unbranched cell at the tip of the aerial filament differentiates and eventually the aerial hyphae develop into chains of spores. Spores are dispersed into the environment and when settled in the soil, start to germinate, which restarts the life cycle (Angert, 2005).

1.9.2 *Streptomyces* S4

In this study, *Streptomyces* S4 was used, which has a symbiotic relationship with the leaf-cutting ants *Acromyrmex octospinosus*. These ants are highly evolved as they cultivate fungus in specialised chambers in their nests (Haeder *et al.* 2009), which they use as a food source. These ants bring leaves to

the nest and cut them into smaller pieces to feed to the fungus, which later becomes their main food source. The ants maintain the fungal gardens and need to protect the garden from microfungal weeds. They do this by removing waste and secreting antifungal compounds from metapleural glands (Haeder *et al.* 2009). There is now evidence that there is a symbiosis between these ants and bacterial strains that produce antifungal compounds. One example of this is the strain *Streptomyces* S4, which is known to produce antifungal agents (Haeder *et al.* 2009).

Streptomyces S4 contains biosynthetic gene clusters which lead to the synthesis of the antifungal compounds candicidin (Barke *et al.* 2010) and antimycin (Seipke *et al.* 2011). Candicidin is a polyene antifungal that is known to be highly effective against *Candida albicans.* Ergosterol is the main sterol in the cytoplasmic membrane of fungi. Candicidin binds to ergosterol, which affects the permeability and membrane integrity. This leads to a rapid efflux of potassium ions within the cell which causes death. Antimycins are a group of compounds that are toxic towards a range of pathogenic fungi including *Candida albicans*. Antimycins inhibit cytochrome c reductase, which is an enzyme in the mitochondrial electron transport chain. This disrupts the entire electron transport chain and inhibits cellular respiration which leads to cell death. Previous evidence has found that *Streptomyces* S4 produces both candicidin and antimycins but it is currently not known how they are released extracellularly.

1.9.3 AmBisome (amphotericin B liposome)

The AmBisome is a drug delivery system used to treat fungal infections. The AmBisome is a liposome which is an artificially made spherical vesicle formed of a phospholipid bilayer (Walker *et al.* 2018). The liposome is decorated with the antifungal agent amphotericin B (see Figure 1.22).

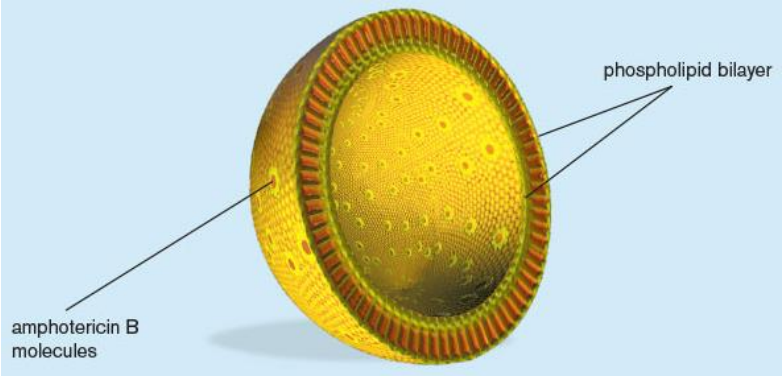


Figure 1.22 The structure of the AmBisome
The AmBisome is a liposome which is an artificially made spherical vesicle formed of a phospholipid bilayer. This liposome is decorated with the antifungal agent amphotericin B. Images sourced from AmBisome website 2018.

The liposomes in the AmBisome preferentially bind to the fungal cell wall. The active amphotericin B is released from the liposome and travels to the fungal cell membrane. Amphotericin B is a polyene antifungal agent which binds to ergosterol in the fungal cell membrane. The exact mechanism of transport of the amphotericin B from the AmBisome to the fungal cell is unknown. However, it is hypothesised that this occurs because amphotericin B has a higher binding affinity for ergosterol compared with cholesterol (the main lipid component in the AmBisome) (Stone *et al.* 2016).

Amphotericin B forms transmembrane channels within the fungal membrane which cause efflux of ions such as potassium, sodium, chloride and hydrogen from the cell. This loss of ions leads to cell death. Due to the known success of the delivery of Amphotericin B within a liposome to fungal cells, it led us to wonder if a similar process occurs in nature. This was tested by purifying membrane vesicles from *Streptomyces* S4 to see if any antifungal agents were present (Chapter 5).

1.10 Therapeutic applications of membrane vesicles

Interest in the MV field is growing and more methods are being developed to use bioengineered MVs for drug delivery and vaccines. MVs are often used by bacteria to secrete virulence factors so blocking MV production could be a target for preventing infection by these pathogens.

1.10.1 Using MVs for development of vaccines

MVs secreted by bacteria are naturally immunogenic and have the potential to be used as vaccines against a range of diseases. MVs also can be manipulated and engineered to display specific antigens needed for the vaccines against particular bacteria. An ideal vaccine should provoke a strong and specific immune response but in a way that is safe and has minimal adverse effects to the host. The immune response is triggered by pathogen-associated molecular patterns (PAMPs). PAMPs are unique and conserved motifs found in different pathogens and are recognised by the immune system as foreign. PAMPs are found on the surface of MVs and can activate the antigen presenting cells of the immune system. Another advantage of using MVs for vaccines is that they are non-replicating and cannot colonise the host or replicate and cause infection. They have also been shown to be thermostable and can withstand chemical treatment (Gerritzen *et al.* 2017). Antigens can be presented on the surface of MVs or within the MVs (luminal) when designing a vaccine. Surface exposed antigens activate antigen-specific B cells of the adaptive immune response. Antigens within the lumen of MVs can be detected by cytotoxic T cells (Gerritzen *et al.* 2017).

OMVs have been developed for use in the vaccination against the *Neisseria meningitidis* serogroup B, which causes bacterial meningitis. There are currently effective vaccines against the meningococcal serogroups A, C, W and Y, but there has been little success with group B. The meningococcal group B polysaccharide is very weakly immunogenic as the antigens have strong similarity to those expressed on human nerve tissues. This means that there is the potential to cause the production of autoantibodies which could be fatal (Hedari *et al.* 2014). This makes vaccines very difficult to produce and different strains can vary in their antigens. In 2013, OMVs were used to produce multicomponent OMV vaccines using antigens from various strains. The OMVs were engineered to express three recombinant proteins from the serogroup B strain and has been predicted to be effective against 78% of the *Neisseria meningitidis* serogroup B strains currently in Europe (Hedari *et al.* 2014). It is also safe to use and can be co-administered with other vaccines (Hedari *et al.* 2014). Findings from this project could be used in the development of vaccines against other pathogenic bacterial strains.

1.10.2 Using MVs for drug delivery

OMVs produced by bacteria can be manipulated to include certain proteins/antigens of interest for drug delivery and vaccines. OMVs produced by various bacterial strains are studied to determine their composition and cargo compared with that of the periplasm, outer membrane and whole cell. Although the exact mechanism is unclear, certain proteins and lipids appear to be selected and excluded from packaging into OMVs. Knowledge of this has allowed the targeting of proteins of interest to OMVs by fusion with proteins known to be incorporated into OMVs.

1.10.2.1 Using *E. coli* OMVs as recombinant protein delivery vehicles

In 2009, a foreign antigen FLAG was targeted to OMVs of an engineered *E. coli* O157:H7 strain. FLAG is a short peptide of 8 amino acids that is used for detection and purification of the recombinant proteins it is added to. It is hydrophilic and its short length allows it to be present on the surface of the protein of interest with minimal disruption to protein function and transport throughout the cell. In this study, the FLAG peptide was fused to the β-barrel domain of OmpA which resided in the OM by chromosomal tagging. The peptide was located to the periplasmic side of the OM and was more protected from extracellular protease degradation (Figure 1.23). The study was successful as the OmpA protein containing the FLAG peptide was successfully delivered to the OMVs (Kim *et al.* 2009). Studies such as these establish models for targeting antigens of interest to OMVs for either drug delivery or vaccines.

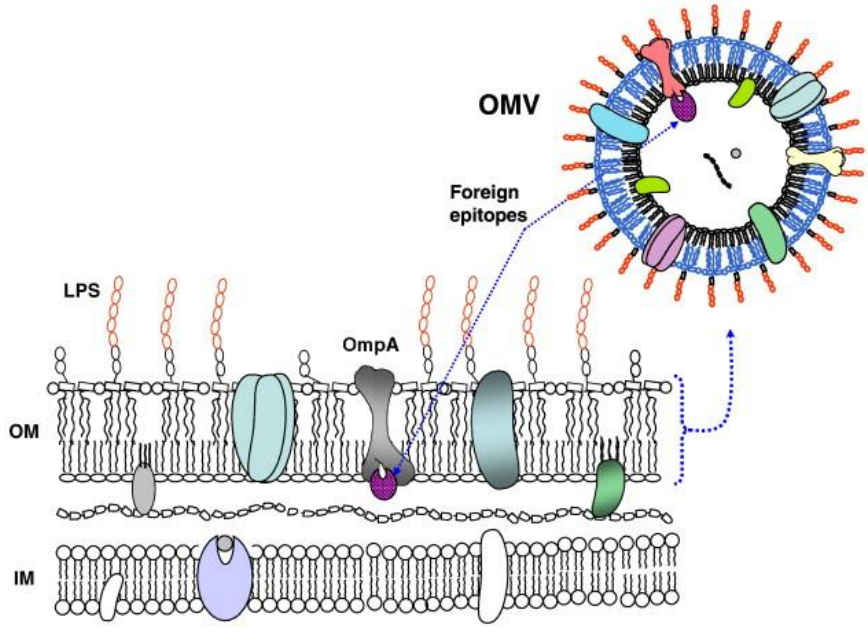


Figure 1.23 The incorporation of OmpA into *E. coli* OMVs
Image sourced from Kim *et al.* 2009. In this paper, a foreign epitope (FLAG) was targeted to *E. coli* OMVs by fusion with OmpA. The polypeptide tag (FLAG) is an artificial antigen commonly used in recombinant DNA and protein technology. FLAG was fused to the β-barrel domain of OmpA which resided in the OM by chromosomal tagging.

1.10.3 Effect of OMVs on the host immune response

1.10.3.1 OMVs can modulate the innate immune response

OMVs can stimulate a proinflammatory response from the host

The innate immune response is the first line of response to prevent infection. The innate immune response is activated when PAMPs are detected. Host cells display pattern recognition receptors (PRRs) that detect specific PAMPs. Recognition of PAMPs leads to activation of the host innate immune response, which includes inflammation, recruitment of phagocytes and the complement cascade. OMVs are formed from the Gram-negative cell envelope and therefore contain PAMPs that are recognised by the hosts' immune system. These include LPS, certain lipids, lipoproteins, virulence factors as well as OM and periplasmic proteins which are conserved in pathogens. PRRs are present on host cells of the innate immune system including dendritic cells and macrophages and so are activated by the presence of OMVs (Kuehn, Kesty. 2005). OMVs are often one of the main factors that initiate an inflammatory response in macrophages and host epithelial cells (Chatterjee, Chaudhuri. 2012).

Interaction of OMVs with the complement cascade

The complement system plays a key role in the immune response. The complement proteins circulate as inactive precursors that are activated in response to PAMPs. A cascade is produced where the binding of one protein causes the binding of the next protein and so on. The final product is a membrane attack complex, which causes cell lysis of bacterial cell membranes. In order to prevent this, OMVs from the Gram-negative bacteria *Morexella* contain the virulence factors $UspA_1$ and $UspA_2$, which bind to one of the essential proteins in the complement system called C3 to inactivate it. Without C3, the complement cascade is inhibited and no membrane attack complex is formed to lyse pathogens (Tan *et al.* 2007)).

In contrast to this, *Neisseria meningitidis* OMVs were found to cause high complement activation compared to LPS on *N. meningitidis* cell OM. This research indicated that the major complement activation with meningococcal septicaemia may be due to the immune response reacting to the OMVs produced by *N. meningitidis* rather than the cells themselves (Bjerre *et al.* 2002). The immune system reacting to the OMVs instead of the cells is beneficial as they can evade the immune system response more easily.

1.10.3.2 OMVs modulate the adaptive immune response

Detection of OMVs

The adaptive immune response is slower to respond to infection than the innate immune response. However, it is a specific response, which is unique to the pathogen. The adaptive immune response is carried out by lymphocytes including B and T cells. B cells are produced in the bone marrow and generate antibodies, which are specific to the pathogen detected. This opsonises the bacterial cells for phagocytosis. T cells can be divided into CD4+ (known as helper T cells) and CD8+ (known as cytotoxic T cells). CD4+ T cells induce other cells of the immune response including B cells and CD8+ T cells to proliferate and respond to the pathogen. CD8+ T cells specifically kill virally infected cells in

the host. The adaptive immune response can react to OMVs in the same way as it would to a pathogen as they contain many of the same PAMPs.

OMVs can contain superantigens

Neisseria lactamica are commensal Gram-negative bacteria that colonise the nasopharynx area. *N. lactamica* have been found to produce OMVs that contain a mitogen on their surface. Mitogens stimulate mitosis and the mitogen associated with *N. lactamica* OMVs was found to activate B cell proliferation, independent of T cells. This mitogen/antigen found on *N. lactamica* OMVs was found to have properties of a superantigen which results in excessive activation of the immune system (Vaughan *et al.* 2010). Superantigens cause activation of high frequencies of T cells or B cells, which are not specific for particular antigens. In this case, naive B cells are stimulated to produce polyclonal antibodies, which have low affinity for removing the OMV-producing pathogen. This leads to a non-specific immune response and allows the OMV-producing bacteria to evade specific adaptive immune responses (Vaughan *et al.* 2010).

1.10.3.3 Example of using of *E. coli* OMVs for cancer therapy

In 2014, OMVs were used in the cell-specific delivery of drugs. A mutant strain of *E. coli* was engineered to produce OMVs which were later loaded with a small interfering RNA (siRNA) by electroporation. The siRNA chosen was known to target kinesin spindle protein mRNA. Kinesin spindle protein plays a critical role in mitosis and its inhibition leads to cell cycle arrest at mitosis then cell death. The OMVs were targeted to cancerous cells using a HER2-specific affibody in the membrane. HER2 is a transmembrane receptor, which is overexpressed in certain cancers such as breast, ovarian and gastric carcinomas (Gujrati *et al.* 2014). HER2 is therefore used as a target for cancer therapies. In order to target the OMVs to the cancerous cells, a genetic fusion was made between the ClyA monomer (known to be packaged into OMVs) and an anti-HER2 affibody. OMVs produced from this strain displayed the HER2 ligand on their surfaces to target them to cancer cells where the siRNA could be delivered. The study appeared to be successful as the OMVs caused cytotoxic effects against cancerous cells that overexpressed HER2.

The *E. coli* OMVs were engineered to have low immunogenicity and minimal endotoxicity to human cells. In order to do this, the *E. coli* K-12 strain used (W3110) had a *msbB* mutation. This mutation causes the cell to produce defective, penta-acylated LPS and reduces the toxicity towards host cells compared to the usual hexa-acylated LPS. These findings agreed with a previous study that reported that hexa-acylated LPS stimulates the production of TNFα from THP1 cells more than penta-acylated LPS (Hajjar, *et al.* 2002). It is also thought that the expression of the HER2 affibody on the surface of the OMVs may have prevented the innate immune response by shielding the OMV PAMPs.

The results of this study were promising as the OMVs appeared to successfully target tumour tissue (due to the HER2 affibody) and release the siRNA. Once released, the siRNA induced cytotoxic effects and inhibited tumour growth. The investigation found that the engineered OMVs were well tolerated by

the host with only very weak immunogenic responses (Gujrati *et al.* 2014). The use of attenuated bacterial strains with minimal virulence factors and toxicity could be used in the future to produce therapeutic OMVs for use in vaccines or drug delivery.

1.11 Project aims and unanswered questions

One of the main project aims was to compare the cargo identified within membrane vesicles from a range of bacterial strains and to speculate on the function of the molecules packaged (ie. are the secretion of these molecules beneficial to the OMV-producing cell?). Another essential aim of the project, was to bioengineer bacterial cells to produce OMVs containing a target protein of interest. This could ultimately be beneficial for therapeutic applications, such as cell-specific drug delivery or development of vaccines.

1.11.1 What are the best methods to purify and characterise OMVs?

The first aim (addressed in Chapter 3) was to develop a cost-effective method to purify OMVs reproducibly, giving the best yield possible. There is no universal protocol for OMV purification in the literature (Klimentová *et al.* 2015) so this needed to be developed. The isolated OMVs should be intact with as few contaminants from the bacterial cell as possible. OMV characterisation methods were sourced and developed from previous studies in the literature (McCaig *et al.* 2013, Klimentová *et al.* 2015). This included using electron microscopy, SDS-PAGE, Western blotting, mass spectrometry, protein quantification and dynamic light scattering.

1.11.2 What are the differences in (O)MV composition, cargo and function from both Gram-negative and Gram-positive bacterial strains?

Membrane vesicles were studied in the Gram-negative bacteria *E. coli* and *P. aeruginosa* and the Gram-positive organism *Streptomyces* S4.

1.11.2.1 Using *E. coli* to study OMV biogenesis

One of the ultimate project aims was to manipulate a bacterial strain of interest into producing OMVs with specific cargo for therapeutic use. The *E. coli* genome has been sequenced and annotated across a broad range of strains and the information is widely available (for example, the EcoCyc database). *E. coli* K-12 strains and *E. coli* B strains are very commonly used as a model organism in the scientific community. This made *E. coli* a good candidate for bioengineering to enable targeted expression of recombinant proteins for delivery and inclusion in OMVs and there are also previous studies that have been successful (Gujrati *et al.* 2014, Kim *et al.* 2009). Additionally, the study may give insight into which molecules pathogenic *E. coli* package into OMVs, which could potentially have clinical relevance.

1.11.2.2 *P. aeruginosa*

P. aeruginosa can form biofilms in the lung of CF patients and are known to communicate during the formation of biofilms by quorum sensing (Eberl *et al.* 2004). *P. aeruginosa* strains are known to

produce OMVs which have been found to contain virulence factors such as toxins and β-lactamases. OMVs from *P. aeruginosa* were studied as a Gram-negative bacterial comparison for *E. coli* OMVs with an aim to compare and contrast OMV cargo in another pathogenic and clinically relevant strain.

1.11.2.3 *Streptomyces* S4

At the outset of the project, there had been reports that Gram-positive bacteria produced extracellular membrane vesicles (MVs). This was originally shown in *Mycobacterium ulcerans* (Marsollier *et al.* 2007) and summarised by Brown *et al.* 2015, Kim *et al.* 2015 and Liu *et al.* 2018. MV production had also been documented in *Streptomyces coelicolor* (Schrempf *et al.* 2011) and *Streptomyces lividans* (Schrempf & Merling 2015). In both publications it was shown that the *Streptomyces* species produced visible exudates that were enriched in MVs and contained a range of proteins, lipids and bioactive molecules such as actinorhodin (a benzoisochromanequinone dimer polyketide antibiotic produced by *S. coelicolor*) and undecylprodigiosin (an alkaloid produced by *S. lividans*).

In this study, *Streptomyces* S4 was used which has a symbiotic relationship with the leaf-cutting ants *Acromyrmex octospinosus*. These attine ants are highly evolved as they cultivate fungus in specialised chambers in their nests (Haeder *et al.* 2009) which they use as a food source. *Streptomyces* S4 contains biosynthetic gene clusters which leads to the synthesis of the antifungal compounds candicidin (Barke *et al.* 2010) and antimycin (Seipke *et al.* 2011). Unlike both *S. lividans* and *S. coelicolor*, S4 does not produce highly coloured and visible exudates but it was known that candicidin and antimycin were secreted extracellularly and produce a visible zone of inhibition when plated with *C. albicans* (Barke *et al.* 2010). We reasoned that the antifungals may be packaged into MVs to facilitate their diffusivity and targeting. This was particularly applicable to candicidin which is a complex and highly insoluble molecule. *Streptomyces* S4 mutants were also available which were unable to produce antimycin (*ΔantC*), candicidin (*ΔfscC*) and a double mutant which was unable to produce antimycin or candicidin (*ΔantC ΔfscC*). This enabled us to investigate whether the biosynthesis of either candicidin or antimycin was specifically linked to MV biogenesis in *Streptomyces* S4.

1.11.3 Can we target a protein of interest to be incorporated into bacterial MVs?

During these studies, *E. coli* K-12 clearly demonstrated an enrichment of FimA into OMVs. For this reason, fusion of GFP and mNeon green to FimA were chosen to trial targeting a chosen protein to the OMVs. However, at the outset of the project, it was not clear whether fusion of a protein to FimA alone was sufficient to facilitate correct targeting. We trialled this with FimA monomer protein which was tagged with either GFP (chromosomal FimA-GFP fusion) or Neon Green (exogenously expressed FimA-mNeon Green fusion using a plasmid) to see which (if any) were successful.

Chapter 2: Materials and Methods

2.1 Materials

Table 2.1 General reagent list

Chemical name	Supplier	Product code
Acetic acid	Fisher Scientifc	A0360
Acetone	Fisher Scientifc	A0560
Agar technical No3	Oxoid	LP0013
Ammonium sulphate	Acros Organics	205870010
Bacto peptone	BD Chemicals	211677
Bacto tryptone	BD Chemicals	211705
Bacto yeast extract	BD Chemicals	212750
Bovine serum albumin	Sigma-Aldrich	A2153
Butanol	Fisher Scientifc	B4850
Calcium chloride, anhydrous	Sigma-Aldrich	C1016
Casein enzymic hydrolate	Sigma-Aldrich	22090
Chloroform	Fisher Scientifc	C4960
D-glucose anhydrous	Fisher Scientifc	G/0500/61
D-Mannitol	Sigma-Aldrich	M9546
Ethanol (absolute, 99.8+%)	Fisher Scientifc	E0650
Glucose	Fisher Scientifc	G05002
Glycerol	Fisher Scientifc	G/0600/17
Glycine	Fisher Scientifc	G/0800/60
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	Melford	B2001
Hydrochloric acid (HCl)	Fisher Scientifc	H1150
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Melford	MB1008
Malt extract	Oxoid	LP0039
Methanol	Fisher Scientifc	M4000
OptiPrep (density gradient medium)	Sigma-Aldrich	D1556
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich	78830
Phosphate buffered saline	Oxoid	BR0014G
Protein assay dye reagent concentrate (Bradford assay)	Bio-rad	5000006
Sodium bicarbonate	Sigma-Aldrich	S5761
Sodium chloride	Fisher	S/3160/60
Sodium dodecyl sulphate (SDS)	Melford	B2008
Sucrose	Fisher	S/8600/60
Trichloroacetic acid (TCA)	Sigma-Aldrich	T4885
Tris (hydroxymethyl methylamine)	Fisher	T/P630/60
Tryptone soy broth	Sigma-Aldrich	22092
Tween 20	Sigma-Aldrich	P9416

Table 2.2 Enzymes, substrates and inhibitors

Enzymes and Substrates	Supplier	Product code
4-Nitrophenyl acetate	Sigma-Aldrich	N8130
Mini Protease Inhibitor Cocktail tablets	Sigma-Aldrich	11836153001 ROCHE
Protease from *Streptomyces griseus*	Sigma-Aldrich	P5147
Proteinase K	Sigma-Aldrich	P6556
Trypsin	Sigma-Aldrich	59430C-100ML

Table 2.3 SDS-PAGE reagents

SDS-PAGE	Supplier	Product code
InstantBlue Protein Stain	Expedeon	ISB1L
NuPAGE 4-12% Bis-Tris Protein Gels, 1.0 mm, 10-well	Thermo-Fisher	NP0321BOX
NuPAGE MOPS SDS Running Buffer 20x	Thermo-Fisher	NP0001
NuPAGEMES SDS Running Buffer 20x	Thermo-Fisher	NP0002
Pierce Silver Stain Kit	Thermo-Fisher	24612
Precision Plus Protein Dual Color Standards	Bio-rad	1610374
Reducing sample buffer (4x)	Invitrogen	NP0008

Table 2.4 Wet transfer/Western blotting reagents

Wet transfer/Western blotting	Supplier	Product code
BCIP/NBT tablets	Sigma	B5655
Milk (Instant Dried Skimmed)	Tesco	N/A
PVDF membrane 0.2 µm pore	Roche	3010040001
PVDF membrane 0.45 µm pore	Thermo-Fisher	88518

Table 2.5 Antibodies used for Western blotting and immunogold labelling

AntibodiesG10C8B2: GB2:G10	Immunogen (molecular weight, kDa)	Supplier and Catalogue number	Clonality	Host	Antibody Specificty
Anti-FimA monoclonal	FimA (18 kDa)	Professor Hultgren, Washington University in St. Louis	Polyclonal	Rabbit	"Gel slice antibodies were made by treating rabbits with material from gel slices containing FimA (from SDS PAGE of UTI89 type 1 pilus preps)"
Anti-FimA polymer	Polymerised FimA found in wells	Professor Hultgren, Washington University in St. Louis	Polyclonal	Rabbit	"Whole pili antibodies were made by treating rabbits with non-denatured UTI89 type 1 pilus preps"
Anti-Flagellin	Flagellin (51 kDa)	Abcam #ab93713	Polyclonal	Rabbit	This antibody is specific for bacterial Flagellin (FliC). Reacts with Flagellin from: *E. coli, Salmonella anatum, Salmonella selandia*
Anti-GFP antibody	GFP (27 kDa)	Professor Gullick, UKC	Monoclonal	Mouse	GFP
Anti-mouse secondary antibody (Alkaline-phosphatase conjugated)	N/A	Promega #S372B	Polyclonal	Goat	Heavy and light chains for all IgG subclasses. Immunoaffinity-purified using immobilized antigens and conjugated to alkaline phosphatase (AP) enzyme
Anti-Neon green antibody	mNeon green (34 kDa)	Chromotek #32F6	Monoclonal	Mouse	The antibody recognizes mNeonGreen at the N-terminus, C-terminus, or internal site of the fusion protein
Anti-OmpA antibody	Native *E.coli* OmpA (37 kDa)	Antibody research corporation #111120	Polyclonal	Rabbit	*E.coli* and other Gram-negative bacteria
Anti-rabbit secondary antibody (Alkaline-phosphatase conjugated)	N/A	Sigma #A3687	Polyclonal	Goat	Purified rabbit IgG as the immunogen

Table 2.6 Reagents for TEM

Electron Microscopy	Supplier	Product code
Gelatin capsules	Agar Scientific	G29208
Glutaraldehyde fixative	Agar Scientific	AGR1011
Immunogold conjugate 10 nm particle size	BBI solutions	EM.GMHL.10
Immunogold conjugate 15 nm particle size	BBI solutions	EM.GAR15
LR White Medium Grade Resin	Agar Scientific	AGR1281
Paraformaldehyde	Agar Scientific	R1018
Uranyl acetate	Agar Scientific	AGR1260A

Table 2.7 Reagents for Confocal Microscopy

Confocal Microscopy	Supplier	Product code
ProLong Gold antifade mountant	Life Technologies	P36930
Lectin from *Triticum vulgaris* (wheat) FITC conjugate	Sigma-Aldrich	L4895

Table 2.8 Reagents for Mass Spectrometry

Mass Spectrometry	Supplier	Product code
4-HCCA matrix solution	Aldrich	14,550-5
Acetonitrile (ULC/MS - CC/SFC)	Biosolve	12041
Ammonium bicarbonate (LCMS grade)	Fluka	40867
DTT	Melford	MB1015
Formic acid (LC/MS grade)	Fisher Scientific	A117-50
Iodoacetamide	Sigma-Aldrich	I6125
Peptide Calibration Standard II	Bruker	8222570
Trypsin (Sequencing Grade Modified Trypsin)	Promega	V511A
Water (HPLC grade)	Fisher	10449380

Table 2.9 Antibiotics/Antifungals used

Antibiotics/Antifungals	Supplier	Product code
Antimycin	Sigma	A8674
Candicidin	Bioaustralis	BIA-C1564
Chloramphenicol	Sigma	C0378

Table 2.10 Reagents used for cloning techniques

Cloning materials	Supplier	Product code
1 KB DNA ladder	Promega	G5711
10x Ligase buffer	Promega	C1268
10x NEB buffer Cutsmart	New England BioLabs	B7204S
10x Promega enzyme buffer	Promega	R9991
10x T4 Polynucleotide Kinase Reaction Buffer	New England BioLabs	B0201S
2x PCRBIO Taq Mix Red	PCR Biosystems	PB10.13
6x loading dye	New England BioLabs	B70245
AscI restriction enzyme	New England Biolabs	R0558S
EcoRI restriction enzyme	New England BioLabs	R0101S
Ethidium bromide	Sigma	E7637
NdeI restriction enzyme	New England BioLabs	R0111S
PstI restriction enzyme	Promega	R611A
Q5® High-Fidelity 2X Master Mix	New England BioLabs	M0492S
T4 DNA ligase	Promega	M180A
T4 Polynucleotide Kinase	New England BioLabs	M0201S

2.2 Equipment list

2.2.1 Autoclave:

Classic Prestige Medical autoclave sterilises by heating contents at 121ºC for 11 mins (1.4 bar pressure).

2.2.2 Vacuum pump

The vacuum pump was used for sterilisation of 150 mL volumes and above.

Table 2.11 Comparison of the different filters used for sterilisation

Pore size (µm)	Filter brand	Membrane type	Volume (mL)	Use
0.2	Nalgene Rapid-Flow	SFCA	150	Sterilise buffers/solutions
0.2	Nalgene Rapid-Flow	PES	500	OMV purification
0.45	Nalgene Rapid-Flow	PES	500	OMV purification

2.2.3 Thermo Scientific Barnstead Easypure II system

Double deionised water was filtered through a Barnstead D3750 irradiated hollow fibre filter (pore size 0.2 µm, resistance 18.2 MΩ.cm). This is referred to as 'MQ water' throughout the thesis.

Table 2.12 Centrifuges used throughout this project

Centrifuge used	Rotor used	Centrifuge tubes/Sample size
Eppendorf Centrifuge 5415 R	F45-24-11	1.5 mL microcentrifuge tubes
Beckman Coulter Optima LE-80K Ultracentrifuge	Type 70 Ti	26.3 mL polycarbonate ultracentrifuge tube
Beckman Coulter TL-100 Ultracentrifuge	TLA-100.3	750 µL sample volume in 3 mL ultracentrifuge tube
Beckman Coulter. Avanti J-265 centrifuge	JLA-16.250	250 mL polycarbonate centrifuge tube
Sigma 2K15 centrifuge	#12149	15 mL or 50 mL Falcon tubes
Thermo Scientific Savant SPD111V230	N/A (fixed rotor)	1.5 mL microcentrifuge tubes

2.2.4 Spectrophotometers

- **UV-Vis spectrophotometer:** Shimadzu UV-1800 spectrophotometer was used to measure the optical density of cultures (at 600 nm) and protein concentration by Bradford assay (at 595 nm). Plastic 1 mL cuvettes were used with a 1cm path length.
- **Cary Spectrophotometer:** The spectrophotometer Agilent Technologies Cary 60 UV-Vis was used to draw UV-Vis spectrum to detect candicidin and antimycin. 500 µL samples were loaded on to the spectrophotometer in UV quartz cuvettes (Sigma-Aldrich Z276723-1EA).
- **NanoDrop Spectrophotometer:** DNA and protein concentration were quantified using the NanoPhotometer 50 (Implen).

2.2.5 SDS-PAGE, protein transfer and Western blotting

- **SDS-PAGE tank:** XCell SureLock Mini-Cell Electrophoresis System
- **Western blot wet transfer tank:** Mini Trans-Blot (Bio-Rad).

- **Agarose gel preparation and tank:** Agarose gels were prepared using the multiSUB Midi electrophoresis unit, 10 x 10 cm UV Tray, 2 x 16 sample combs, loading guides and dams. Gels were run on the Fisherbrand multiSUB Midi Horizontal Gel System.
- **SDS-PAGE gel and Western blot photos:** Photos were taken using the G:Box Chemi XX6 machine by SynGene and associated software.

2.2.6 Mass Spectrometry

Proteins of interest on silver stained gels were identified using Bruker ultrafleXtreme MALDI-TOF/TOF mass spectrometer and associated software.

2.2.7 Dynamic Light Scattering (DLS)

The Litesizer 500 Anton Paar was used to characterise the OMVs using the DLS mode.

2.2.8 Sonication

Sonication was performed using the Soniprep 150 (MSE).

2.2.9 Microplate reader

Growth curves were produced by culturing *E. coli* in CELLSTAR 48 Well Cell Culture Plates (Greiner Bio-One, #677 180). Two identical microplate readers were used to generate growth curves at 25ºC and 37ºC concurrently. The microplate readers used were SPECTROstar Nano (BMG Labtech) with associated SPECTROstar Nano software.

2.2.10 Microscopy

- **Electron microscopy:** Jeol transmission electron microscope model JEM 1230. Photos taken using a Gatan multiscan digital camera and operated at an accelerating voltage of 80 kV.
- **Confocal microscopy:** Zeiss lsm 880 with airscan with associated Zen Black software.
- **Light microscopy:** GXM L2800 Premium Compound Microscope at 400x magnification and photos were taken using the associated camera.

2.2.11 Imaging of agar plates

Photos of plates taken using aCOLyte (Synbiosis).

2.2.12 Cloning

- **UV transilluminator:** BioView UV transilluminator was used to locate DNA bands for gel extraction.
- **PCR machine:** PCR reactions were performed in Veriti 96 Well Thermocycler (Applied Biosystems).

2.3 Media and buffers

All media and buffers were stored at 4ºC unless otherwise stated.

2.3.1 MQ water

In all solutions prepared for experiments, double deionised water was used. This was filtered further using Thermo Scientific Barnstead Easypure II system.

2.3.2 Media and buffer sterilisation

All media and PBS buffers were autoclaved or filter sterilised using filters of 0.2 µm pore size (Cole-Parmer). For volumes over 100 mL, Nalgene filters with a 0.2 µm pore size and a vacuum pump were used.

2.3.3 Media

All media described below were prepared by addition of components listed to the desired volume of MQ water, mixed well then autoclaved. Agar technical No 3 was added (20 g/L) to media before autoclaving for preparation of agar plates.

Table 2.13 Media recipes

Media name	Media recipe and preparation details
Lysogeny broth (LB)-Lennox	10 g/L Bacto tryptone, 5 g/L Bacto yeast extract, 5 g/L sodium chloride
Yeast Extract Peptone Dextrose (YPD)	20 g/L glucose, 20 g/L Bacto peptone, 10 g/L Bacto yeast extract
Tryptone Soy Broth (TSB)	30 g/L TSB pre-made powder
Yeast Extract-Malt Extract Medium (YEME)	3 g/L Bacto yeast extract, 3 g/L Malt extract, 5 g/L Bacto peptone, 10 g/L glucose
Mannitol Soya (MS) Agar plates	20 g/L soya flour (Holland and Barrett), 20 g/L mannitol, 20 g agar. Media was autoclaved twice before pouring to ensure there were no spores
Milk (casein) agar plates	28 g/L skimmed milk powder, 5 g/L casein enzymic hydrolysate, 2.5 g/L yeast extract, dextrose 1 g/L dextrose, 15 g/L agar, final pH at 25ºC 7.0. Heat to boiling to dissolve then sterilise by autoclaving. Mix well after autoclaving and pour.

Table 2.14 Media supplements

Supplement	Supplier & Catalogue number	Stock concentration	Sterilisation	Working concentration
Chloramphenicol	Sigma #C0378	25 mg/mL in ethanol, absolute	N/A	25 µg/mL
IPTG	Melford #MB1008	1 M in sterile MQ water	Filter sterilised (0.2 µm pore size)	0.5 mM

Media supplement stocks were stored at -20ºC. Chloramphenicol stock was mixed into ‘hand hot’ LB agar then plates were poured and left to set for 20-30 mins. Agar plates were left to dry next to a Bunsen burner for a minimum of 30 mins and stored at 4ºC.

2.3.4 Buffer and Solution preparations

Table 2.15 Buffer and solution recipes

Buffer category	Buffer name	Buffer preparation details
General use buffers	HEPES buffer	10 mM HEPES/0.85% NaCl, adjusted to pH 7.4 and filter sterilised
	HEPES buffer with $CaCl_2$	10 mM HEPES/0.85% NaCl/20 mM $CaCl_2$, adjusted to pH 7.4 and filter sterilised
	Phosphate buffered saline (PBS)	1 tablet dissolved in 100 mL MQ water and autoclaved
SDS-PAGE, protein transfer and Western blotting	100% TCA stock	A 100% stock solution of TCA was prepared by adding 227 mL sterile MQ water to 500 g powder as described by the Trichloroacetic acid SigmaUltra datasheet (T9159)
	Western transfer buffer	25 mM Tris and 192 mM glycine (no pH adjustment)
	TBS	137 mM sodium chloride, 10 mM Tris and 0.1% Tween 20 pH 7.4.
	TBST	137 mM sodium chloride, 10 mM Tris and 0.1% Tween 20 pH 7.4.
TEM	TBST for immunogold labelling	20 mM Tris, 500 mM NaCl, 0.1% BSA, 0.05% Tween 20, pH 7.2. Made fresh on the day
Proteinase K test	PMSF stocks	200 mM stocks of PMSF in absolute ethanol were prepared and stored at -80ºC. Immediately prior to use in a Proteinase K test, stocks were diluted to 50 mM using HEPES buffer
	SDS stocks	Various concentrations of SDS were prepared in MQ water
Outer membrane and periplasmic protein purification	Tris-sucrose-EDTA (TSE buffer)	200 mM Tris-HCl, 500 mM sucrose, 1 mM EDTA, pH 8.0. Add protease inhibitor cocktail tablet immediately before use and store at 4ºC
Cloning reagents (prepared by Dr Alex Moores)	T salts	75 mM $CaCl_2$, 6 mM $MgCl_2$ and 15% glycerol in MQ water (filter sterilised)
	Magnesium chloride	0.1 M $MgCl_2$, 15% glycerol in MQ water (filter sterilised)
	Annealing buffer for Oligos	0.1 M Tris, 10 mM EDTA, 0.5 M $NaCl_2$ in MQ water, pH 7.6-7.8
Agarose gel	50x Tris-Acetate-EDTA buffer (TAE) buffer	1 M Tris, 50 mM EDTA, 28.6 mL glacial acetic acid, pH 8.27

2.3.4.1 Preparation of 4% (w/v) formaldehyde fix for EM

To prepare 4% (w/v) formaldehyde in PBS, 8% (w/v) paraformaldehyde was prepared then mixed 1:1 with 2X concentrated PBS. 2 g paraformaldehyde was dissolved in 20 mL water total in a glass beaker. The mixture was heated to 65-70ºC with continuous stirring. When condensation had formed on the beaker, 2-3 drops of 1 M sodium hydroxide in water was added until the solution changed from white and cloudy to clear. Once dissolved, the solution was left to cool to room temperature. MQ water was added to make the total volume 25 mL then 25 mL 2X PBS was added to make a final solution of 4% (w/v) formaldehyde in 1X PBS. Fix was stored at 4ºC.

2.4 Microbial strains

Table 2.16 Microbial strains used in this study

Strain name	Strain characteristics and additional information	Reference/Source for further information
Burkholderia cenocepacia J2315	Pathogenic strain originally isolated from a cystic fibrosis patient	Dr. Vittorio Venturi, International Centre for Genetic Engineering (Holden *et al.* 2009)
Candida albicans SC5314	Commonly used *Candida albicans* wild type strain from which most laboratory strains are derived (pathogenic)	Dr Luisa Sordi, University of Kent (Candida Genome Database)
Clinical isolate 1 (*E.coli* MS207)	Bacteraemia isolate belonging to clonal group ST685	Strain originates from East Kent Hospitals University NHS Foundation
Clinical isolate 2 (*E.coli* MS10)	EEC958: Uropathogenic strain (UPEC) strain belonging to clonal group ST131	Dr Mark Shepherd, University of Kent (Totsika *et al.* 2011)
Clinical isolate 3 (*E.coli* MS1)	CFT073: Uropathogenic strain (UPEC) belonging to clonal group ST73	Dr Mark Shepherd, University of Kent (Welch *et al.* 2002
Clinical isolate 4 (*E.coli* MS343)	83972: Asymptomatic bacteriuria strain	Dr Mark Shepherd, University of Kent. (Klemm *et al* . 2006)
Clinical isolate 5 (*E.coli* MS190)	Bacteraemia isolate belonging to clonal group ST162	Strain originates from East Kent Hospitals University NHS Foundation
Clinical isolate 6 (*E.coli* MS234)	Bacteraemia isolate belonging to clonal group ST69	Strain originates from East Kent Hospitals University NHS Foundation
E. coli B, wildtype	Wild type (Parental) strain of *E. coli* B strains such as BL21	Keio collection #2507 (Baba *et al.* 2006)
E.coli Δ*fimA* (JW4277-1)	BW25113 *ΔfimA782::kan*	Keio collection #11065 (Baba *et al.* 2006)
E.coli Δ*fimB (*JW4275-1)	BW25113 *ΔfimB780::kan*	Keio collection #11063 (Baba *et al.* 2006)
E.coli Δ*fimC* (JW4279-1)	BW25113 *ΔfimC784::kan*	Keio collection #11066 (Baba *et al.* 2006)
E.coli Δ*fimD (*JW5780-1)	BW25113 *ΔfimD785::kan*	Keio collection #11607 (Baba *et al* . 2006)
E.coli Δ*fimE (*JW4276-1)	BW25113 *ΔfimE781::kan*	Keio collection #11064 (Baba *et al.* 2006)
E.coli Δ*fimF (*JW4281-1)	BW25113 *ΔfimF786::kan*	Keio collection #11067 (Baba *et al.* 2006)
E.coli Δ*fimG* (JW4282-2)	BW25113 *ΔfimG787::kan*	Keio collection #11770 (Baba *et al.* 2006)
E.coli Δ*fimH* (JW4283-3)	BW25113 *ΔfimH788::kan*	Keio collection #11068 (Baba *et al.* 2006)
E.coli Δ*fimI* (JW5779-1)	BW25113 *ΔfimI783::kan*	Keio collection #11573 (Baba *et al.* 2006)
E.coli Δ*fimZ* (JW5073-1)	BW25113 *ΔfimZ745::kan*	Keio collection #11159 (Baba *et al* . 2006)
E.coli Δ*fliC (*JW1908-1)	BW25113 *ΔfliC769::kan*	Keio collection #9586 (Baba *et al.* 2006)
E.coli AAEC278	*E.coli* MG1655 Δ*(fimBE)-sacB Neo* . Allelic exchange intermediate strain produced in the construction of '*E.coli* with fimbriae locked on' strain. In this strain, the invertible element (*fimS*) is in the 'on' position to produce T1F. The *fim* fragment has been replaced by the *sacB* Neo gene cassette	Dr. Ian Blomfield, University of Kent, #AAEC278 (McClain et al. 1993)
E.coli BL21	*E.coli* B strain derivative. *fhuA2 [lon] ompT gal [dcm] ΔhsdS.* Competent *E.coli* B strain for routine non-T7 expression	New England BioLabs #C2530H
E.coli BL21 (DE3)	*E.coli* B strain derivative. *fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS.* ***λ DE3*** *= λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5* Identical to *E.coli* BL21 except this strain contains the λDE3 lysogen that carries the gene for T7 RNA polymerase under control of the lacUV5 promoter	New England BioLabs #C25271
E.coli DH5α	NEB® 5-alpha Competent *E. coli* (High Efficiency). Derivative of DH5α cells used for routine non-T7 expression	New England BioLabs #C2987I

Strain name	Strain characteristics and additional information	Reference/Source for further information
E.coli FimB-LacZ fusion	*E.coli* WT MG1655 Δ*lacZYA* Δ*fimB* (-457 to +209 relative to fimB ORF) FimB-LacZ translational fusion. This strain contains a deletion of the Lac operon (*lacZYA*) and *fimB* and an insertion of a FimB-LacZ fusion protein. T1F production locked off is FimB is no longer functional.	Dr. Ian Blomfield, University of Kent, #BGEC056 (El-Labany *et al.* 2003)
E.coli fimbriae locked on	*E.coli* MG1655 *fimB-am6 fimE-aml8* (produced from intermediate strain AAEC278). T1F production locked on. The invertible element (*fimS*) is locked on due to the modifications in the parental intermediate strain (AAEC278)	Dr. Ian Blomfield, University of Kent, #AAEC356 (McClain *et al.* 1993)
E.coli parental (BW25113)	*E. coli* K-12 strain derivative. Contains six mutations: *Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, Δ(rhaD-rhaB)568, hsdR514.* The *E. coli* K-12 BW25113 parent strain is the common background genotype used for the generation of the *E. coli* Keio Knockout Collection	Keio collection #7636 (Baba *et al.* 2006)
E.coli WT MG1655	*E.coli* K-12 derivative. Contains 2 mutations: *λ-, rph-1* . Used as the wild-type strain to produce *E.coli* FimB-LacZ fusion strain and *E.coli* with fimbriae locked on	Keio collection #6300 (Baba *et al.* 2006)
E.coli ΔflhA (JW1868-1)	BW25113 *ΔflhA732::kan*	Keio collection # 9554 (Baba *et al.* 2006)
E.coli ΔfliD (JW1909-1)	BW25113 *ΔfliD770::kan*	Keio collection #9587 (Baba *et al.* 2006)
E.coli ΔfliS (JW1910-1)	BW25113 *ΔfliS771::kan*	Keio collection #9588 (Baba *et al.* 2006)
E.coli Δlon (JW0429-1)	BW25113 *Δlon-725::kan*	Keio collection #8592 (Baba *et al.* 2006)
*E.coli ΔlrhA (*JW2284-6)	BW25113 *ΔlrhA771::kan*	*Keio collection #11785* (Baba *et al.* 2006)
E.coli ΔompT (JW0554-1)	BW25113 *ΔompT774::kan*	Keio collection #8680 (Baba *et al.* 2006)
MG1655 with GFP-FimA protein fusion	ASC129 (MG1655 *fimAΩGFPmut2*)	Professor Sander Tans, AMOLF, The Netherlands (Tans *et al.* 2009)
Pseudomonas aeurginosa PA01	Clinical isolate. Two *P. aeruginosa* pathogenicity islands (PAPI-1 and PAPI-2) are absent from *PA01* which means it is less virulent than *PA14.*	Dr Luisa De Sordi, University of Kent (He *et al.* 2004)
Pseudomonas aeurginosa PA14	Clinical isolate. Two *P. aeruginosa* pathogenicity islands (PAPI-1 and PAPI-2) are present in the genome of *PA14* which is a highly virulent clinical isolate	Dr Luisa De Sordi, University of Kent (He et al. 2004)
Saccharomyces cerevisiae BY4741	Commonly used laboratory strain derived from *S. cerevisiae* S288C	EuroSCARF #Y00000
Streptomyces S4 Δ*fscC*	S4 *fscC* null mutant: this strain does not produce Candicidin	Professor Matt Hutchings UEA (Seipke *et al.* 2011)
Streptomyces S4 Δ*antC*	S4 *antC* disruption mutant: this strain does not produce Antimycin	Professor Matt Hutchings UEA (Seipke *et al.* 2011)
Streptomyces S4 Δ*antC* Δ*fscC*	S4 strain containing knockouts of both *fscC* and *antC* genes meaning that there is no production of Candicidin or Antimycin	Professor Matt Hutchings UEA (Seipke *et al.* 2011)
Streptomyces S4 WT	Wild type *Streptomyces* S4 strain: originally isolated from attine ant nests	Professor Matt Hutchings UEA (Seipke *et al.* 2011)

2.5 Microbial growth and storage conditions

2.5.1 Preparation of bacterial glycerol stocks

50% autoclaved glycerol (in MQ water) was mixed with stationary phase bacterial culture in a sterile cryotube vial in a 1:1 ratio. All strains were stored at -80ºC.

2.5.2 Preparation of *Streptomyces* S4 spore stocks

Streptomyces S4 strains were supplied by Professor Matt Hutchings from the University of East Anglia. Strains were spread on MS agar plates and incubated for 1 week at 30ºC to form a lawn of spores. 1 mL of sterile 10% (v/v) glycerol (in MQ water) was added to the surface of the plate and mixed using a sterile swab. Any residual liquid from the plate was removed to form the -20ºC spore stock. 1 mL 10% (v/v) glycerol was also used to rinse any spores from the sterile swab. This stock was split into aliquots and stored at -20ºC.

2.5.3 Culture conditions

Cultures were grown in conical flasks containing at least one-fifth of their own volume of medium or 500 mL to 1 L of medium in a 2 L baffled flask. Cultures were incubated at 37ºC, shaking at 180 RPM for 18 hrs unless otherwise stated. Cultures were inoculated into LB from either a -80 °C glycerol stock or from colonies on a freshly streaked agar plate. Bacteria were streaked from -80 °C glycerol stocks and agar plates were inverted and incubated overnight at 37ºC unless otherwise stated.

2.5.4 Measuring optical density

Culture optical density was measured at 600 nm using a Shimadzu UV-1800 spectrophotometer in plastic 1 mL cuvettes with a 1 cm path length.

2.5.5 *E. coli* growth curves on the microplate reader (25ºC and 37ºC)

Growth curves were produced for the following *E. coli* strains: BL21 (DE3), Parental BW25113, FimB-LacZ fusion, Δ*fimA* and Δ*fimC*. Each strain was grown to stationary phase at 37ºC, 180 RPM overnight. **Two 48 well plates were prepared in duplicate containing the following samples:**

- **Growth curves in LB:** Each strain was diluted to OD600 0.1 using LB before addition of 500 µL to each well. There were 4 repeats for each strain.
- **Growth curves in LB + 1.5 M ammonium sulphate:** Ammonium sulphate was slowly added to *E. coli* strains at OD600 0.1 so that cells were cultured in LB with 1.5 M ammonium sulphate. 500 µL was added to each well and each sample was done in triplicate.
- **Growth curves in chloramphenicol:** Chloramphenicol stock was added to *E. coli* strains at OD600 0.1 so that cells were cultured in LB + 25 µg/mL chloramphenicol. Only 1 repeat for each strain.

One of the 48 well plates was run on the microplate reader at 37ºC and the other was at 25ºC. OD600 readings were taken every 8 mins and the growth curve graphs were generated using Microsoft Excel.

2.6 OMV standard purification protocol

2.6.1 Standard protocol for purifying OMVs from Gram-negative bacteria

The strain of interest was inoculated into 500 mL to 1L LB media and incubated at 37°C, 180 RPM for 18 hrs in 2 litre baffled flasks. The bacterial culture was pelleted by centrifugation at 12,000 RPM (14,515 x g) for 10 min at 4°C. The supernatant (containing OMVs) was extracted and filtered through a 0.2 µm PES membrane filter for *Pseudomonas aeruginosa* strains or 0.45 µm PES membrane filter for *E. coli* strains. This was to remove any whole bacterial cells or large bacterial fragments. To ensure that all live bacterial cells had been removed, 500 µL-1 mL of filtered supernatant was spread onto LB agar plates and incubated for 24-48 hrs at 37°C to check for growth. OMVs were precipitated out of solution by slowly adding 1.5 M ammonium sulphate then incubated overnight at 4°C with gentle stirring. The OMVs were pelleted by centrifugation at 16,000 RPM (25,805 x g) for 30 mins at 4°C. The resulting OMV pellets were resuspended in 1-5 mL 10 mM HEPES/0.85% NaCl, pH 7.4 for further analysis. *P. aeruginosa* OMV samples were filter sterilised once more using 0.2 µm pores to give a cleaner sample. OMVs resuspended in 10 mM HEPES buffer were stored at 4ºC then at -20ºC for future use in SDS-PAGE gels or Western blotting.

2.6.2 Standard protocol for purifying OMVs from competent cells containing desired plasmid

1 colony from a successful transformation was inoculated into 50 mL LB containing 25 µg/mL chloramphenicol in a 250 mL flask then incubated at 37°C, 180 RPM overnight. The OD600 of cultures of interest were recorded and flasks were prepared as described in Table 2.17. Flasks were incubated at 37ºC, with shaking at 180 RPM for 18 hrs.

Table 2.17 Set up of *E. coli* cultures for induction with pJB005 plasmid

Flask name	Volume of LB (mL)	Volume of starter culture added (mL)	Volume of 25mg/mL chloramphenicol added (mL)	Volume of 1M IPTG added (µL)
1A - Parental strain + IPTG	500	5	0.5	250
1B - Parental strain - IPTG	500	5	0.5	0
2A) *ΔfimA* strain + IPTG	500	5	0.5	250
2b) *ΔfimA* strain - IPTG	500	5	0.5	0

The OD600 was monitored and cells were induced with 0.4 mM IPTG at an OD600 of 0.25-0.3 (early stationary phase). Cells were grown to mid-late exponential phase (Figure 2.1). When the induced cells had reached an OD600 of approximately 1.0, OMVs were purified using the standard protocol for Gram-negative bacteria (Section 2.6.1). Flask 1B is a negative control of Flask 1A and Flask 2B is a negative control of Flask 2A with no addition of IPTG. Once Flasks 1A and Flask 2A had reached the correct OD600, OMVs were purified immediately and with their respective negative controls.

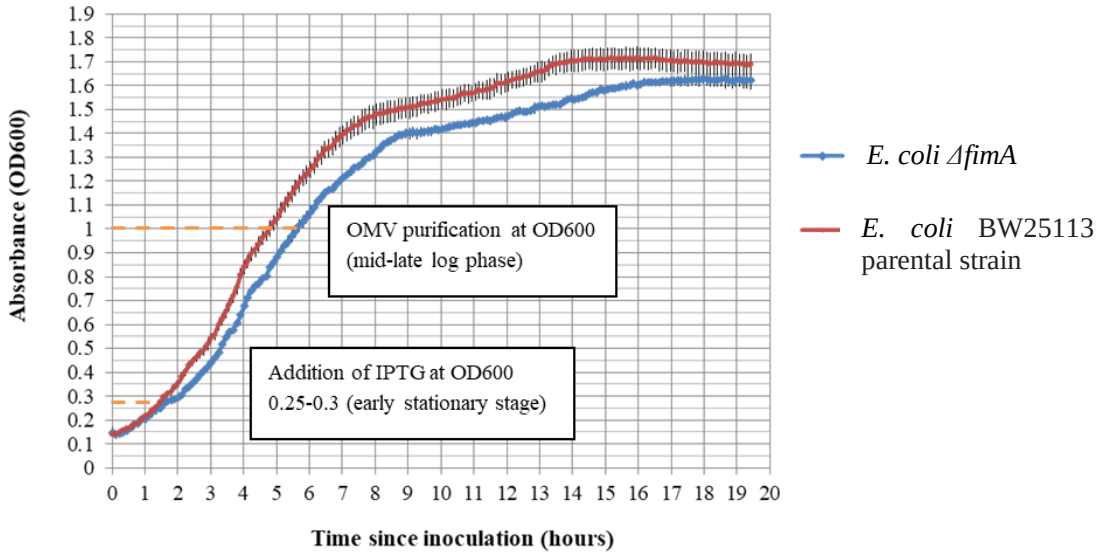


Figure 2.1 Growth curve of *E. coli* parental strain and *ΔfimA* at 37 °C. Strains were grown in triplicate, error bars represent 1 Standard Deviation from the mean

2.6.3 Standard protocol for purifying MVs from *Streptomyces* S4 strains

20 µL of spore stock was added to 1 mL sterile MQ water and spread on to a sterile MS agar plate. The plate was incubated at 30ºC for 5-7 days. Colonies were inoculated into 12.5 mL YEME:TSB (both media mixed 1:1) and incubated at 30ºC, 180 RPM for 72 hrs. 5 mL of each culture was inoculated into 500 mL LB in 2 litre baffled flasks then incubated at 30°C, 180 RPM for 72 hrs. OD600 of each culture was determined using a range of dilutions and confirmed to be approximately 3.0 for each strain. The bacterial culture was pelleted by centrifugation at 5000 x *g* for 30 mins at 4ºC and the supernatant was removed to be filtered through a 0.45 µm PES membrane filter. MV purification protocol from this point onwards was as described above in Section 2.6.1.

2.6.4 Ultracentifugation

For ultracentrifugation, the OMV pellets were resuspended in 4.5 mL 45% (v/v) OptiPrep in 10 mM HEPES/0.85% NaCl, pH 7.4. 4 mL was layered on to the bottom of a 26.3 mL polycarbonate ultracentrifuge tube (Beckman). A gradient of 4 mL of 40%, 35%, 30%, 25% and 20% (v/v) OptiPrep in 10 mM HEPES buffer was prepared and slowly added in layers on top of the OMVs in 45% OptiPrep. Tubes were balanced by addition/removal of the 20% OptiPrep layer. Ultracentrifugation was performed at 111,000 x *g* for 2 hrs at 4 °C for *P. aeruginosa* strains or 50,000 RPM (183,960 x *g*) for 3 hrs at 4ºC for *E. coli* strains. Ultracentrifuge tubes were incubated at 4ºC overnight to allow each layer

to settle. Without disturbing the ultracentrifuge tube, each layer was removed sequentially then kept at 4°C for further analysis. 240 µL of the sample of interest was precipitated using 60 µL TCA using the standard protocol prior to loading on an SDS-PAGE gel. In order to visualise the OMVs by TEM, the OptiPrep needed to be removed from the samples. Each sample of interest was diluted with PBS so that the percentage of OptiPrep was now 2-3 % in each sample. The OptiPrep was then removed by centrifugation at 16,000 RPM (25,805 x *g*) for 3 hrs at 4ºC. Each pellet was resuspended in 1.5 mL 10 mM HEPES buffer. 1.2 mL OMVs in HEPES was centrifuged at 13,200 RPM (14,220 x *g*) for 30 mins at 4ºC and finally resuspended in 12 µL 10 mM HEPES buffer. 10 µL of this sample was added to the EM grids for visualisation of the OMVs.

2.6.5 Alternative techniques to remove flagella

OMVs were purified from 500 mL culture using the standard protocol and were resuspended in 5 mL 10 mM HEPES buffer.

The following methods were compared:

1. Standard method: 1 mL OMVs in 10 mM HEPES buffer were centrifuged at 4ºC for 30 mins, 13,200 RPM. The resulting OMV pellet was resuspended in 30 µL 10 mM HEPES buffer.

2. Low speed spin: 1 mL OMVs in 10 mM HEPES buffer was centrifuged at 6000 x *g* at 4ºC for 30 mins. The resulting OMV pellet was resuspended in 30 µL 10 mM HEPES buffer.

3. Proteinase K: 900 µL OMV sample was mixed with 100 µL Proteinase K sample (100 µg/mL in HEPES /CaCl2 buffer). Samples were incubated at 37ºC for 30 mins. 5 mM PMSF was added to inhibit the protease and incubated at 37ºC for another 30 mins. OMV were centrifuged at 13,200 RPM (14,220 x *g*) for 30 mins at 4 °C. The resulting OMV pellet was resuspended in 30 µL 10 mM HEPES buffer.

4. OMVs before filtration through a 0.45 µm membrane filter: 2 mL OMVs in 10 mM HEPES buffer were centrifuged at 4ºC for 30 mins, 13,200 RPM. The pellet was resuspended in 30 µL 10 mM HEPES buffer.

5. OMVs after filtration through a 0.45 µm membrane filter: 2 mL OMVs in 10 mM HEPES buffer was filtered through a 0.45 µm membrane. The sample was centrifuged at 4ºC for 30 mins, 13,200 RPM. The pellet was resuspended in 30 µL 10 mM HEPES buffer.

6. Addition of proteases from *Streptomyces griseus.* A protease stock of 10% (w/v) was prepared in 10 mM HEPES buffer with $CaCl_2$. This was then diluted to give other concentrations of proteases from 1% to 0.1%. OMVs resuspended in HEPES were incubated with proteases of the relevant concentrations at 37ºC for 30 mins. The samples (450 µL) were centrifuged at 4ºC for 30 mins, 13,200 RPM (14,220 x *g*) and the pellet was resuspended in 20 µL 10 mM HEPES buffer in preparation for TEM.

2.6.6 Alternative techniques to remove fimbriae

As ultracentrifugation was unsuccessful, alternative techniques were compared to see if OMVs could be isolated from the co-purified fimbriae in *E. coli* K-12 strains. OMVs were purified from 500 mL culture using the standard protocol and were resuspended in 5 mL 10 mM HEPES buffer.

The following methods were compared:

1. **OMVs untreated:** 500 µL OMV in 10 mM HEPES buffer
2. **OMVs heat treated at 95ºC for 30 mins:** 500 µL OMVs in 10 mM HEPES buffer were heated at 95ºC for 30 mins
3. **OMVs sonicated:** OMVs were subject to 6 cycles of sonication for 30 seconds then resting for 30 seconds at 30% power (maximum is 40%)

2.6.7 Lipid extraction

Lipids and proteins were separated in OMV samples using chloroform-methanol extraction method (Ferrez, *et al.* 2003). 1 mL *E. coli* OMVs were mixed with 4 mL chloroform-methanol mixture (2:1 v/v). Briefly, the mixture was vortexed for 20 seconds then centrifuged at 2500 x *g* for 10 mins. The aqueous layer (top layer, mostly methanol and water) was discarded. The interphase layer (containing OMV proteins) was extracted and transferred to a new 1.5 mL microfuge tube. Samples were spun in a vacuum centrifuge to remove all surrounding liquid and OMV proteins were resuspended in 10 mM HEPES buffer for Proteinase K test. As a control, 1 mL 100 µg/mL BSA was added to 4 mL of chloroform-methanol mixture and was treated in the same way as the OMVs.

2.7 Outer membrane and periplasmic protein extractions

Outer membrane proteins and periplasmic proteins were isolated using Tris-sucrose-EDTA (TSE) buffer extraction (Quan *et al.* 2013). This method was chosen as it appears to be an efficient method of extracting outer membrane and periplasmic proteins with minimal contamination of proteins from the inner membrane and cytoplasm (Quan *et al.* 2013). Isolation of periplasmic proteins, OM proteins and OMVs were performed on the same *E. coli* culture concurrently for direct comparison. In each case, the colony of interest was inoculated into 750 mL LB and grown overnight. 500 mL of this culture was used to purify OMVs and 100 mL was used for the periplasmic and OM extraction.

Briefly, 100 mL fresh overnight culture was diluted to be OD600 1.0. The bacterial cells were harvested by centrifugation at 3000 x *g* for 20 mins at 4ºC and the supernatant was discarded. The cell pellet was carefully resuspended into 1 mL TSE buffer then incubated on ice for 30 mins. This sample was then centrifuged at 16,000 RPM for 30 mins at 4ºC and the supernatant was transferred to a new microfuge tube suitable for ultracentrifugation. The supernatant at this stage contained the soluble extracted bacterial envelope proteins. To separate outer membrane proteins from periplasmic proteins, the supernatant was centrifuged at 43,000 RPM for 1 h at 4 °C (TL-100 Ultracentrifuge rotor Beckman). The pellet from this step contained the outer membrane proteins and the supernatant contained the soluble periplasmic proteins (Quan *et al.* 2013).

2.8 Protein manipulation techniques

2.8.1 Bradford assay

The concentration of protein in both cells and OMV samples were determined using a Bradford assay. A standard curve was produced using a range of BSA concentrations between 0-1000 µg/mL diluted in MQ water. 20 µL of the BSA standard at each concentration was vortexed with 1 mL 1x Bradford reagent and incubated at room temperature for 15 mins. The Absorbance of the samples were read at a wavelength of 595 nm and a standard curve was constructed. The Bradford assay was performed using the same procedure as for the BSA standards and the protein concentration (µg/mL) was calculated from the standard curve. Standards and OMV/cell samples were performed in triplicate and an average was taken.

2.8.2 Standardisation of protein samples for SDS-PAGE gel

All samples were standardised to the same protein concentration prior to loading on an SDS-PAGE gel unless otherwise stated. A Bradford assay was performed to determine the protein concentration of all samples. The sample with the lowest concentration of protein was determined and all other samples were diluted to be the same. Once all samples were the same protein concentration, they were either loaded on to an SDS-PAGE gel or TCA precipitated to concentrate (see method below). Any empty lanes on an SDS-PAGE gel were filled with 10 mM HEPES buffer (details in Section 2.15) and 4x reducing sample buffer (RSB).

2.8.3 TCA-precipitation of OMVs

Purified OMVs (resuspended in HEPES) were vortexed with cold 100% TCA stock solution to make a final concentration of 20% TCA. Samples were incubated on ice for 30 mins then centrifuged for 30 mins, 13,200 RPM (14,220 x *g*) at 4 °C. The supernatant was removed and 0.5 mL cold acetone was added. After briefly vortexing, the samples were centrifuged for 15 mins, 13,200 RPM (14,220 x *g*) at 4 °C. The supernatant was removed and each OMV pellet resuspended in 10 mM HEPES buffer (details in Section 2.15) and 4x RSB in a 3:1 ratio.

2.8.4 SDS-PAGE

SDS-PAGE gels were run using the Novex Xcell II Mini-Cell system for Electrophoresis with pre-cast 10 well 4-12% Bis-Tris gels. SDS-PAGE gels were run using 1x MOPS or 1x MES SDS running buffer. Each sample was mixed with the appropriate volume of 4X reducing sample buffer (RSB) and heated to 95 °C for 5 mins prior to loading. 20 µL of each sample was loaded into each well. 0.2 µL markers were used each time to estimate protein size when the SDS-PAGE was due to be silver stained. 5-10 µL markers were used when staining with Coomassie. Gels were run at 165 V for 48 mins (MES running buffer) or 55 mins (MOPS running buffer).

2.8.4.1 Detection of proteins via Coomassie

SDS-PAGE gel was placed in 25 mL InstantBlue and left on a shaker for 1 hour at room temperature.

2.8.4.2 Detection of proteins via silver staining

SDS-PAGE gels were developed using the Pierce Silver Stain kit as described in the manufacturer's protocol. All steps below were carried out on an orbital shaker. Briefly, SDS-PAGE gels were subject to 2 x 5 min washes in MQ water then 2 x 15 min washes minimum in Gel Fix (30% ethanol, 10% acetic acid, 60% MQ water). Gels were washed for 2 x 5 mins in 10% (v/v) ethanol then 2 x 5 mins in MQ water. Gels were sensitised for 1 min (in 50 µL sensitiser in 25 mL MQ water) then washed 2 x with MQ water for 1 min. Gels were stained for 30 mins minimum in Staining Solution (0.5 mL Enhancer with 25 mL Silver Stain solution). After 2 x 20 second washes with MQ water, gels were developed using Developer Solution (0.5 mL Enhancer with 25 mL Developer solution) for 1-3 mins. Once bands appeared, the reaction was stopped using 5% (v/v) acetic acid for 10 mins. After imaging, gels were stored long term in 5% (v/v) acetic acid or MQ water at 4ºC.

2.8.4.3 Imaging of SDS-PAGE gels and Western blots

Gels and blots were imaged using Syngene G:BOX and associated software.

2.8.4.4 Densitometry analysis of protein profiles on SDS-PAGE gel

Densitometry analysis was performed using Image J (Miller, 2010).

2.8.4.5 Extraction of FimA monomer protein from an SDS-PAGE gel

An SDS-PAGE gel was run containing protein markers and OMVs from *E. coli* with fimbriae locked on strains. After running, the SDS-PAGE gel was cut in half. One half (containing 1 lane of protein markers and 1 lane of the OMV sample) was silver stained so that the FimA protein could be visualised (at 18 kDa). This was called the 'stained FimA' sample. The other half of the gel containing 1 lane of protein markers and 1 lane of OMVs was not stained. However, using the protein markers and other stained half of the gel, the location of FimA at 18 kDa could be estimated and this was extracted. This sample was called the 'unstained FimA' sample as no silver staining had taken place. The excised gel pieces were cut as small as possible and were incubated with 1 mL elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA, pH 7.5) at 30 °C, shaking at 180 RPM. The samples were centrifuged at 5000 x *g* for 10 minutes and the supernatant, (containing the extracted protein), was removed.

2.8.5 Wet transfer

An SDS-PAGE gel was run of the samples as described above including 5-10 µL protein markers. Transfer from the SDS-PAGE gel to PVDF membrane was performed using the Bio-Rad electro transfer cell equipment using the manufacturer's instructions.

2.8.5.1 Western blot protocol for anti-OmpA antibody

All steps below were carried out on an orbital shaker. After transfer, the membrane was blocked in 10% BSA in TBST for 30 mins then incubated overnight at 4ºC with anti-OmpA antibody (diluted 1:50,000

in 5% (w/v) BSA in TBST). Membranes were then subject to 3 x 15 min washes in 1X TBST then incubated for 1 hour with 10 mL secondary antibody (diluted in 5% BSA in TBST). Membranes were then subject to 3 x 5 min washes in 1X TBST then 2 x 5 min washes in 1X TBS. Bands were developed in the dark using BCIP/NBT substrate for 1-10 mins and the reaction was stopped by washing in MQ water. See Table 2.18 for further information about PVDF membranes and secondary antibodies used for each primary antibody.

2.8.5.2 Western blot protocol for all other antibodies

All steps below were carried out on an orbital shaker. After transfer, the membrane was blocked in 5% (w/v) milk in TBST for 30 mins then incubated overnight at 4ºC with primary antibody diluted in 5% milk in TBST (see Table 2.18 for details of dilutions for each primary antibody). Membranes were then subject to 4 x 5 min washes in 1X TBST then incubated for 1 hour with of secondary antibody (diluted in 5% milk in TBST). Membranes were then subject to 4 x 5 min washes in 1X TBST. Bands were developed in the dark using BCIP/NBT substrate for 1-10 mins and the reaction was stopped by washing in MQ water.

Table 2.18 Dilutions for primary and secondary antibodies during Western blotting and PVDF membrane types used for transfer

Antibody name	Dilution used in Western blots	Host	Secondary alkaline-phosphatase conjugated antibody used	Dilution used in Western blots	PVDF Membrane pore size used with antibody (µm)
Anti-FimA monoclonal	1 in 5000	Rabbit	Anti-rabbit (Sigma)	1 in 5000	0.2
Anti-FimA polymer	1 in 5000	Rabbit	Anti-rabbit (Sigma)	1 in 5000	0.45
Anti-Flagellin	1 in 10,000	Rabbit	Anti-rabbit (Sigma)	1 in 5000	0.45
Anti-GFP antibody	1 in 3333	Mouse	Anti-mouse secondary antibody (Promega)	1 in 5000	0.45
Anti-Neon green antibody	1 in 1000	Mouse	Anti-mouse (Promega)	1 in 5000	0.45
Anti-OmpA antibody	1 in 50,000	Rabbit	Anti-rabbit (Sigma)	1 in 5000	0.45

2.8.5.3 Imaging of Western blots

Gels and blots were imaged using Syngene G:BOX and associated software.

2.8.6 Mass spectrometry (matrix-assisted laser desorption/ionisation, MALDI)

All preparation for the mass spectrometry described below was performed in Kevin Howland's laboratory (UKC). **Microfuge tube preparation:** Before use microfuge tubes used in this procedure were rinsed with ethanol and air-dried overnight in a foil covered beaker to reduce keratin contamination. **Band excision:** SDS-PAGE gels containing the bands of interest were subject to 2 x 10 min washes with MQ water. Bands of interest were then carefully excised from the SDS-PAGE with a clean washed scalpel and cut further into 1 mm x 1 mm squares. The extracted gel pieces were incubated overnight at 4ºC in 500 µL MQ water in a clean microfuge tube. **Reduction and alkylation**: All solutions used were prepared on the day and all centrifugation steps were performed at 5000 RPM for 1 min at room temperature. Firstly, the gel pieces were centrifuged and the MQ water was removed.

Gel pieces were incubated in 1 mL 50 mM NH_4HCO_3:acetonitrile (1:1) for 15 mins then spun down to remove liquid. Gel pieces were shrunk by addition of 100 µL acetonitrile for 15 mins then centrifuged and all liquid removed. 50 µL 10 mM DTT in NH_4HCO_3 was added to cover the gel pieces then incubated at 56ºC for 30 mins. Gel pieces were spun down and any excess liquid was removed. The gel pieces were then shrunk briefly by incubation in acetonitrile for 2 mins. Acetonitrile was removed and 55 mM iodoacetamide in 50 mM NH_4HCO_3 was added. Iodoacetamide is light sensitive so gel pieces were incubated for 20 mins in the dark. Gel pieces were then washed in 100 µL 50 mM NH_4HCO_3:acetonitrile (1:1) then 50 mM NH_4HCO_3 then acetonitrile in the same way as described above and all excess liquid was removed. **In-gel Digestion:** Gel pieces were rehydrated in 20 µL digestion buffer (25 mM NH_4HCO_3, 10% acetonitrile and 10 ng/µL Trypsin) and incubated on ice for 30 mins. Any remaining liquid was removed and gel pieces were rehydrated in 10 µL 25 mM NH_4HCO_3, 10% acetonitrile (no Trypsin) and incubated at room temperature overnight for digestion. **Extraction of peptides:** 5 µL acetonitrile was added to the gel pieces and sonicated for 15 mins in an ultrasound bath. Gel pieces were briefly centrifuged and the supernatant was collected and transferred to a fresh microfuge tube. 10 µL 50% acetonitrile, 5% formic acid solution was added to the gel pieces and sonicated for 15 mins in an ultrasound bath. Gel pieces were spun down and the supernatant was transferred to the same microfuge tube as in the previous step. These combined supernatants were stored at 4ºC if samples were to be analysed in the next 24 hrs or stored at -20ºC.

Analysis of samples

Proteins were identified using Bruker ultrafleXtreme MALDI-TOF/TOF mass spectrometer and associated software. Samples to be identified were added to the MTP Anchorchip MALDI-TOF plate (Bruker). 0.5 µL sample (prepared above) was added to the MALDI-TOF plates in known coordinates and left to air dry. 1 µL matrix solution (0.7 mg/mL α-Cyano-4-hydroxycinnamic acid dissolved in solvent mixture 85% acetonitrile, 15% water, 0.1% TFA and 1 mM $NH_4H_2PO_4$) was then added on top of each sample and left to air dry. 0.5 µL Peptide Calibration Standard solution was added to the plate and left to air dry. The plate was loaded on to the instrument and the following settings were used: **Polarity:** positive, **Laser frequency:** 2 kHz, **Ion sources:** 25 kV and 22.35 kV, **Lens:** 7.5 kV, **Pulsed ion extraction:** 80 nS, **Peptide Calibration Range:** 700-3500 Da, **Data sampling rate:** 4 Gs/s. For each sample 3500 shots were summed and saved. Protein was identified by a Peptide Mass Fingerprint (PMF) search in the Mascot database. A match is significant if it has a score greater than 70.

2.9 Microscopy

2.9.1 Transmission Electron Microscopy (TEM)

2.9.1.1 Preparation of copper and gold grids used for TEM

EM grids (Table 2.19) used for TEM and were prepared and supplied by Ian Brown (UKC).

Table 2.19 Comparison of grids used for TEM

TEM grid material	Coating	Mesh size	Use
Copper	Formvar/carbon	400	Used to visualise purified (O)MVs. Default grid type used unless otherwise stated
Gold	Formvar/carbon	400	Immunogold labelling (O)MVs
Gold	None	400	Immunogold labelling cells and (O)MVs embedded in resin

2.9.1.2 Standard TEM protocol to visualise OMVs

OMVs resuspended in 10 mM HEPES buffer were concentrated for EM by centrifugation at 13,200 RPM (14,220 x *g*) for 30 mins at 4°C. The OMV pellets were then resuspended in 10-15 μL 10 mM HEPES buffer. 10 μL purified OMVs were added to a copper EM grid and left to settle for 10 mins. OMVs were then fixed by adding 10 μL of 4% (w/v) formaldehyde in PBS and the grids were left for 10 mins. The grids were subject to 4 x 1 min washes in MQ water then negatively stained using 2% (w/v) uranyl acetate in PBS. Grids were air dried for 20 mins and loaded on to the EM for analysis.

2.9.1.3 Time lapse experiment to observe OMV biogenesis

Colonies of *P. aeruginosa PA14* and *E. coli* BL21 (DE3) were inoculated into 25 mL LB and incubated for 18 hrs at 37ºC, 180 RPM. 15 mL was centrifuged at 3000 RPM for 8 mins at room temperature. The bacterial pellets were resuspended in 15 mL PBS then subject to two washes in PBS (3000 RPM for 8 mins). The bacterial pellet was resuspended in 10 mL fresh LB and the OD600 of the culture was measured. Cells were diluted in LB to give an OD600 of 0.1. 10 μL of cells in LB was added to gold carbon coated EM grids and incubated at 37ºC. Grids were fixed at various time points over 22 hrs by adding 10 μL 5% (v/v) glutaraldehyde in 200 mM CAB (sodium cacodylate arsenic pH 7.2) for 5 mins. The grids were then washed in 20 μL 2.5% (v/v) glutaraldehyde in 100 mM CAB for 2 mins then 30 mins. The grids were then subject to 3 x 1 min water washes. Grids were air dried for 20 mins then loaded on to the EM for analysis (JEOL 1230) at 80 KV.

2.9.1.4 Immunolabelling OMVs using anti-OmpA antibody

Purified *E. coli* BL21 (DE3) OMVs were concentrated 10x by centrifugation at 13,200 RPM (14,220 x *g*) for 30 mins at 4ºC then resuspended in 10 mM HEPES buffer. 10 μL OMVs were added to each grid and left to settle for 10 mins. Grids were fixed by washes in 20 μL 2% (w/v) formaldehyde + 0.5% (v/v) glutaraldehyde in 100 mM CAB pH 7.2 for 1 min then 30 mins. Grids were then subject to 2 x 5 min washes in 100 mM CAB then 2 x 5 min washes in TBST. Grids were washed in 2% (w/v) BSA in TBST for 1 min then 30 mins. Grids were then incubated with anti-OmpA antibody (Antibody Research Corporation 111120) diluted 1 in 50 in TBST. An identical grid was used as a negative control and was incubated in TBST only. Grids were left in primary antibody at 4ºC overnight. The next day, grids were washed 5x in TBST for 1 min then grids were incubated in immunogold conjugated anti-rabbit antibody

(BBI solutions EM.GAR15) which was diluted 1 in 50 in TBST. Grids were washed in secondary antibody for 1 min then left for 30 mins. Grids were subject to 5 x 1 min washes in TBST then 5 x 1 min washes in Milli-Q water. Lastly, grids were negatively stained using 2% (w/v) uranyl acetate in PBS then air dried for 20 mins. Grids were loaded on to the EM for analysis (JEOL 1230) at 80 KV.

2.9.1.5 Embedding bacterial cells in resin for immunogold labelling and TEM analysis

Colonies were inoculated into 12.5 mL LB and incubated at 37 °C, 180 RPM for 20 hrs. 1 mL overnight culture was harvested by centrifugation at 13,000 RPM for 2 mins. Cells were fixed in 1.5 mL 2.5% (v/v) gluteraldehyde in 100 mM sodium cacodylate buffer pH 7.2 (CAB) for 3 hrs with gentle rotation at 20 RPM. Fixed cells were pelleted by centrifugation at 13,000 RPM for 2 mins then washed twice in 100 mM CAB buffer, followed by final resuspension in 1.5 mL CAB. Cells were pelleted by centrifugation at 13,000 RPM for 2 mins then resuspended in 1.5 mL 50% (v/v) ethanol. Resuspended cells were mixed with gentle rotation at 20 RPM for 10 mins. This was repeated so that the cells were washed in 70% (v/v) ethanol, 90% (v/v) ethanol and 3x washes in 100% ethanol to remove all water. Cell pellet was finally resuspended in 1.5 mL LR White Resin Medium Grade (Agar scientific, AGR1281) and left spinning on the rotor overnight at room temperature. Cells were pelleted by centrifugation at 13,000 RPM for 2 mins, the supernatant was removed and the cells were resuspended in fresh LR White Resin (Agar Scientific). This was repeated once more. Cells resuspended in resin were left spinning at 20 RPM at room temperature for 5 hrs. Cells were then added to Gelatin capsules (Agar Scientific G29208). They allow resin polymerisation in the absence of oxygen as they are sealed. Cells were pelleted (13,000 RPM for 2 mins) and resuspended in 0.5 mL resin. The cells (resuspended in resin) were then added to the capsules and fresh resin was used to top up the capsules to the point of overflowing to leave as little air is in the capsule as possible. The capsules were placed into new 1.5 mL Eppendorfs (cut in half) so they could be microfuged at 1000 RPM for 5 mins so that the cells were at the bottom of the capsule. Capsules were incubated at 60ºC for 22 hrs to allow polymerisation of resin inside the gelatin capsules. The gelatin capsules were cut off the resin using a Teflon coated razor blade. The bottom of the resin capsule contained the cell sample (was visible as a LB-coloured pellet). The top part of the resin (away from the sample) was filed down to the flat then the sample was labelled with permanent marker. The resin pieces were stored in a Petri dish for sectioning and EM at a later date.

2.9.1.6 Embedding OMVs in resin for immunogold labelling and TEM analysis

E. coli strains were grown in 750 mL LB and purified using the standard OMV purification protocol for *E. coli.* The final OMV pellet was resuspended in 1 mL 10 mM HEPES buffer. 800 µL *E. coli* OMVs were harvested by centrifugation at 13,200 RPM (14,220 x *g*) for 30 mins at 4 ºC. OMVs were then fixed in 1.5 mL 2.5% gluteraldehyde in 100 mM sodium cacodylate buffer pH 7.2 (CAB) for 1.5 hrs with gentle rotation. Fixed OMVs were pelleted by centrifugation at 13,200 RPM (14,220 x *g*) for 30 mins then washed twice in 100 mM CAB, followed by final resuspension in 25 µL CAB. 25 µL of fixed OMVs were added to PCR tubes immersed in a 54°C water bath. After warming, 25 µL of pre-heated 3% (w/v) agarose solution in 100 mM CAB was added to the OMV suspension and mixed thoroughly by careful pipetting. Pre-heated agarose was mixed 1:1 with sample. The agarose-cell suspension was

then transferred into a pre-warmed frame constructed from two glass microscope slides separated by an acetate gasket, held together by bulldog clips. The slides were incubated at 4ºC for 10 mins to set. The microscope slides were separated and the agarose gel was cut into small (~2 mm) squares with a razorblade. The agarose pieces were transferred to a petri dish into a droplet of 0.1% Alcian blue in 1% acetic acid (v/v). Agarose squares were then transferred to a glass vial and washed twice in 3 mL 100 mM CAB. Using this process, agarose pieces were washed in 50% (v/v) ethanol, 70% (v/v) ethanol, 90% (v/v) ethanol and finally 3x in 100% ethanol. All 100% ethanol was removed and agarose pieces were left in approximately 3 mL resin overnight at room temperature so that all remaining ethanol evaporated. All resin liquid was removed around the agarose pieces then fresh resin was added. This was repeated and the agarose pieces were left in resin for 5 hrs. This was then added to gelatin capsules which were filled with resin to the top until overflowing. 1 blue agarose piece was added to the bottom of the gelatin capsule filled with resin. Capsules were incubated at 60ºC for 22 hrs to allow polymerisation. The gelatin capsules were cut off the resin using a Teflon-coated razor blade. The bottom of the resin capsule contained the OMV sample (visible due to the blue dye) and the top part of the resin (away from the sample) was filed down to the flat then labelled with permanent marker. The resin pieces were stored in a Petri dish for sectioning and EM at a later date.

2.9.1.7 Sectioning and visualisation of embedded samples

Sectioning was performed by Ian Brown (UKC) on a RMC MT-XL ultra-microtome with a diamond knife. Sections were added to un-coated 300 mesh gold grids (Ian Brown). Grids were washed in 2% (w/v) BSA in TBST for 1 min then 30 mins. Grids were incubated in 15 µL primary antibody in TBST at 4°C overnight. See Table 2.20 for further details on the primary and secondary antibodies used.

Table 2.20 Description of all antibodies used for immunogold labelling

Primary antibody name	Dilution of primary antibody used in (TBST)	Host	Secondary antibody used	Dilution of secondary antibody used (in TBST)
Anti-FimA monoclonal	1 in 50	Rabbit	Anti-rabbit 15 nm gold (BBI solutions)	1 in 50
Anti-FimA monomer and Anti-GFP antibody mix	1 µL FimA antibody, 1 µL GFP, 48 µL TBST	Rabbit & Mouse	Anti-mouse 10 nm gold (BBI solutions) **then** anti-rabbit 15 nm gold	1 in 50
Anti-FimA polymer	1 in 50	Rabbit	Anti-rabbit 15 nm gold (BBI solutions)	1 in 50
Anti-Flagellin	1 in 50	Rabbit	Anti-rabbit 15 nm gold (BBI solutions)	1 in 50
Anti-GFP antibody	1 in 50	Mouse	Anti-mouse 10 nm gold (BBI solutions)	1 in 50
TBST only (anti-mouse negative control)	N/A	N/A	Anti-mouse 10 nm gold (BBI solutions)	1 in 50
TBST only (anti-rabbit negative control)	N/A	N/A	Anti-rabbit 15 nm gold (BBI solutions)	1 in 50

Grids were subject to 5 x 1 min washes in TBST then incubated in secondary immunogold conjugated antibody for 30 mins. For the samples probed with both anti-FimA monomer and anti-GFP primary antibodies, grids were incubated in anti-mouse secondary antibody for 15 mins then anti-rabbit secondary antibody for 15 mins. Grids were subject to 5 x 1 min washes in TBST then 5 x 1 min washes in TBST. Grids were incubated in 4.5% (v/v) uranyl acetate in 1% (v/v) acetic acid solution for 15 mins then washed in a stream of MQ water. Lastly, grids were stained by incubation in Reynolds lead citrate for 3 mins then washed in a stream of MQ water. Grids were air dried for 30 mins then loaded on to the EM for analysis. For further details about antibodies used, see Section 2.1.

2.9.2 Confocal microscopy

Confocal microscopy for *Streptomyces* S4 cells and MVs

Streptomyces S4 cells were inoculated into 12.5 mL YEME:TSB (two media mixed in a 1:1 ratio) and incubated at 30ºC, 180 RPM for 48 hrs. 1 mL cells were pelleted by centrifugation at 13,200 RPM (14,220 x *g*) for 30 mins so that both cells and OMVs were pelleted together. The supernatant was removed and 1 mL 2% (w/v) paraformaldehyde in PBS was used to fix cells. Cells were pelleted again and 1 mL 100 µg/mL WGA-FITC (Sigma L4895) was mixed with the cells and incubated in the dark for 1 hour. 15 µL cells were added onto a 1.5 mm thickness coverslip before being inverted into a drop of ProLong Gold antifade mountant (Life Technologies, P36930) on a glass slide. Slides were incubated at room temperature in the dark overnight to cure. Samples were visualised the next day by confocal microscopy (Zeiss lsm 880 with airscan with associated Zen Black software) under the supervision of Matt Lee. A scale bar was added to images using Fiji (Image J). The only modification to the protocol when using *Streptomyces* S4 MVs was that there was an additional concentration step after the incubation with WGA-FITC for 1 hour. MVs were pelleted by centrifugation at 13,200 RPM (14,220 x *g*) for 30 mins then resuspended in 15 µL PBS which was added to the glass slide in the same way as above.

2.9.3 Light microscopy

Light microscopy for fimbriae agglutination assay

Each *E. coli* strain was inoculated into LB and incubated at 37ºC for 18-24 hrs, 180 RPM. *Saccharomyces cerevisiae* was inoculated into YPD and incubated at 30ºC for 18-24 hrs, 180 RPM. 5 µL of overnight *E. coli* strain was mixed with 5 µL *Saccharomyces cerevisiae* on a glass microscope slide. Yeast cell agglutination was visualised using GXM L2800 Premium Compound Microscope at 400x magnification and photos were taken using the associated camera.

2.10 Protease studies

2.10.1 Proteinase K test

Purified OMV samples were treated with a working concentration of 10 µg/mL Proteinase K (resuspended in HEPES/$CaCl_2$ buffer, details in Section 2.15) and/or varying concentrations of SDS (in MQ water). The OMVs were incubated in the presence and absence of Proteinase K and SDS for 30 mins while shaking at 37°C, 180 RPM. PMSF was added to every sample to inhibit Proteinase K (0.5 mM working concentration) then samples were incubated for another 30 mins while shaking at 37°C, 180 RPM. Samples were then TCA precipitated to concentrate then run on an SDS-PAGE gel for analysis. For pre-lysed OMVs, OMVs were heated for 95°C for 30 mins then left at room temperature to cool before Proteinase K addition.

Table 2.21 Description of the contents of each sample in a Proteinase K test

Lane number	Lane description	OMVs (or substituted with 10 mM HEPES buffer)	100µg/mL Proteinase K (or substituted with 10 mM HEPES/CaCl2 buffer)	SDS stock (w/v) added (or substituted with MQ water)
2	OMVs - Prot K - SDS	168 µL OMVs	24 µL HEPES/CaCl2	48 µL water
3	OMVs + Prot K - SDS	168 µL OMVs	24 µL Proteinase K	48 µL water
4	OMVs - Prot K + SDS	168 µL OMVs	24 µL HEPES/CaCl2	48 µL 20% SDS
5	OMVs heated to 95°C + Prot K	168 µL OMVs	24 µL Proteinase K	48 µL water
6	OMVs + Prot K + SDS 0.02%	168 µL OMVs	24 µL Proteinase K	48 µL 0.02% SDS
7	OMVs + Prot K + SDS 1%	168 µL OMVs	24 µL Proteinase K	48 µL 5% SDS
8	OMVs + Prot K + SDS 2%	168 µL OMVs	24 µL Proteinase K	48 µL 10% SDS
9	OMVs + Prot K + SDS 4%	168 µL OMVs	24 µL Proteinase K	48 µL 20% SDS
10	Proteinase K + SDS only	168 µL HEPES	24 µL Proteinase K	48 µL 20% SDS

2.11 Detergent studies

2.11.1 *E. coli* and *Streptomyces* S4 detergent studies

Various concentrations of SDS were added to *E. coli*/*Streptomyces* S4 MVs then incubated for 60 mins at 37 ºC, shaking at 180 RPM. Samples were TCA precipitated and finally resuspended in 30 µL 10 mM HEPES buffer and 10 µL 4x RSB prior to loading on to an SDS-PAGE gel.

2.12 Cloning / DNA manipulation techniques

2.12.1 Plasmid design and cloning overview

The plasmid pJB005 was donated by Dr Alex Moores (UKC) for use in cloning. See Figure 2.2 for plasmid map and details. The vector is a derivative of the pCA24N plasmid with mNeonGreen fused at the C-terminal. The pCA24N vector originated from Japan's coli stocks.

Reference: https://shigen.nig.ac.jp/ecoli/strain/locale/change?lang=en

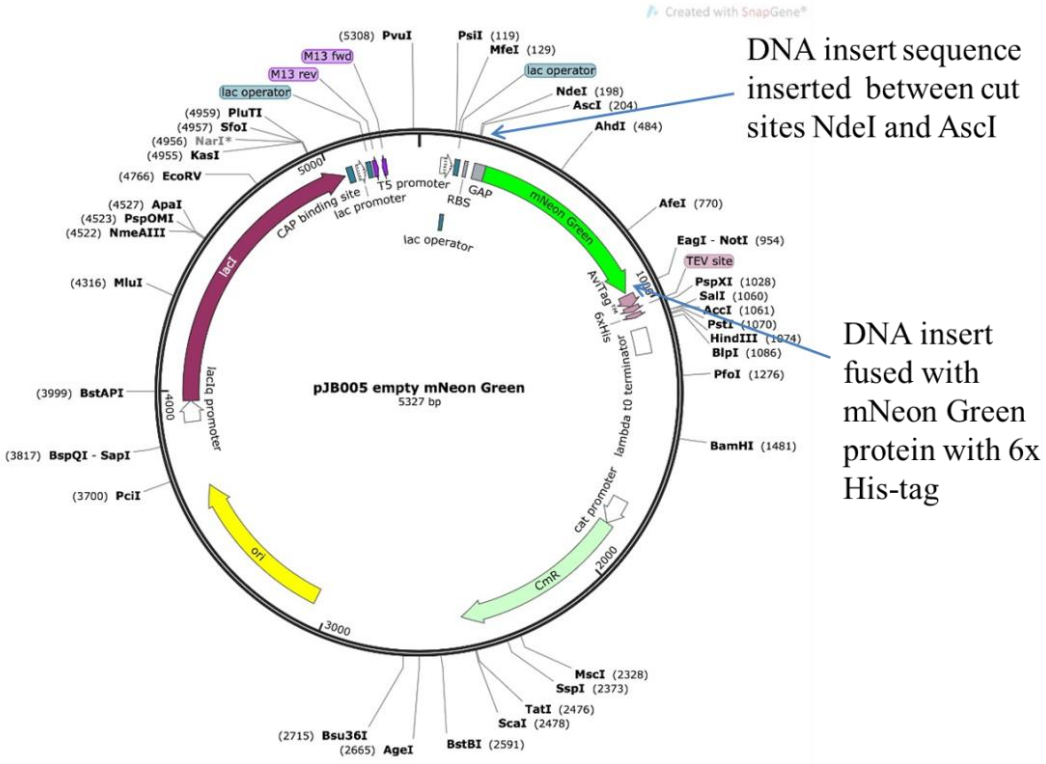


Figure 2.2 pJB005 plasmid map used for cloning. Plasmid map generated using SnapGene.

Aim: To re-introduce the *fimA* and *fimC* genes back into *E. coli ΔfimA/ΔfimC* strains using the pJB005 plasmid. The resulting proteins are a FimA-mNeon Green fusion protein and a FimC-mNeon Green fusion protein. Figure 2.3 gives an overview of the cloning procedure.

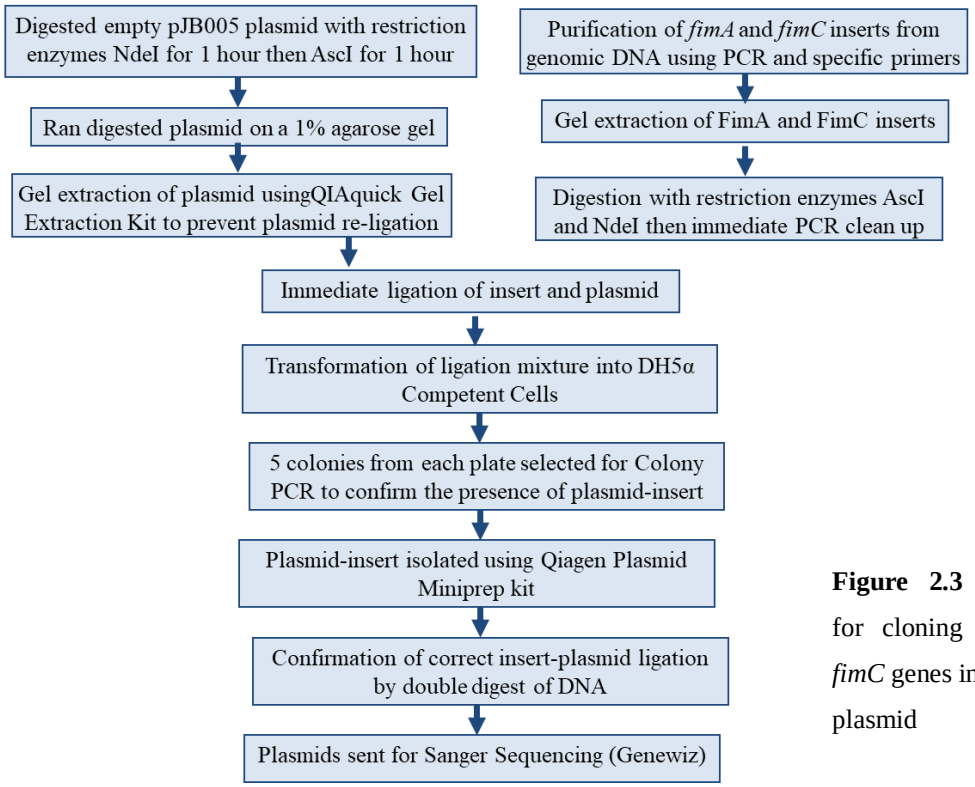


Figure 2.3 Overview for cloning *fimA* and *fimC* genes into pJB005 plasmid

2.12.1.2 Cloning Oligos into pJB005 plasmid overview

Aim: To create an mNeon Green protein with the FimA signal peptide sequence on the N-terminus of the protein. Figure 2.4 gives an overview of the cloning procedure.

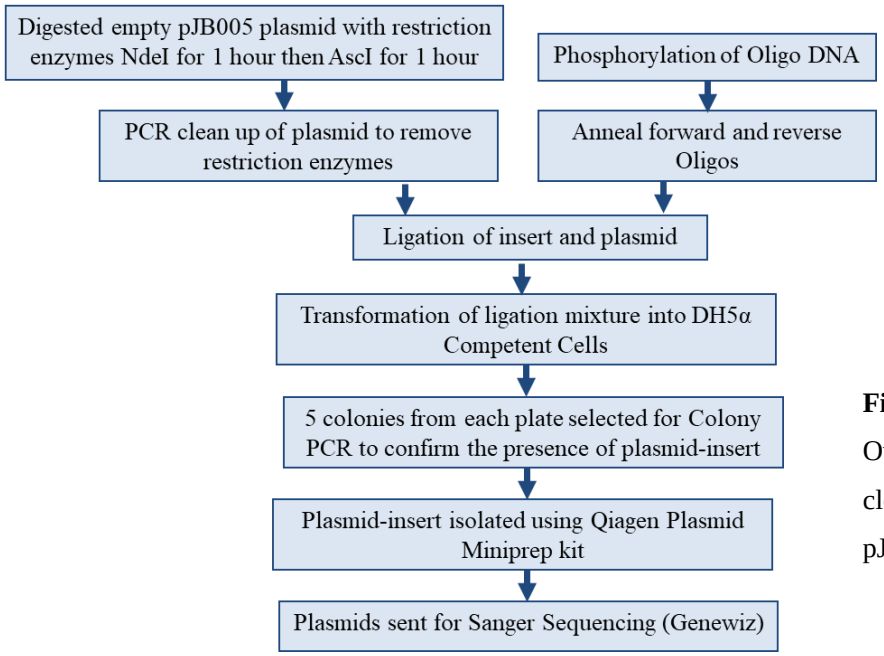


Figure 2.4 Overview for cloning Oligos into pJB005 plasmid

2.12.2 Primer design and preparation

The following primers (listed in Table 2.22) were ordered from Integrated DNA Technologies (IDT). Further information of primer design can be found in Appendix A.1-A.2.

Table 2.22 List of primers ordered from IDT with brief descriptions of their use

Primer name	Primer description	Primer sequence (5' - 3')
pJB.gene.seq.f	Forward primer for insert in plasmid pJB005	GTGAGCGGATAACAATTATAATAG
pJB.fluro.seq.r	Reverse primer for insert in plasmid pJB005 including mNeon green protein	CTAATTAAGCTTGGCTGCAGGT
fimA.full.f	Forward primer for full *fimA* gene in *E.coli* MG1655	TTTTCATATGAAAATTAAAACTCTGGCAATCGTTG
fimA.full.r	Reverse primer for full *fimA* gene in *E.coli* MG1655	TTTTGGCGCGCCTTGATACTGAACCTTGAAGG
fimC.full.f	Forward primer for full *fimC* gene in *E.coli* MG1655	TTTTCATATGAGTAATAAAAACGTCAATGTAAGG
fimC.full.r	Reverse primer for full *fimC* gene in *E.coli* MG1655	TTTTGGCGCGCCTTCCATTACGCCCGTCATTTTG
Oligo.FimA.sp.f	Oligo - FimA signal peptide sequence - Forward sequence	TATGATGAAAATTAAAACTCTGGCAATCGTTGTTCTGTCGGCTCTGTCCCTCAGTTCTACAGCGGCTCTGGCCGG
Oligo.FimA.sp.r	Oligo - FimA signal peptide sequence - Reverse sequence	CGCGCCGGCCAGAGCCGCTGTAGAACTGAGGGACAGAGCCGACAGAACAACGATTGCCAGAGTTTTAATTTTCATCA

2.12.2.1 Primer stocks (-80 ºC freezer)

DNA was resuspended in sterile MQ water to make a 100 µM stock solution (stored at -80ºC).

2.12.2.2 Working stocks of DNA (-20 °C)

-80°C primer stocks were diluted to be 10 µM DNA which were then stored at -20°C.

2.12.3 Transformation of DNA

2.12.3.1 Preparation of competent cells

The following strains from The Coli Genetic Stock Center (Keio collection) were made chemically competent with the guidance of Dr Alex Moores (UKC). The desired strain was inoculated into 10 mL LB and grown overnight at 37°C, 180 RPM. 50 µL of this culture was inoculated into 50 mL LB and was incubated at 37°C, 180 RPM until an OD600 0.5 was reached. 3.75 mL pre-warmed sterile 100% glycerol was added slowly to the flask 5 mins before reaching OD600 0.5. After OD600 0.5 was reached, the cells were chilled on ice for 10 mins. The cells were centrifuged for 10 mins at 4000 RPM, 4°C and the supernatant discarded. Cells were resuspended gently in 50 mL of ice-cold magnesium chloride solution (see Section 2.15 for details). Cells were pelleted by centrifugation for 8 mins at 3800 RPM and the supernatant was discarded. The cell pellet was resuspended gently in 12.5 mL ice cold T

salts (containing $CaCl_2$ and $MgCl_2$, see Section 2.15 for details) and incubated on ice for 20 mins with occasional mixing. Cells were pelleted at 4ºC at 3600 RPM for 6 mins then the cells were finally resuspended gently into 2.5 mL T salts. Competent cells were split into aliquots of 200 µL into pre-chilled tubes and stored immediately at -80 ºC. All steps above were performed in a sterile environment and cells remained on ice for as long as possible.

2.12.3.2 Transformation protocol

Competent cells were thawed on ice for 20-30 mins before addition of exogenous DNA. 1 µL of purified plasmid (approximately 150 ng/µL) was mixed with 50 µL competent cells then incubated for 30 mins on ice. 50 µL competent cells only were used as a negative control. The cells were then heat shocked at 42ºC for 45 seconds to allow uptake of exogenous DNA through the disrupted membrane. Cells were then incubated on ice for 2 mins for recovery and retention of the exogenous DNA. 500 µL sterile LB was added to each reaction then incubated at 37ºC for 1 hour. 50 µL was then spread on a LB plate containing 25 µg/mL chloramphenicol for selection of cells containing the desired plasmid. To concentrate the cells further, 450 µL cells were centrifuged at 3500 RPM for 3 mins. The supernatant was removed and cells were resuspended in 50 µL LB and spread on to LB plate containing 25 µg/mL chloramphenicol. All plates were incubated at 37ºC overnight.

2.12.4 DNA isolation

2.12.4.1 Isolation of genomic DNA

Genomic DNA from *E. coli* MG1655 was supplied by Dr Alex Moores (UKC).

2.12.4.2 Isolation of *fimA* and *fimC* inserts from genomic DNA

Table 2.23 PCR to isolate *fimA* and *fimC* genes from genomic DNA

Volume (µL)	Component
25	2x Q5 Master Mix
2.5	Forward primer (10 µM)
2.5	Reverse primer (10 µM)
19	MQ water
1	Genomic DNA from *E.coli* MG1655 (201 ng/L)

2.12.4.3 Isolation of plasmid DNA

A sterile pipette tip was used to pick up a colony of interest from a successful transformation. This pipette tip containing the colony was then used as described in Figure 2.5.

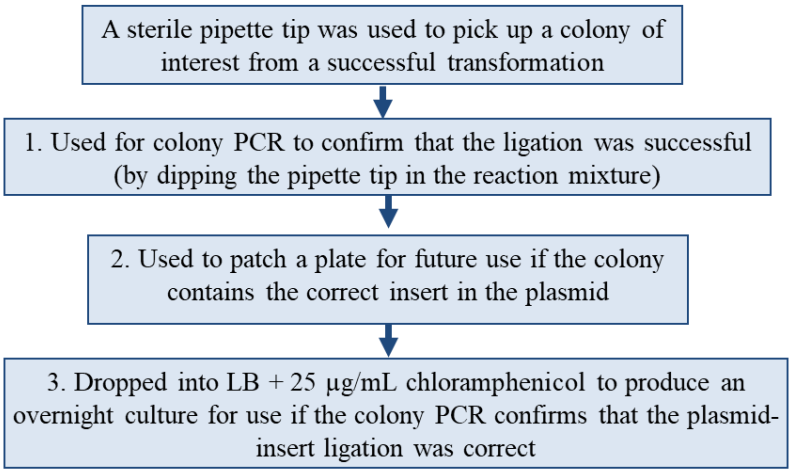


Figure 2.5 Procedure for the isolation of DNA for colony PCR

Cells were grown in LB with 25 µg/mL chloramphenicol overnight (37ºC, shaking at 180 RPM). 5 mL of culture was used and plasmid DNA was isolated using QIAprep® Spin Miniprep Kit (Qiagen). Isolated plasmid DNA was eluted using either 50 µL sterile MQ water or 50 µL supplied EB buffer (10 mM Tris·HCl, pH 8.5). DNA concentration was determined by using a NanoDrop Spectrophotometer.

2.12.5 Preparation of plasmid and inserts for ligation (*fimA/fimC* inserts)

2.12.5.1 Preparation of pJB005 plasmid for ligation

Undigested pJB005 plasmid was provided by Dr Alex Moores (UKC).

2.12.5.2 Plasmid digestion with restriction enzymes NdeI and AscI

The reaction was set up as follows (Table 2.24). NdeI restriction enzyme was added and the mixture was incubated for 1 hour at 37°C. AscI was then added and incubated for another 1 hour at 37°C. NdeI enzyme needs 3 base pairs or more on each side of the cleavage site to recognise the sequence (NEB catalogue 2013-2014, page 330). For this reason, NdeI was added first then AscI was added 1 hour later.

Table 2.24 Digestion of plasmid with restriction enzymes NdeI and AscI

Volume (µL)	Component
3	10x NEB Cutsmart enzyme buffer
7	pJB005 plasmid (to make 1 µg DNA)
18	Sterile MQ water
1	NdeI restriction enzyme (20 Units/µL)
1	AscI restriction enzyme added after 1 hour (10 Units/µL)

2.12.5.3 Gel extraction of plasmid to prevent re-ligation

6 µL 6x loading dye was added to the 30 µL reaction above and ran on a 1% agarose gel. 12 µL was loaded into 3 consecutive wells. The location of the plasmid was determined using a UV box and the bands from all 3 lanes were extracted using a scalpel. The plasmid was extracted using QIA quick gel extraction kit (Qiagen) and quantified using the NanoDrop spectrophotometer. The extracted plasmid was used immediately for ligation.

2.12.5.4 Preparation of *fimA* and *fimC* inserts for ligation (restriction digest)

Gel extraction of *fimA* and *fimC* insert DNA

fimA and *fimC* inserts were extracted from genomic DNA as described above. In order to purify *fimA/fimC* only (and no other genomic DNA), a gel extraction was performed. 30 µL of the PCR reaction was mixed with 6 µL 6x loading dye and ran on a 1% (w/v) agarose gel. 15 µL was loaded into 2 consecutive wells. The location of the DNA was determined using a UV box and the bands from both lanes were extracted using a scalpel. The plasmid was extracted using QIA quick gel extraction kit (Qiagen) and quantified using the NanoDrop spectrophotometer. The extracted insert DNA was used immediately for ligation.

Digestion with restriction enzymes NdeI and AscI

The reaction was set up as follows (Table 2.25) and incubated for 2 hrs at 37 °C.

Table 2.25 Digestion of inserts with restriction enzymes NdeI and AscI

Volume (µL)	Component
6	10x NEB Cutsmart enzyme buffer
45	*fimA/fimC* extracted DNA (to make 1 µg)
7	Sterile MQ water
1	NdeI restriction enzyme (20 Units/µL)
1	AscI restriction enzyme (10 Units/µL)

PCR clean up

Immediately after digestion, restriction enzymes were removed using QIAquick PCR Purification Kit.

2.12.6 Preparation of plasmid and inserts for ligation (Oligo insert)

2.12.6.1 Preparation of pJB005 plasmid for ligation

Plasmid digestion with restriction enzymes NdeI and AscI

The reaction was set up as follows (Table 2.26). NdeI restriction enzyme was added and the mixture was incubated for 1 hour at 37 °C. AscI was then added and incubated for another 1 hour at 37 °C.

Table 2.26 Restriction digest of pJB005 plasmid with restriction enzymes NdeI and AscI

Volume (µL)	Component
3	10x NEB Cutsmart enzyme buffer
7	pJB005 plasmid (to make 1 µg DNA)
18	Sterile MQ water
1	NdeI restriction enzyme (20 Units/µL)
1	AscI restriction enzyme added after 1 hour (10 Units/µL)

PCR clean up

Restrictions enzymes were removed from the DNA after digestion using the QIAquick PCR Purification Kit (Qiagen) using the manufacturer's instructions.

2.12.6.2 Preparation of Oligos for ligation

Phosphorylation

Oligos were phosphorylated using the reagents listed in Table 2.27 and incubated at 37°C for 45 mins.

Table 2.27 Phosphorylation of Oligo DNA

Volume (µL)	Component
6	Oligo DNA (10 µM)
5	10 mM ATP
5	10x T4 Polynucleotide Kinase Reaction Buffer
37	Sterile MQ water
2	T4 Polynucleotide Kinase

Annealing forward and reverse Oligos

50 µL forward primer oligo for FimA signal peptide was mixed with 50 µL associated reverse primer oligo for FimA signal peptide. 11 µL annealing buffer was added and the tube was added to a heat block and incubated at 95ºC for 5 mins. The heat block was then turned off and left to cool to room temperature

2.12.7 Ligation protocol

2.12.7.1 Ligation of plasmid with *fimA* and *fimC* insert

The following ligation mixtures were prepared with varying ratios of Insert:Vector (see Table 2.28).

Tables 2.28 Ligation mixtures prepared with differing ratios of Insert:Vector

0:1 Insert:Vector

Volume (µL)	Component
0	Digested PCR insert
1	Digested vector (5 ng/µL)
1	10x ligase buffer (Promega C1268)
1	T4 DNA ligase (Promega M180A)
7	Sterile MQ water

1:1 Insert:Vector

Volume (µL)	Component
1	Digested PCR insert
1	Digested vector (5 ng/µL)
1	10x ligase buffer (Promega C1268)
1	T4 DNA ligase (Promega M180A)
6	Sterile MQ water

3:1 Insert:Vector

Volume (µL)	Component
3	Digested PCR insert
1	Digested vector (5 ng/µL)
1	10x ligase buffer (Promega C1268)
1	T4 DNA ligase (Promega M180A)
4	Sterile MQ water

5:1 Insert:Vector

Volume (µL)	Component
5	Digested PCR insert
1	Digested vector (5 ng/µL)
1	10x ligase buffer (Promega C1268)
1	T4 DNA ligase (Promega M180A)
2	Sterile MQ water

All tubes were centrifuged briefly to spin down then incubated at 4ºC overnight. 50 µL DH5α competent cells were transformed with 5 µL ligation reaction.

2.12.7.2 Ligation of plasmid with Oligo DNA

Ligation of plasmid with Oligo DNA was performed as described below in Table 2.29.

Table 2.29 Ligation of plasmid with annealed Oligo DNA

Volume (µL)	Component
0.5	Annealed Oligos (annealed Oligos diluted 1 in 6 with sterile MQ water)
0.5	Digested pJB005 plasmid (5 ng/µL DNA)
1	10x ligase buffer (Promega C1268)
1	T4 DNA ligase (Promega M180A)
7	Sterile MQ water

Negative control

Volume (µL)	Component
0.5	Digested pJB005 plasmid (5 ng/µL DNA)
1	10x ligase buffer (Promega C1268)
1	T4 DNA ligase (Promega M180A)
7.5	Sterile MQ water

All tubes were centrifuged briefly to spin down then incubated at 4ºC overnight. 50 µL DH5α competent cells were transformed with 5 µL ligation reaction.

2.12.8 DNA analysis techniques

2.12.8.1 Colony PCR

Colony PCR reactions were set up as described in the Table 2.30 below. A negative control was run using undigested plasmid only (no colonies). See Appendix A.3-A.4 for results.

Tables 2.30 Reaction mixtures set up for colony PCR

Volume (µL)	Component
12.5	2x PCRBIO Taq Mix Red (PCR Biosystems)
1.25	Forward primer for plasmid (10 µM)
1.25	Reverse primer for plasmid (10 µM)
10	MQ water
0	Colony of interest (colony taken from patch and mixed straight into the colony PCR reaction)

Negative control:

Volume (µL)	Component
12.5	2x PCRBIO Taq Mix Red (PCR Biosystems)
1.25	Forward primer for plasmid (10 µM)
1.25	Reverse primer for plasmid (10 µM)
9	MQ water
1	Undigested plasmid (pJB005)

Colony PCR reactions were added to the PCR machine and ran using settings in Table 2.31 below.

Table 2.31 PCR reaction program used for colony PCR

Stage of PCR	Temperature (°C)	Time (minutes)	Number of cycles
1. Initial denaturation	95	3	1
2a. Denaturation	95	1	30
2b. Annealing	53	1	
2c. Elongation	72	2	
3. Final extension time	72	5	1
4. Storage	4	∞	N/A

2.12.8.2 Agarose gel electrophoresis

1% or 2% (w/v) agarose gels were made by dissolving agarose in 1x TAE buffer by heating in a microwave. Once fully dissolved, this was then poured into a gel mould (Fisher Scientific) and left to set for 20-30 mins. 5 µL DNA 1KB ladder (Promega) was loaded into the first well of all gels to estimate DNA fragment sizes. 6x loading dye was added to samples (unless the reaction contained Taq polymerase) and 5 µL was loaded into each well unless otherwise specified. Gels were run at 150 V for 25 mins then stained for 30 mins in 0.5 µg/mL ethidium bromide in MQ water. Bands were visualised using G:Box machine by SynGene and associated software.

2.12.8.3 Double digest of plasmid to confirm insert

pJB005 plasmid containing *fimA* insert

The reactions were set up as follows (Table 2.32) and incubated for 1 hour at 37 °C.

Table 2.32 Double digest of plasmids containing inserts to confirm the gene has been inserted correctly

Volume (µL)	Component
1	pJB005 with *fimA* insert (160 ng/µL)
1	10x Promega enzyme buffer
1	PstI restriction enzyme (10 Units/µL)
1	NdeI restriction enzyme (20 Units/µL)
6	Sterile MQ water

Volume (µL)	Component
1	pJB005 only (empty vector)
1	10x Promega enzyme buffer
1	PstI restriction enzyme (10 Units/µL)
1	NdeI restriction enzyme (20 Units/µL)
6	Sterile MQ water

pJB005 plasmid containing *fimC* insert

The reactions were set up as follows (Table 2.33) and incubated for 1 hour at 37 °C.

Table 2.33 Double digest of plasmids containing inserts to confirm the gene has been inserted correctly

Volume (µL)	Component
1	pJB005 with *fimC* insert (214 ng/µL)
1	10x Promega enzyme buffer
1	AscI restriction enzyme (10 Units/µL)
1	EcoRI restriction enzyme (20 Units/µL)
6	Sterile MQ water

Volume (µL)	Component
1	pJB005 only (empty vector)
1	10x Promega enzyme buffer
1	AscI restriction enzyme (10 Units/µL)
1	EcoRI restriction enzyme (20 Units/µL)
6	Sterile MQ water

2 µL 6x loading dye was added to each sample. 5 µL was then loaded into each well of a 1% agarose gel and stained using ethidium bromide. See Appendix A.5-A.6 for results.

2.12.8.4 DNA sequencing

Samples were shipped to Genewiz for Sanger Sequencing. Plasmids to be sequenced were diluted to 100 ng/µL using autoclaved MQ water. These were sent with the relevant primers which were diluted to 5 µm (5 pmol/µL). All inserts were confirmed as the correct sequence. See Appendix A for results.

2.13 *Streptomyces* S4 specific techniques

2.13.1 Bioassay of *Streptomyces* S4 cells and MVs on *C. albicans*

Preparation of MVs for ZOI plates was outlined in Figure 2.6.

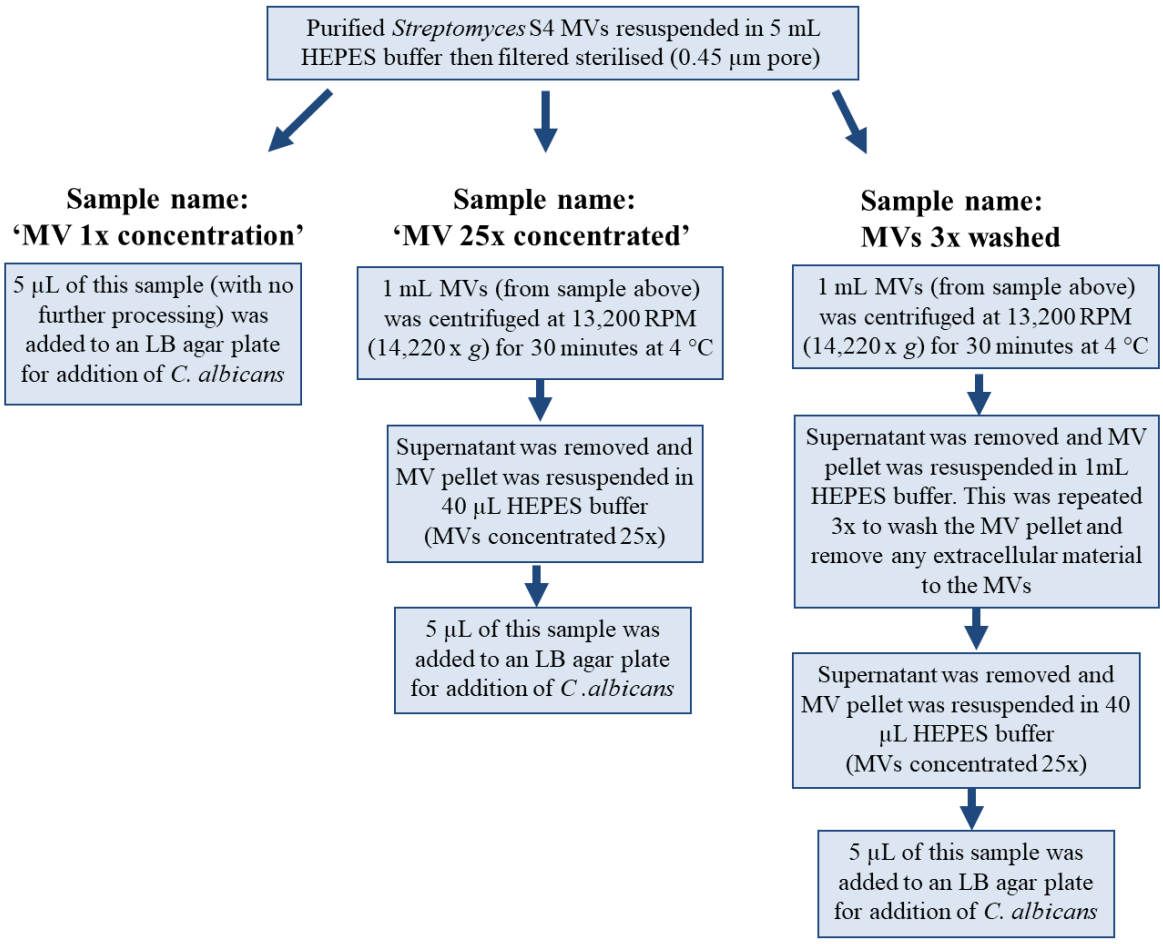


Figure 2.6 Preparation of MVs for zone of inhibition plates with *C. albicans*

2.13.1.1 Preparation of *Streptomyces* S4 WT MVs with various number of washes in 10 mM HEPES buffer (Figure 2.7).

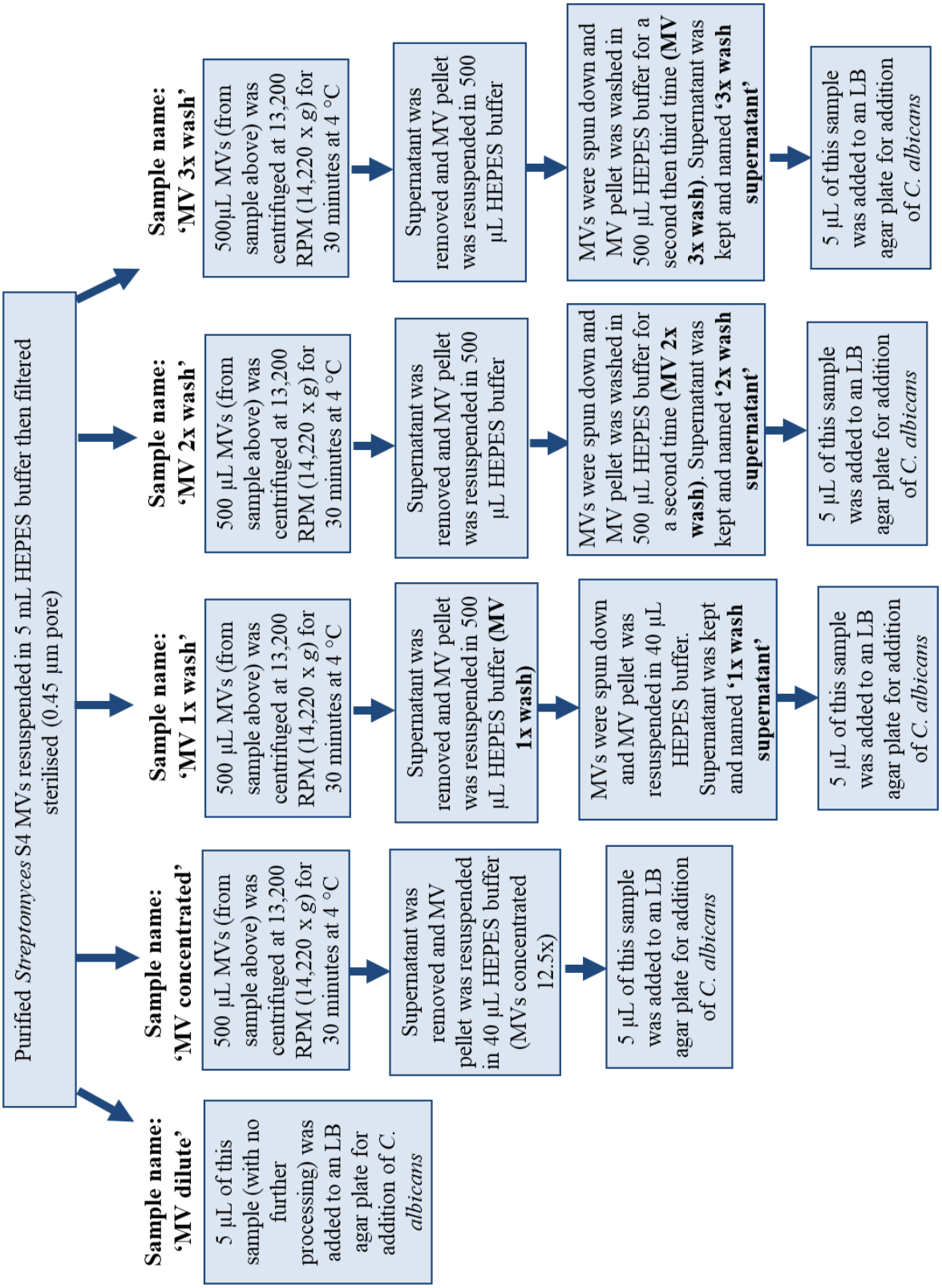


Figure 2.7 Preparation of MVs with various numbers of wash steps for zone of inhibition plates with *C. albicans*

2.13.1.2 Effect of *Streptomyces* S4 cells on *C. albicans* growth

Streptomyces S4 colonies (from MS plates) were inoculated into TSB:YEME (two media mixed together in a 1:1 ratio) and incubated at 30ºC, 180 RPM for 72 hrs. 5 µL of this culture was spotted at the centre of an LB agar plate and left to soak/dry for 2 hrs. Plates were incubated at 30ºC for 72 hrs. *C. albicans* was added to the plates and the zones of inhibition (ZOI) were measured. All plates were prepared in triplicate and the average ZOI and standard deviation was calculated for each strain.

2.13.1.3 Effect of candicidin and antimycin on *C. albicans* growth

Candicidin (Bioaustralis) and antimycin (Sigma) were resuspended in ethanol to give 1 mg/mL concentration. These stocks were then diluted with ethanol to give various concentrations ranging from 1 mg/mL to 1 µg/mL. All candicidin stocks were stored at -20ºC and antimycin at 4ºC. 10 µL candicidin/antimycin at each concentration was added to LB plates and left to soak/dry for a minimum of 2 hrs at room temperature. 10 µL ethanol only was used as a negative control. *C. albicans* was added to the plates and the zones of inhibition (ZOI) were measured. All plates were prepared in triplicate and the average ZOI and standard deviation was calculated for each concentration of antifungal.

2.13.1.4 Effect of *Streptomyces* S4 MVs on *C. albicans* growth

5 µL or 10 µL purified MVs (depending on experiment) were added to LB plates and left to soak/dry for a minimum of 2 hrs at room temperature. *C. albicans* was added to the plates and the zones of inhibition (ZOI) were measured.

2.13.1.5 *C. albicans* addition to plate

C. albicans was streaked from the -80 ºC glycerol stock on to a YPD agar plate and incubated for 18-24 hrs at 37°C. Colonies were inoculated into YPD media and incubated at 37ºC for 18-24 hrs in preparation for the experiment. 5 µL MVs/cells/antifungals were added to LB plates and left to soak/dry for a minimum of 2 hrs at room temperature. The OD600 of *C. albicans* was measured then diluted to OD600 1.0. Cells were centrifuged at 5000 RPM for 5 mins and the supernatant was discarded. *C. albicans* cell pellets were resuspended into 50 mL 'hand hot' LB agar (0.5% w/v) then 10 mL was slowly added to each plate. Plates were incubated at 37ºC for 18 hrs and the diameter of the ZOI measured using a ruler in mm. All plates were prepared in triplicate and the average ZOI and standard deviation was calculated for MVs from each strain.

2.13.2 Identification of candicidin in *Streptomyces* S4 MVs

2.13.2.1 Butanol extraction

Streptomyces S4 MVs were purified from all 4 strains using the usual protocol (Section 2.6.3). After the final spin at 16,000 RPM (25, 805 x *g*) to pellet the MVs, the following 3 methods were used to extract the antifungal components for further analysis (see Figure 2.8).

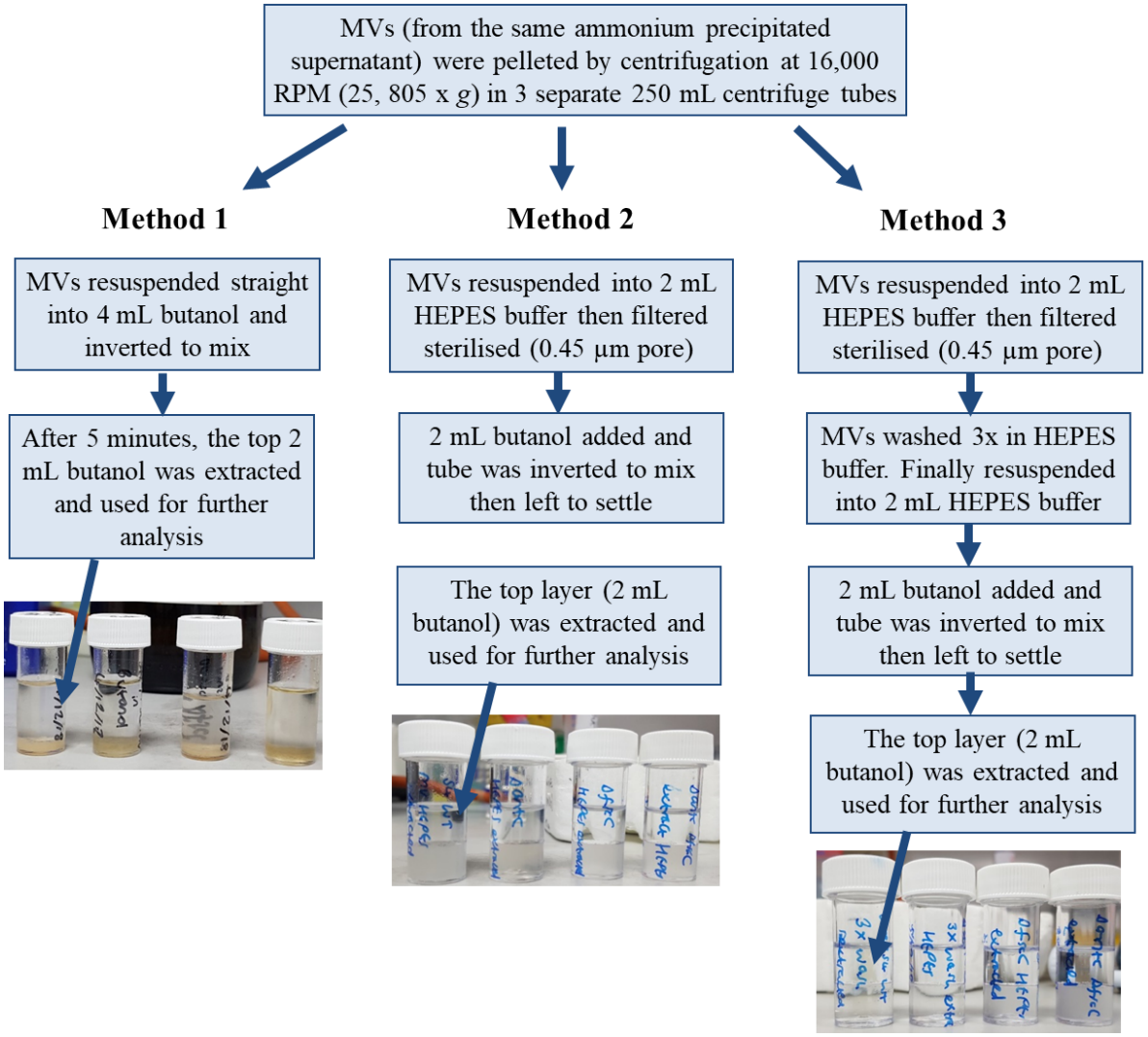


Figure 2.8 Different methods of butanol extraction trialled on *Streptomyces* S4 MVs to identify candicidin

Analysis of candicidin/antimycin by UV-Vis spectrophotometer

UV-Vis spectrum was determined using the Cary spectrophotometer.

Chapter 3

Optimisation of techniques for OMV purification and characterisation

3.1 Introduction

3.1.1 Optimisation of OMV purification procedure

In order to complete the project aims outlined in Section 1.11, optimisation of OMV isolation and characterisation was essential. The aim was to optimise a method to purify OMVs reproducibly, giving the best yield possible. The purified OMVs should be intact with as few contaminants from the bacterial cell as possible. Methods were also developed to characterise the purified OMVs and determine the differences in OMV composition and cargo between different bacterial strains and compared with the whole cell. Various methods are used to purify OMVs within the literature and this is summarised in Figure 3.1.

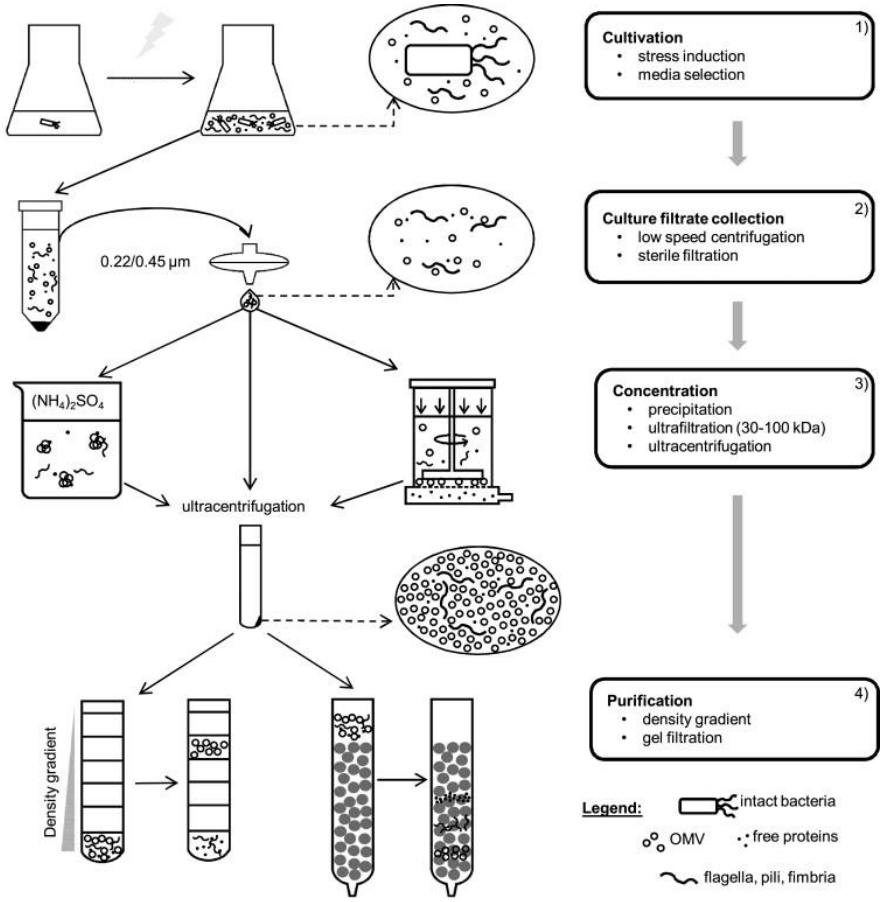


Figure 3.1 Summary of different methods of OMV purification used in the literature
Bacterial strains are cultured (1) then centrifuged to remove whole cells. OMV production can be increased if the cells are under stress. The supernatant from the centrifugation is filtered to remove any whole cells (2). OMVs can be concentrated by precipitation (for example with ammonium sulphate) or ultrafiltration (3). Lastly, OMVs can be purified further using either gel filtration or density gradient ultrafiltration (4). Image sourced from Klimentová *et al.* 2015.

The starting point to develop this protocol was to optimise one that had been successfully used to purify OMVs by a previous student of Dr Gary Robinson (Dr Luisa de Sordi). This protocol involved growing the bacteria of interest overnight in LB, centrifuging the culture then filtering the supernatant through a PES membrane filter to remove any remaining bacterial cells. The supernatant was treated with 1.5 M ammonium sulphate to precipitate the OMVs so that they could be concentrated with another centrifugation step at 16,000 RPM (25,805 x *g*). Lastly, any remaining contaminants were removed using buoyant density ultracentrifugation. Ultracentrifugation is often the final step in OMV isolation protocols as it separates the OMVs from any contaminating proteins or extracellular appendages which may have been co-purified with the OMVs (such as Type 1 fimbriae or flagella). If performed correctly, OMVs settle into a lower-density region of the gradient whereas any contaminating proteins, flagella or fimbriae remain in the bottom layer.

3.1.2 Methods of OMV characterisation

During this study, OMVs were purified from one *Burkholderia cenocepacia* strain, two *Pseudomonas aeruginosa* strains (Chapter 3), thirty-three *E. coli* strains (Chapters 3-4) and four *Streptomyces* S4 strains (Chapter 5). In order to ultimately manipulate membrane vesicles for therapeutic purposes, the OMVs must first be characterised from each strain using the techniques described below. Evidence is also needed to show that the purified OMVs are genuine, entire and that the cargo within is still functional (e.g. treatment with proteases).

3.1.2.1 Visualisation of OMVs by Transmission Electron Microscopy TEM

Purified OMVs were visualised using electron microscopy (EM) and compared to those found in the literature to confirm that they were the correct size, shape and appearance. To observe OMV biogenesis, *E. coli* BL21 (DE3) cells and *P. aeruginosa* (*PA14*) cells were grown in LB on gold EM grids and fixed at various time points over 24 hours. The cells were examined using TEM and photos were taken at each time point. Lastly, immunolabelling using an anti-OmpA antibody was applied to OMVs purified from *E. coli* BL21 (DE3). OmpA is a major protein in the outer membrane of *E. coli* and is known to be found on the surface of *E. coli* OMVs (Figure 3.2). Detecting OmpA on the surface of purified OMVs is evidence of successful isolation.

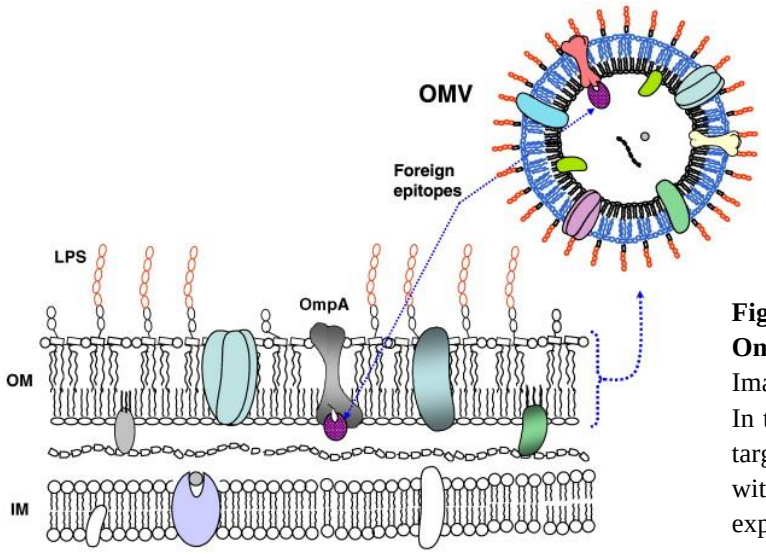


Figure 3.2 The incorporation of OmpA into *E. coli* OMVs
Image sourced from Kim *et al.* 2009. In this paper, proteins of interest were targeted to *E. coli* OMVs by fusion with OmpA. See Figure 1.23 for full explanation.

3.1.2.2 Proteome analysis of OMVs

SDS-PAGE

The protein profiles of OMVs purified from various bacterial strains were visualised using SDS-PAGE gels, which were silver stained. The protein profile of the purified OMVs was compared to that of the whole cell, periplasmic proteins and outer membrane proteins. This provides evidence that the OMVs have their own protein composition and shows which proteins are enriched and excluded from the OMVs.

Mass Spectrometry

Bands of interest in OMVs were excised from the SDS-PAGE gels and identified by mass spectrometry.

Western blotting

Western blots were performed to detect OmpA in purified OMVs from various strains of *E. coli.* This evidence complements the immunogold labelling images of *E. coli* OMVs using the anti-OmpA antibody.

3.1.2.3 Evidence that purified OMVs are intact: Proteinase K treatment of OMVs

The Proteinase K test was used to confirm that isolated OMVs are intact and to distinguish which proteins are outside the OMVs or within the lumen. This test is based on an experiment found in the literature (Figure 3.3). The principle is that the enzyme Proteinase K will degrade most proteins outside of the OMVs. However, proteins within the lumen of the OMVs will be protected from Proteinase K degradation. OMVs were incubated with Proteinase K in the presence and absence of SDS. SDS is a detergent and lyses OMVs which allows Proteinase K into the interior lumen of the OMVs where it can degrade proteins.

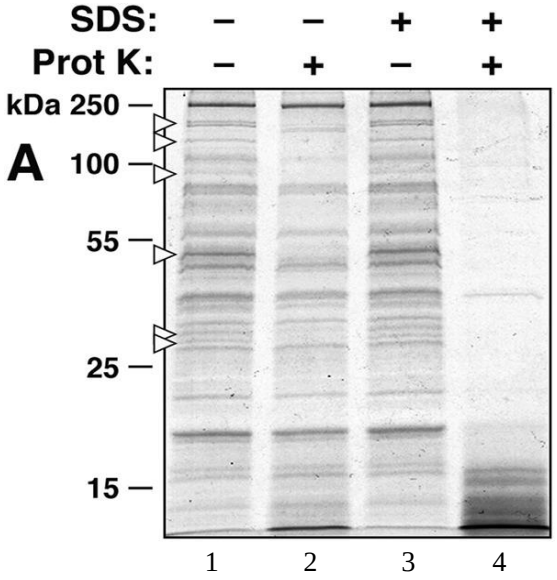


Figure 3.3 Proteinase K test on OMVs from *Francisella novicida* in the literature
OMVs were treated (or untreated) with 10 μg/ml Proteinase K in the presence or absence of 0.02% (w/v) SDS to disrupt vesicle integrity. OMVs were incubated for 1 hour at room temperature then 0.1 mM PMSF was added to inhibit the protease. The white arrows indicate which proteins in the OMVs are susceptible to Proteinase K digestion when no SDS is added (comparing lanes 1 and 2). Experiment, methodology and image sourced from McCaig *et al.* 2013

3.1.2.4 Quantification of OMVs

Bradford assay

OMVs can be quantified by measuring either the protein or lipid components in the sample. Proteins in OMV samples can be quantified using a Bradford assay (Bradford, 1976). This method was used throughout the project to quantify OMVs and standardise the samples prior to loading on an SDS-PAGE gel.

3.1.2.5 Dynamic light scattering (DLS)

DLS is used to determine the number, intensity and size distribution of nanosized particles in a solution. Brownian motion is the random movement of particles in a solution due to constantly colliding with solvent molecules around them. Smaller particles move faster than larger molecules in solution. DLS is based on measuring the Brownian motion of particles in a solution which can be used to determine particle size.

During DLS, the sample in the cuvette is illuminated by a laser beam. The particles (for example OMVs resuspended in buffer) scatter the light of the laser beam in all directions. The scattered light is detected at a certain angle which is known as the scattering angle θ. Here, a photon detector analyses the fluctuation of the scattered light (Figure 3.4 **a**). The DLS can analyse the scattered light and use this information to estimate particle size distribution and numbers. Smaller particles give faster fluctuations of scattered light than larger particles Figure 3.4 **b**.

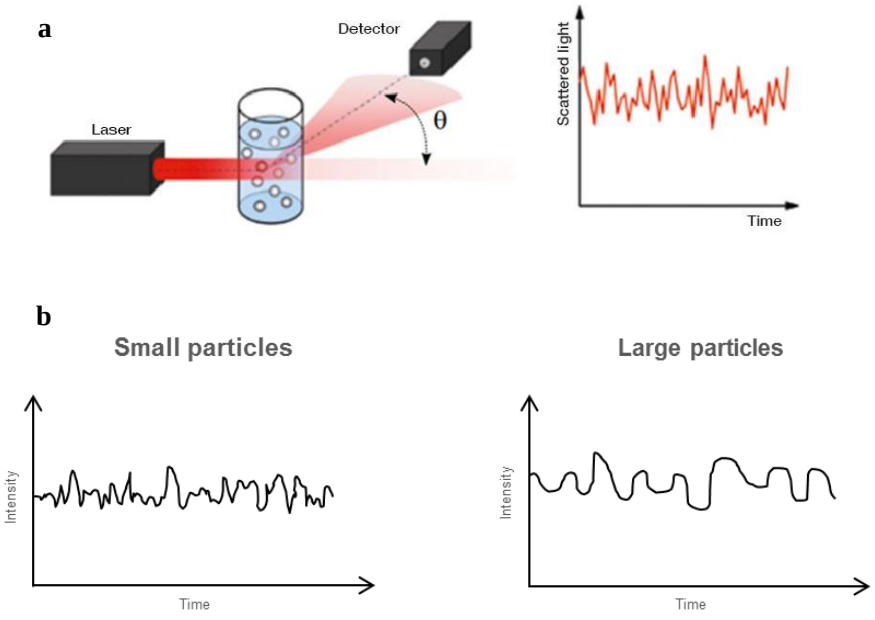


Figure 3.4 Diagram showing the main principle of DLS technique
Diagram to show the main components of Dynamic Light Scattering (DLS). Firstly, the sample in the cuvette is illuminated by a laser beam. The particles scatter the light of the laser beam in all directions. A photon detector analyses the fluctuation of the scattered light (**a**). Smaller particles give faster fluctuations of scattered light than larger particles (**b**). Image sourced from Anton Paar website, 2019

This technique has been used to estimate the size of *E. coli* OMVs in the literature (Bielaszewska *et al.* 2017). The Litesizer 500 (Anton Paar) used in this study gives a value called the Mean Intensity for each sample run using the DLS setting. This gave the mean light intensity detected in kcounts/s and was trialled to quantify the OMVs.

3.1.3 Bacterial strains used to study OMVs

Table 3.1 Brief introduction to the bacterial strains used in Chapter 3. See Section 1.11 for rationale for using these strains.

Strain name	Strain characteristics/additional information
B. cenocepacia J2315	Pathogenic strain isolated from a cystic fibrosis patient
E. coli B Parental (B strain)	*E. coli* B wildtype strain. Parental strain of *E. coli* BL21 and BL21 (DE3)
E.coli BL21 (B strain)	Competent *E.coli* B strain for routine non-T7 expression. Deficient in proteases Lon and OmpT. No flagella or fimbriae produced. Resistant to phage T1
E.coli BL21 (DE3) (B strain)	Identical to *E.coli* BL21 except this strain contains the λDE3 lysogen that carries the gene for T7 RNA polymerase under control of the lacUV5 promoter
E.coli FimB-LacZ fusion (K-12 strain)	This strain contains a deletion of the Lac operon (*lacZYA*) and *fimB* and an insertion of a FimB-LacZ fusion protein. **Fimbriae production is locked off** as FimB is no longer functional
E.coli fimbriae locked on (K-12 strain)	**Fimbriae production locked on.** The invertible element (*fimS*) is locked on due to the modifications in the parental intermediate strain
E.coli WT MG1655 (K-12 strain)	Used as the wild type strain to produce *E.coli* FimB-LacZ fusion strain and *E.coli* with fimbriae locked on
E.coli WT Parental BW25113 (K-12 strain)	The parental strain of the Coli Genetic Stock Center Keio collection (from which the knock-out strains are derived)
E.coli ΔfimA (K-12 strain)	Strain contains a knockout of the protein FimA which is the main structural subunit of Type 1 fimbriae
E.coli ΔfliC (K-12 strain)	Strain contains a knockout of the protein Flagellin (FliC) which is the main structural subunit of flagella
P. aeurginosa PA01	Two *P. aeruginosa* pathogenicity islands (PAPI-1 and PAPI-2) are absent from *PA01* which is less virulent than *PA14*
P. aeurginosa PA14	Two *P. aeruginosa* pathogenicity islands (PAPI-1 and PAPI-2) in the genome of *PA14* which is a highly virulent clinical isolate

3.1.4 Main chapter aims

1. To determine a reproducible and cost-effective method of isolating OMVs, which can be applied to all Gram-negative bacterial strains
2. To determine if OMVs can be purified with no contaminants from the bacterial cell. For example, can co-purification of proteins, flagella or fimbriae from the bacterial supernatant be prevented?
3. To gain strong evidence that the purified OMVs are genuine, entire and still functional
4. To compare the composition and cargo of OMVs between bacterial strains
5. To determine which proteins are enriched and excluded in OMVs from a range of strains

3.2 Results

3.2.1 Optimisation steps for OMV purification

The initial OMV purification protocol was taken from the thesis of Dr Luisa de Sordi. The protocol was optimised to give the standard OMV purification protocol below (Figure 3.5). After centrifugation at 16,000 RPM (25,805 x *g*), an OMV pellet is produced which is often visible. This pellet was either resuspended in 10 mM HEPES buffer for further analysis or resuspended into 45% (v/v) OptiPrep so the OMVs could be purified further by ultracentrifugation.

3.2.1.1 OMV purification protocol summary

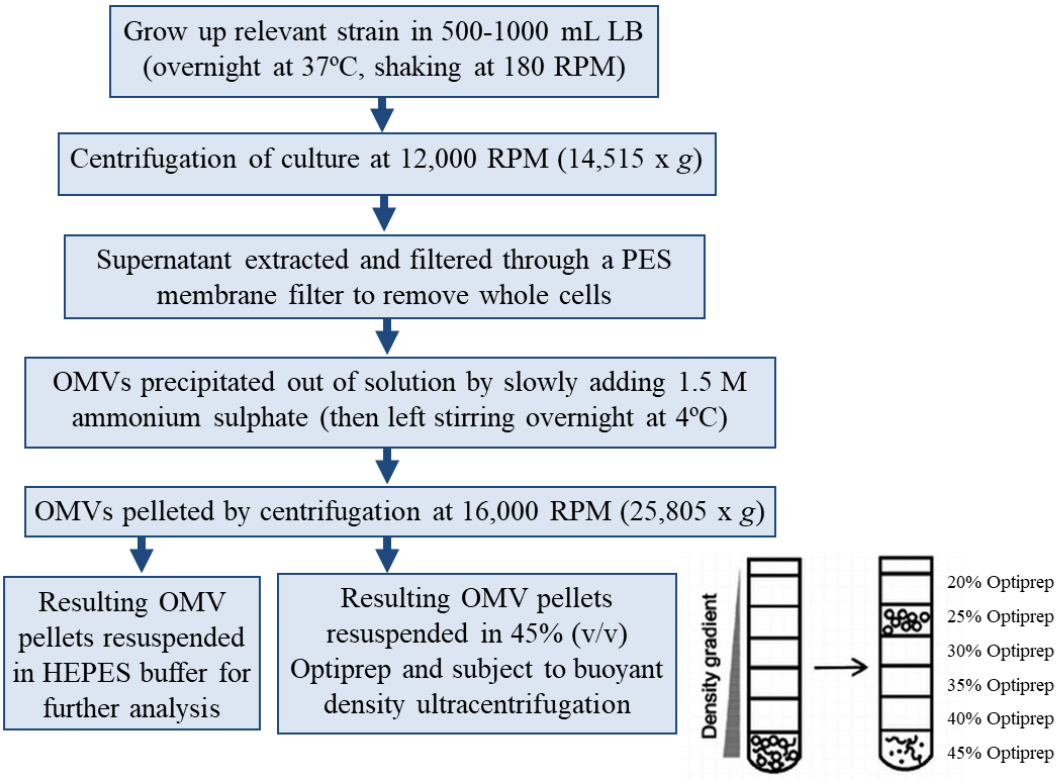


Figure 3.5 Standard OMV purification protocol

An OMV purification protocol was optimised and used as the standard for all experiments unless otherwise stated. This flow chart is a simplified summary of the OMV purification procedure. Ultracentrifugation diagram was adapted from: (Klimentová, Stulik 2015)

3.2.1.2 How reproducible is the OMV purification protocol?

OMVs purified from *E. coli* BL21 (DE3) on five different dates were compared. This was to confirm that the OMV purification protocol developed was reproducible. Firstly, the TEM images of the OMVs were compared (Figure 3.6) and then the protein profile of the purified OMVs (Figure 3.7**a**). Protein densitometry plots were also generated using Fiji (Image J) to compare the protein profile of the MVs from each strain (Figure 3.7**b**).

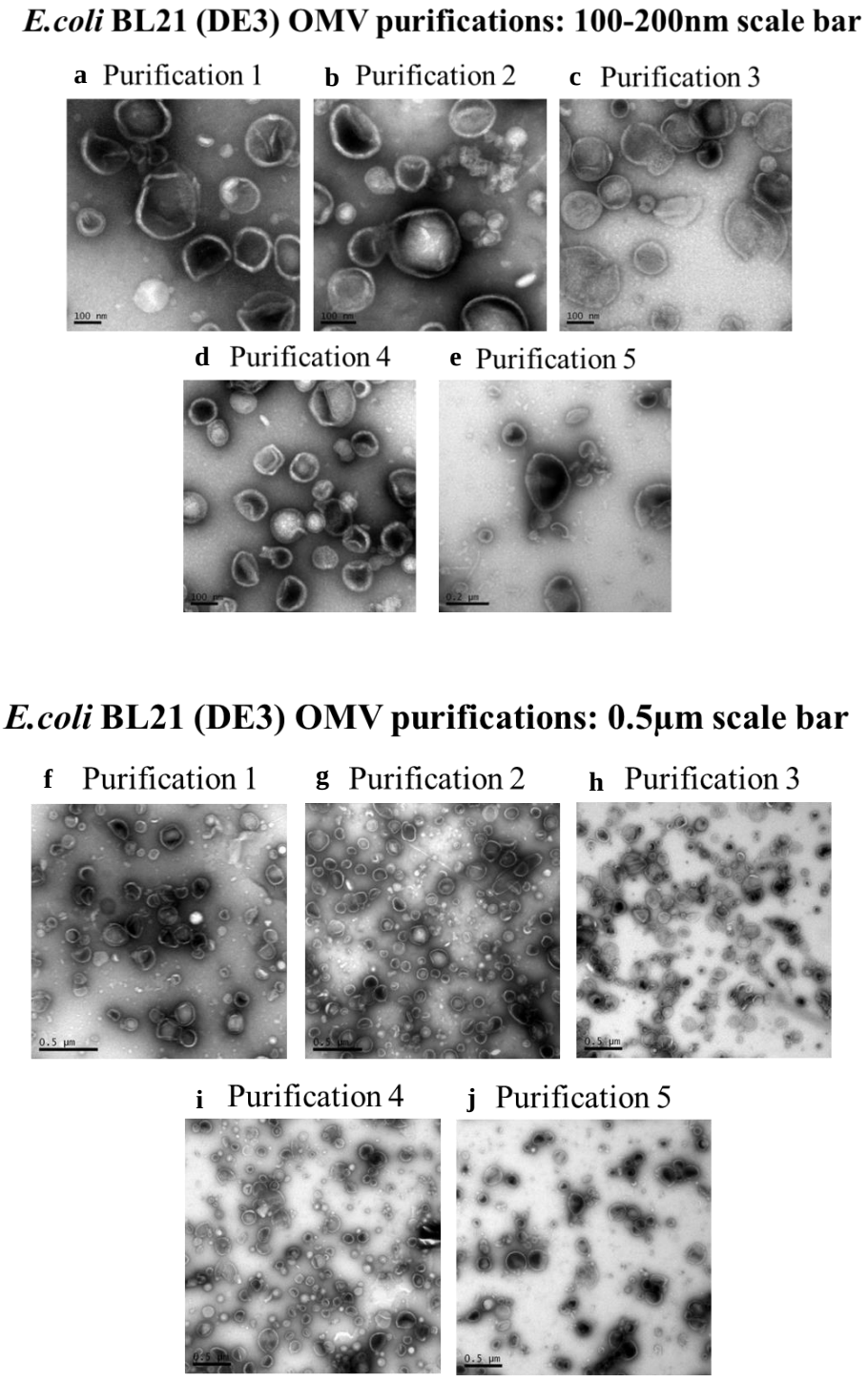
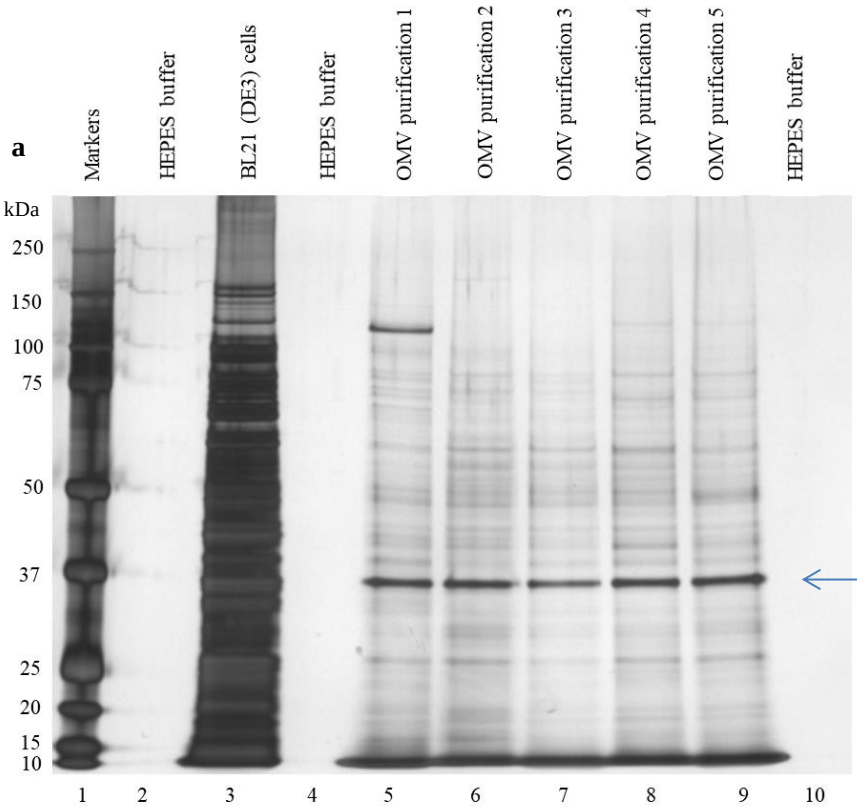


Figure 3.6 Comparison of OMVs purified from *E. coli* BL21 (DE3) on five separate occasions
TEM images of OMVs from *E. coli* BL21 (DE3) purified on five separate occasions. 'Purification 1' was performed on 30/06/16 (**a, f**). 'Purification 2' was performed on 14/02/17 (**b, g**). 'Purification 3' was performed on 23/01/17 (**c, h**). 'Purification 4' was performed on 06/12/16 (**d, i**). 'Purification 5' was performed on 17/01/17 (**e, j**).

a



b

Lane number on SDS-PAGE gel (Figure 3.6a)	Protein concentration (µg/mL)	OD600 after 18 hours growth at 37°C
5	26	5.07
6	35	4.75
7	21	4.74
8	22	4.86
9	25	4.89

Figure 3.7 Comparison of the protein profiles of OMVs purified from *E. coli* BL21 (DE3) on five separate occasions

OMVs from *E. coli* BL21 (DE3) purified on five separate occasions. 'Purification 1' was performed on 30/06/16. 'Purification 2' was performed on 14/02/17. 'Purification 3' was performed on 23/01/17. 'Purification 4' was performed on 06/12/16. 'Purification 5' was performed on 17/01/17. A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the MV protein profile (**a**). The prominent band at around 37 kDa had previously been identified as OmpF (see Appendix B.1 for details). OMVs were quantified using a Bradford assay for comparison of yield (**b**).

The SDS-PAGE gels showed that the OMV protein profile is different to that of the cells. The protein profile is almost identical each time OMVs are purified from *E. coli* BL21 (DE3) meaning that the purification protocol is reproducible. Each time, *the E. coli* cells grew to an OD600 of approximately 5.0 and produced OMVs with an average protein concentration of 26 µg/mL (Figure 3.7 **b**).

The only major difference in the protein profiles is the extra band between 100 and 150 kDa in Purification 1 and is faintly visible in Purifications 4 and 5, which has not been identified. The prominent band in the *E. coli* BL21 (DE3) OMV protein profile at around 37 kDa had previously been identified as OmpF (see Appendix B.1). OmpF is a porin protein located in the outer membrane. One of its functions is to allow diffusion of small hydrophilic molecules across the membrane and into the periplasm (Duval *et al.* 2009). An enrichment of OmpF in OMVs is expected as it is an outer membrane protein.

3.2.1.3 Are any live bacterial cells present in the purified OMV sample when using this protocol?

During the OMV purification procedure, the cells are centrifuged at 12,000 RPM (14,515 x *g*) and the supernatant (containing the OMVs) is filtered through a PES membrane filter to remove any whole bacterial cells. Each time, 1 mL of filtered supernatant was spread on to a LB agar plate and incubated at 37°C for 24-48 hours to confirm that there was no growth. 1.5 M ammonium sulphate was then slowly added to the supernatant to precipitate the OMVs. The addition of 1.5 M ammonium sulphate to LB was shown to fully inhibit the growth of five strains of *E. coli* (Figure 3.8). These two checks strongly indicate that the OMV samples used in every study did not contain any live bacterial cells.

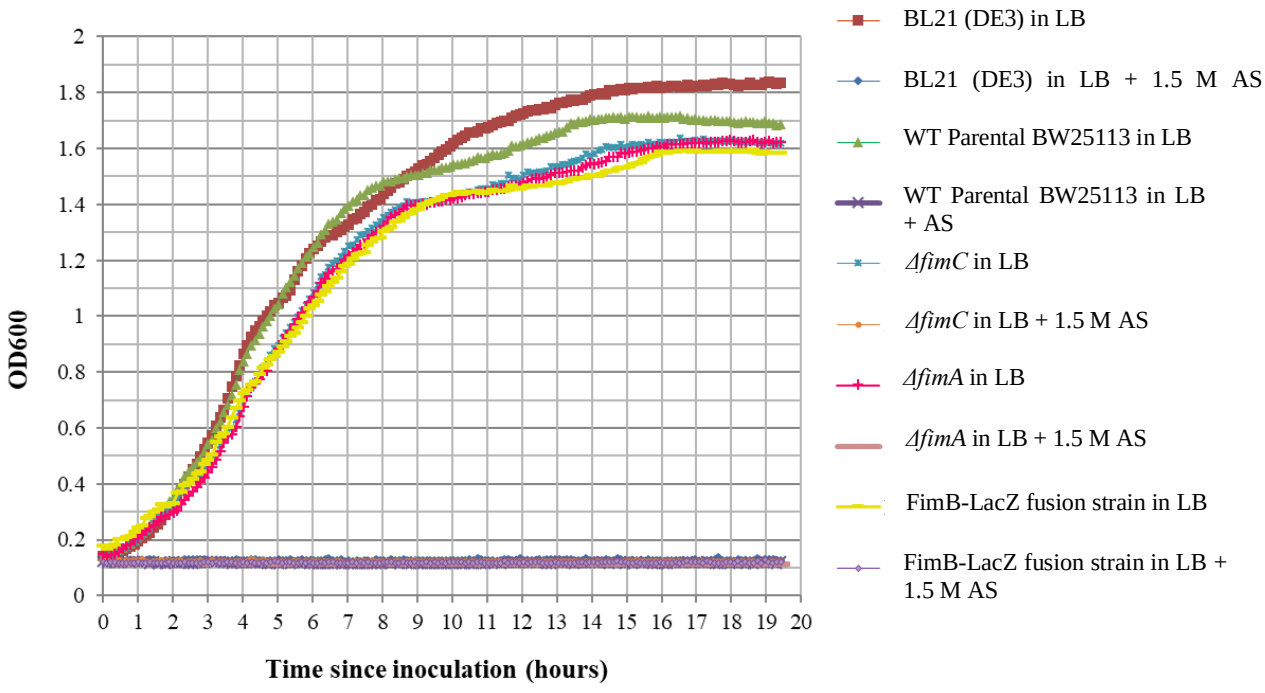


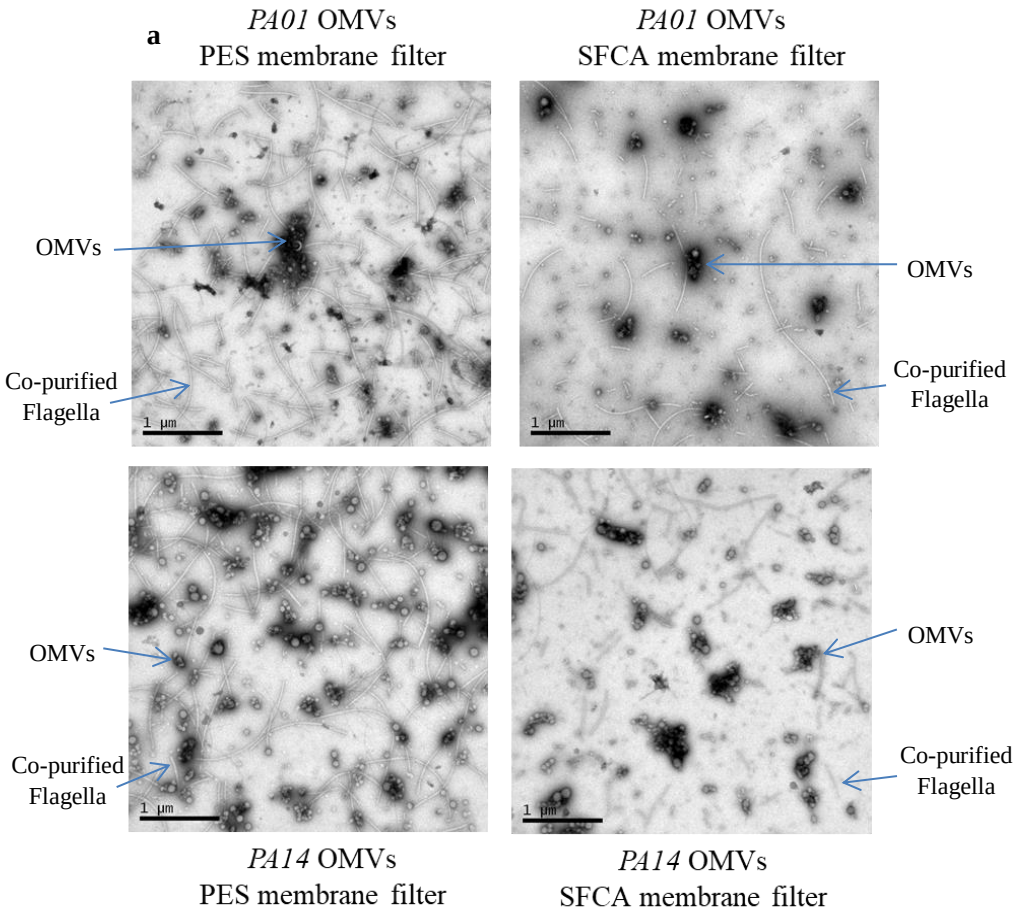
Figure 3.8 Addition of 1.5 M ammonium sulphate to LB inhibits the growth of five *E. coli* strains

Growth curves were produced by culturing *E. coli* in 48 well cell culture plates. Two identical microplate readers were used to generate growth curves at 25ºC and 37ºC concurrently. Addition of 1.5 M ammonium sulphate (AS) to LB was shown to inhibit the growth of the following *E. coli* strains at 25ºC: BL21 (DE3), WT Parental BW25113, *ΔfimA*, *ΔfimC* and FimB-LacZ fusion. For graphs with error bars of 1 standard deviation and growth curves at 37ºC see Appendix B.2.

3.2.1.4 Does the type of membrane filter used affect the OMVs purified?

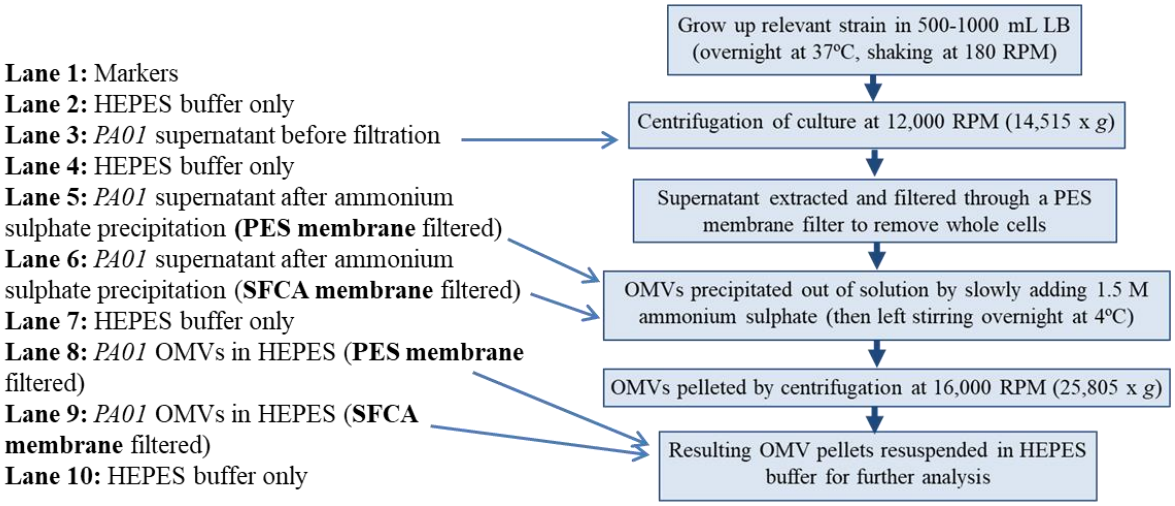
In most OMV purification protocols, there is a filtration of the bacterial supernatant through a membrane filter of pore size 0.2 µm and 0.45 µm to remove any remaining live cells. 0.2 µm pore size was used with OMVs from *P. aeruginosa* as the OMVs were less than 200 nm in diameter. The larger pore size of 0.45 µm was used for *E. coli* cells as the OMVs found were often between 200-400 nm in diameter. A comparison of the OMVs purified using a polyethersulfone (PES) membrane filter was compared to a surfactant-free cellulose acetate (SFCA) membrane filter. PES membrane filters are commonly used for cell culture media and allow rapid filtration. SFCA membrane filters, however, are described as having the lowest protein binding so may improve the purity of the OMVs (Thermo-Fisher Nalgene Filter Brochure).

To compare the OMV profile produced when using PES and SFCA membrane filters, 1 L of *PA01* and *PA14* culture supernatants were split into 2 x 500 mL. One half of the supernatant was filtered through a PES filter (Nalgene rapid-flow, Fisher 10300461) and the other half was filtered through a SFCA membrane filter (Nalgene rapid-flow, Fisher 10201371). After this step, the standard OMV purification protocol was then followed as usual and the OMVs were resuspended in 10 mM HEPES buffer (details in Section 2.15). The TEM images of the OMVs were first compared (Figure 3.9**a**) to assess the purity of the OMV sample (ie. to determine whether flagella and other debris were also present in the OMV sample). Next, the protein profiles of the OMVs were compared after purification with PES and SFCA membranes (Figure 3.9**b-f**).

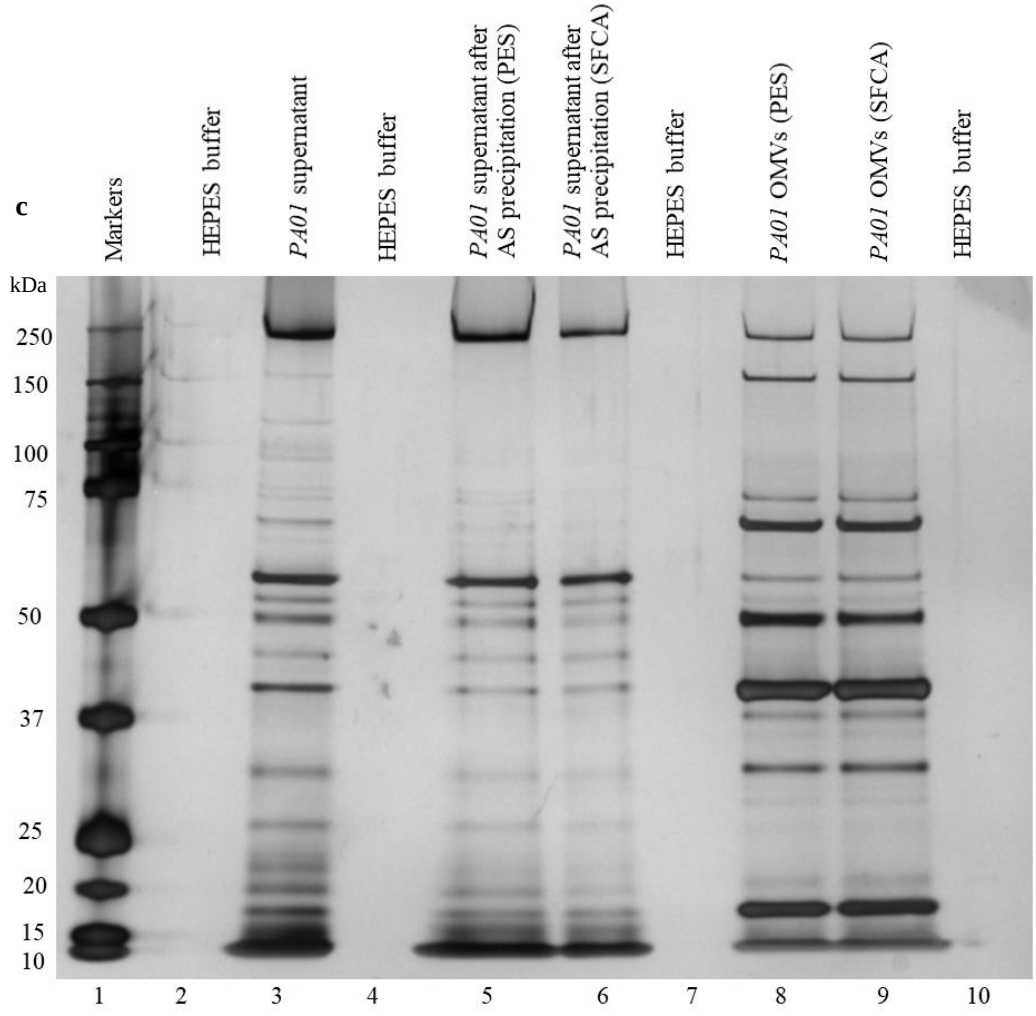


b

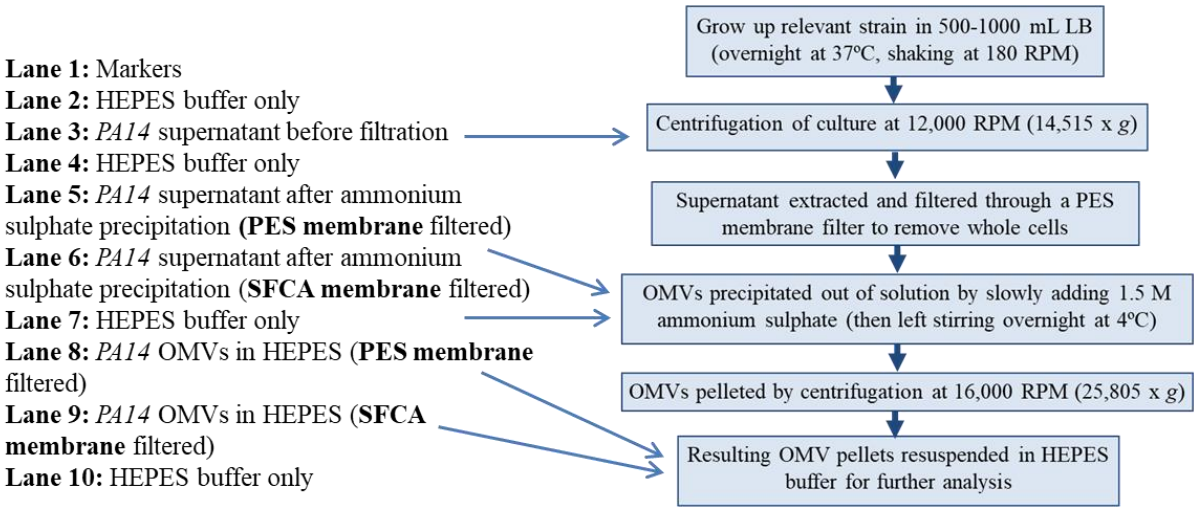
Lane 1: Markers
Lane 2: HEPES buffer only
Lane 3: *PA01* supernatant before filtration
Lane 4: HEPES buffer only
Lane 5: *PA01* supernatant after ammonium sulphate precipitation **(PES membrane** filtered)
Lane 6: *PA01* supernatant after ammonium sulphate precipitation (**SFCA membrane** filtered)
Lane 7: HEPES buffer only
Lane 8: *PA01* OMVs in HEPES (**PES membrane** filtered)
Lane 9: *PA01* OMVs in HEPES (**SFCA membrane** filtered)
Lane 10: HEPES buffer only



c



d



e

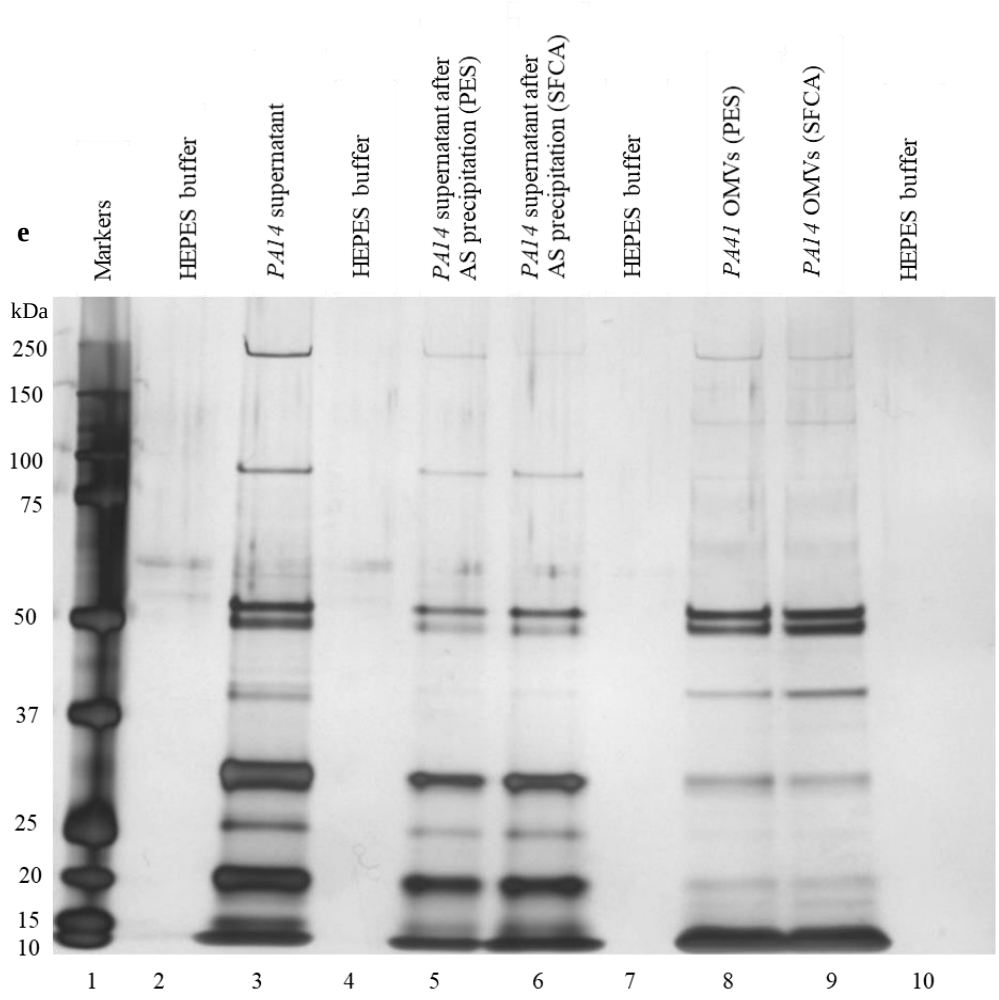


Figure 3.9 Comparing the use of PES vs. SFCA membranes during the OMV purification process
PA01 and *PA14* cultures were grown overnight in 1 L LB. The cells were pelleted and the supernatant was extracted and split into two. One half of the supernatant was filtered through a PES membrane filter and the other half was filtered through a SFCA membrane filter. After this step, the standard OMV purification protocol was followed as usual and the OMVs were resuspended in 3 mL 10 mM HEPES buffer. The OMVs were concentrated then visualised using TEM (**a**). Samples were taken from various steps of the OMV purification procedure which is explained in **b** and **d**. A Bradford assay was performed and all samples from each strain were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run for *PA01* (**c**) and *PA14* (**e**) samples then silver stained to visualise the OMV protein profile.

The TEM images (Figure 3.9 **a**), showed no difference in the purity of the OMVs as there was flagella co-purified when using both filters. There was also no difference in the protein profile when using either filter (**b-e**). After reviewing the data, PES membranes were used for the OMV purification protocol as they are most cost effective and have a faster flow rate than SFCA membrane filters.

3.2.1.5 Can flagella and fimbriae co-purified with OMVs be removed by buoyant density ultracentrifugation?

Flagella and fimbriae can be co-purified with OMVs and this was initially studied in 3 strains (Figure 3.10). TEM images were compared to see if it was possible to distinguish flagella and fimbriae in OMV samples be eye (Figure 3.10**a-b**). *E. coli* WT MG1655 is a K-12 strain which was studied extensively during this project and has been reported previously to express Type 1 fimbriae (Blumer *et al.* 2005). An *E. coli* FimB-LacZ (translational) fusion strain was used as a positive control for visualising flagella as fimbriae production is locked off. Also, an *E. coli* strain with fimbriae production locked on was used as a positive control for fimbriae production as there should be excessive fimbriae.

These images (Figure 3.10**b**) can be used as a reference point for later decisions on how to differentiate between the two by eye. These images show that flagella are thicker and longer than fimbriae. They are also curvy/wavy whereas fimbriae are short, thin and straight appendages. As a final test, OMVs purified from *E. coli* WT BW25113 (a strain very closely related to MG1655 were fixed on to an EM grid. They were then immunogold labelled using an anti-polymerised FimA antibody and Flagellin. This confirmed the presence of fimbriae surrounding the OMVs and not flagella (Figure 3.10**c**). Methods of differentiating between fimbriae and flagella were re-visited and optimised in Section 4.2.6.

a

Strain name	Strain characteristics/additional information
E.coli WT MG1655	Used as the wild-type strain to produce *E.coli* FimB-LacZ fusion strain and *E.coli* with fimbriae locked on
E.coli FimB-LacZ fusion	This strain contains a deletion of the Lac operon (*lacZYA*) and *fimB* and an insertion of a FimB-LacZ fusion protein. **Fimbriae production is locked off** as FimB is no longer functional.
E.coli fimbriae locked on	**Fimbriae production locked on.** The invertible element (*fimS*) is locked on due to the modifications in the parental intermediate strain (see Strain information in Chapter 2)

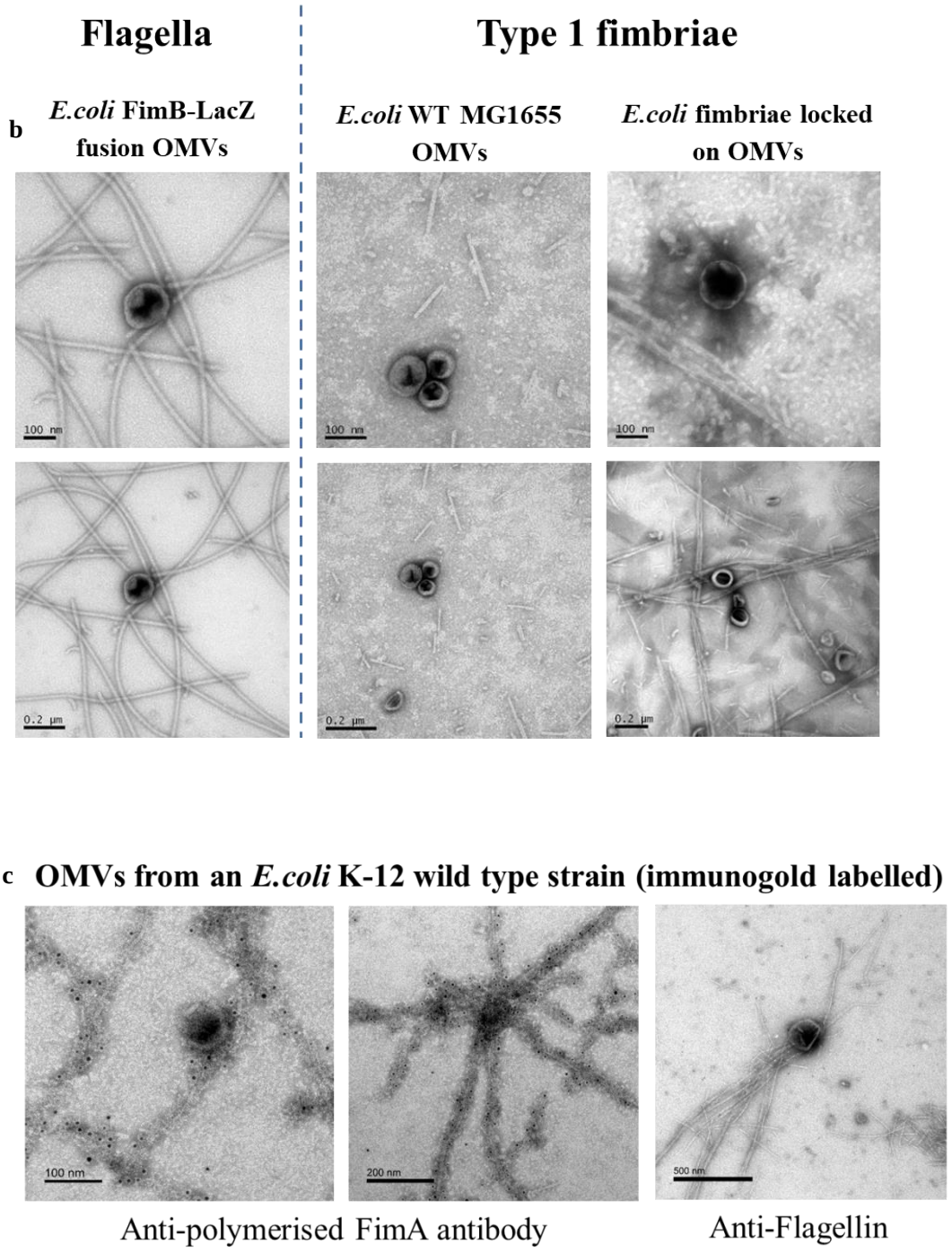


Figure 3.10 TEM images of OMVs purified from three *E. coli* strains to compare co-purification of flagella and fimbriae

The TEM images of OMVs from three *E. coli* strains were compared: *E. coli* MG1655, *E. coli* FimB-LacZ fusion and *E. coli* with fimbriae locked on (**a**). These images (**b**) can be used as a reference point for later decisions on how to differentiate between flagella and fimbriae by eye. As a final test, OMVs purified from *E. coli* WT BW25113 (a strain very closely related to MG1655) and were fixed on to an EM grid. They were then immunogold labelled using an anti-polymerised FimA antibody and Flagellin. This confirmed the presence of fimbriae surrounding the OMVs and not flagella (**c**).

Ultracentrifugation to remove flagella co-purified with *P. aeruginosa* and *E. coli* OMVs

The TEM images in Figures 3.9 and 3.10 indicate that OMVs are co-purified with flagella in the following strains: *PA01, PA14* and *E. coli* FimB-LacZ fusion. OMVs were subject to buoyant density gradient ultracentrifugation by flotation through layers of OptiPrep. OMVs were resuspended in 45% (v/v) OptiPrep then the following layers of OptiPrep were added: 40%, 35%, 30%, 25% and 20% (v/v). If performed correctly, OMVs settle into a lower-density region of the gradient whereas any contaminating flagella or fimbriae remain in the bottom layer. After ultracentrifugation, a thin orange band could be seen within the ultracentrifuge tube (Figure 3.11**a**) which was suspected to contain the OMVs (blue arrow). All six layers of OptiPrep were extracted and then run on an SDS-PAGE gel to identify which layer contained the OMVs (Figure 3.11**b**). The OptiPrep layers of interest were then visualised by TEM (Figure 3.11**c**).

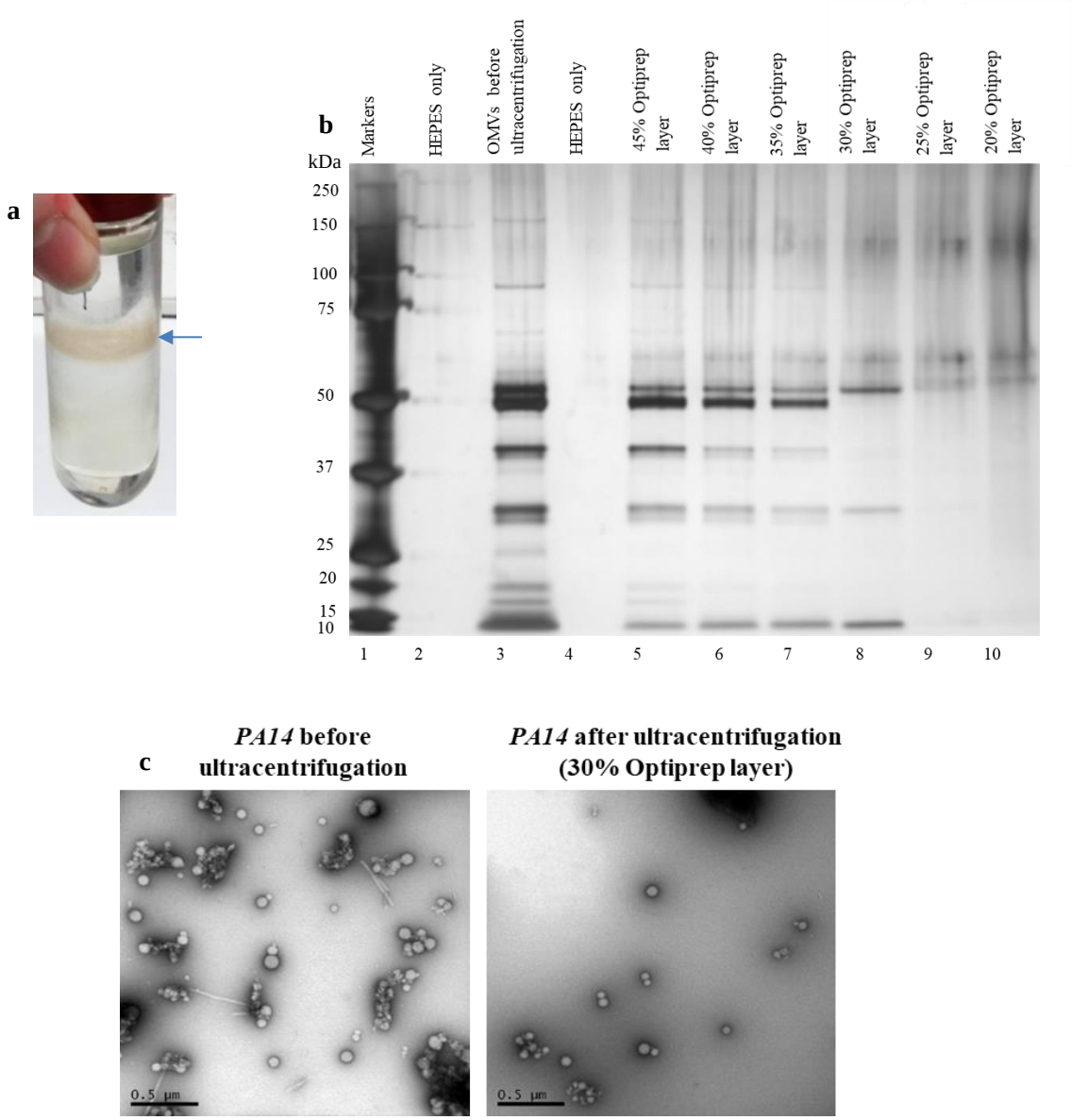


Figure 3.11 Comparing the purity of OMV samples before and after buoyant density ultracentrifugation

PA14 OMVs were subject to buoyant density ultracentrifugation using OptiPrep to separate them from any contaminating flagella. After ultracentrifugation, a thin orange band could be seen within the ultracentrifuge tube (**a**) which was suspected to contain the OMVs (blue arrow). All six layers of OptiPrep were extracted and a portion of the sample was TCA precipitated. The concentrated samples were run on an SDS-PAGE gel and silver stained to identify which layer contained the OMVs (**b**). The OptiPrep layers of interest were then visualised by TEM and the purity of the sample was compared before and after ultracentrifugation (**c**).

Figure 3.11 shows the *PA14* OMVs before and after ultracentrifugation. The layer of 30% OptiPrep contains purified OMVs without the contaminating flagella. This was also successful with *PA01* OMVs (see Appendix B.3). Although this technique was successful with OMVs from *P. aeruginosa*, this technique was not successful at separating *E. coli* WT OMVs from contaminating flagella (Figure 3.12).

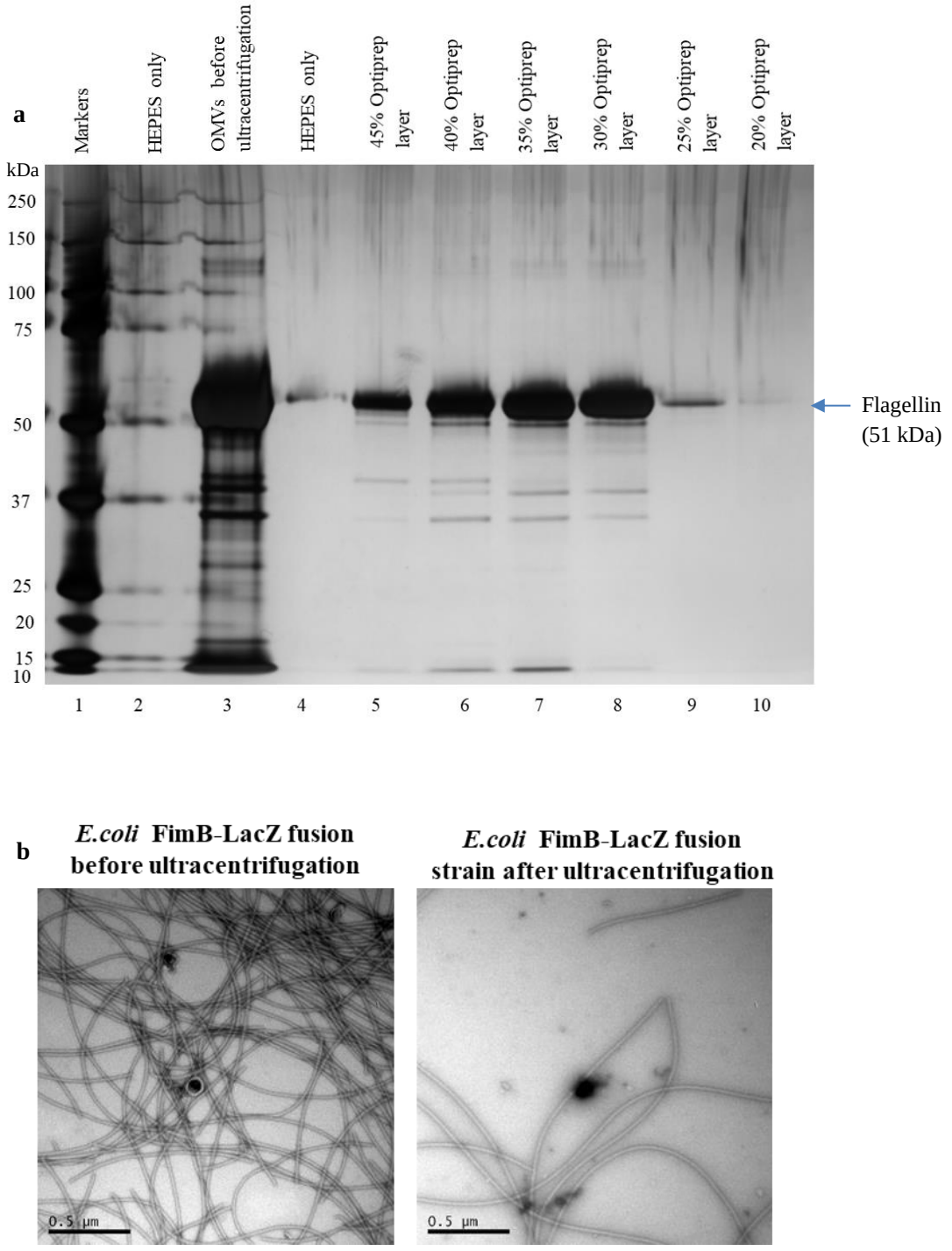


Figure 3.12 Comparing the purity of OMV samples before and after buoyant density ultracentrifugation

E. coli FimB-LacZ fusion strain OMVs were subject to buoyant density ultracentrifugation using OptiPrep to separate them from any contaminating flagella. All six layers of OptiPrep were extracted and a portion of the sample was TCA precipitated. The concentrated samples were run on an SDS-PAGE gel and silver stained to identify which layer contained the OMVs (**a**). The OptiPrep layers of interest were then visualised by TEM and the purity of the sample was compared before and after ultracentrifugation (**b**).

Instead of the OMVs settling into one layer of the OptiPrep gradient, the OMVs were spread across almost all OptiPrep layers and the contaminating flagella was still present. This would lead to a decrease in OMV yield if only one layer was chosen.

3.2.1.6 Alternative methods to remove flagella from purified OMV samples

Although ultracentrifugation was successful for separating *P. aeruginosa* OMVs from contaminating flagella, this was not successful for *E. coli* flagella. Three new methods were trialled to remove the contaminating flagella. The first method was an additional low speed spin of the purified OMVs at 6000 x *g* for 30 minutes (Figure 3.13**a**). The second was addition of Proteinase K at 10 µg/mL (**a**). This concentration was chosen as it is known to degrade extracellular proteins without affecting the OMVs themselves (McCaig *et al.* 2013). The purified OMVs resuspended in 10 mM HEPES buffer (details in Section 2.15) were also subject to filtration through a membrane with 0.45 µm pore size to see if the flagella contamination reduced (**b**).

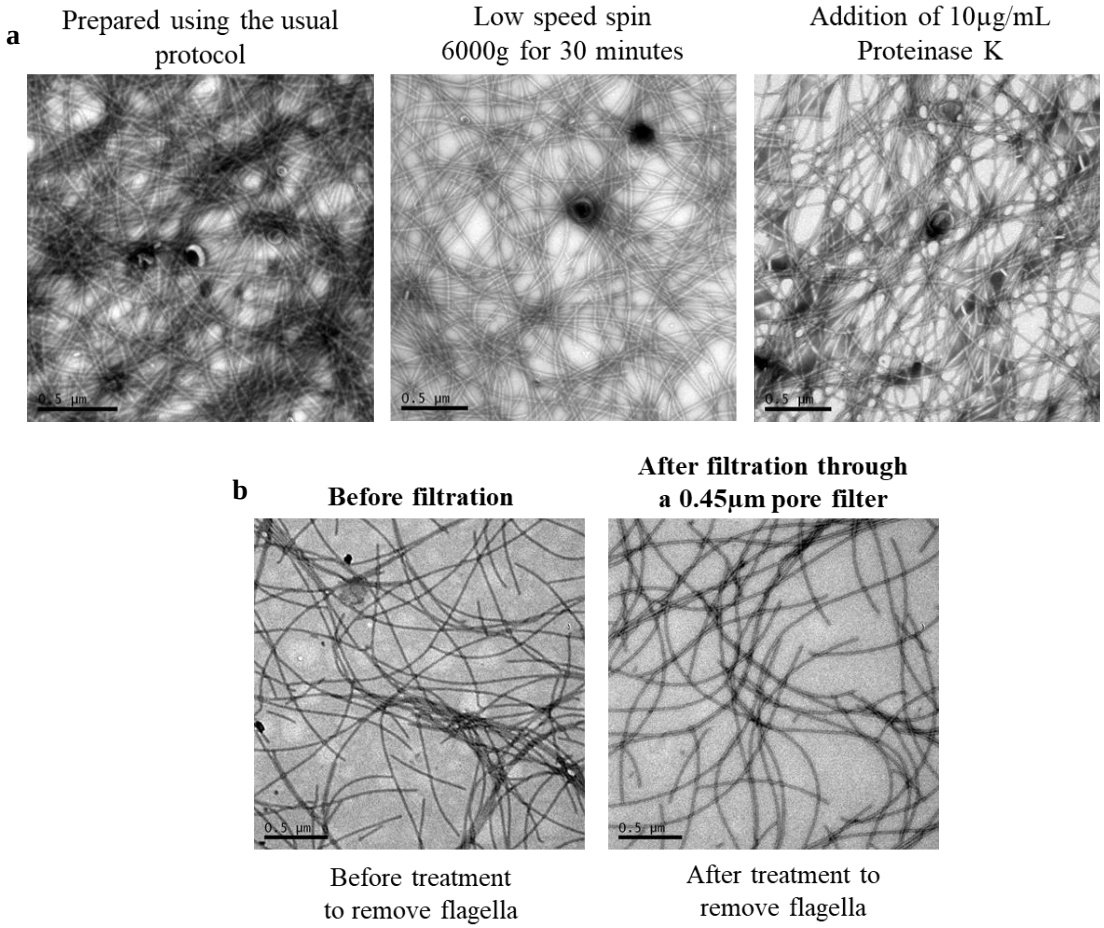


Figure 3.13 Comparison of methods to remove the co-purified flagella in *E. coli* K-12 OMV samples OMVs were purified from 500 mL culture using the standard protocol and were resuspended in 5 mL 10 mM HEPES buffer. **The following methods were compared (shown in a): 1. Usual method:** 1 mL OMVs in 10 mM HEPES buffer were centrifuged at 4ºC for 30 mins, 13,200 RPM. The resulting OMV pellet was resuspended in 30µL 10 mM HEPES buffer. **2. Low speed spin:** 1 mL OMVs in 10 mM HEPES buffer was centrifuged at 6000 x *g* at 4ºC for 30 mins. The resulting OMV pellet was resuspended in 30µL 10 mM HEPES buffer. **3. Proteinase K:** 900µL OMV sample was mixed with 100µL Proteinase K sample (100 µg/mL in HEPES/CaCl2 buffer). Samples were incubated at 37ºC for 30 mins then the protease was inhibited with PMSF. OMVs were centrifuged at 13,200 RPM (14,220 x *g*) for 30 mins at 4°C. The resulting OMV pellet was resuspended in 30µL 10 mM HEPES buffer. **The following methods were compared (shown in b):** OMVs in 10 mM HEPES buffer were compared when additional filtration step through a 0.45 µm membrane was added and were concentrated for TEM as usual.

None of the methods trialled to separate OMVs from flagella in Figure 3.13 were successful. Lastly, OMVs were treated with a mix of proteases from *Streptomyces griseus* at varying concentrations (Figure 3.14).

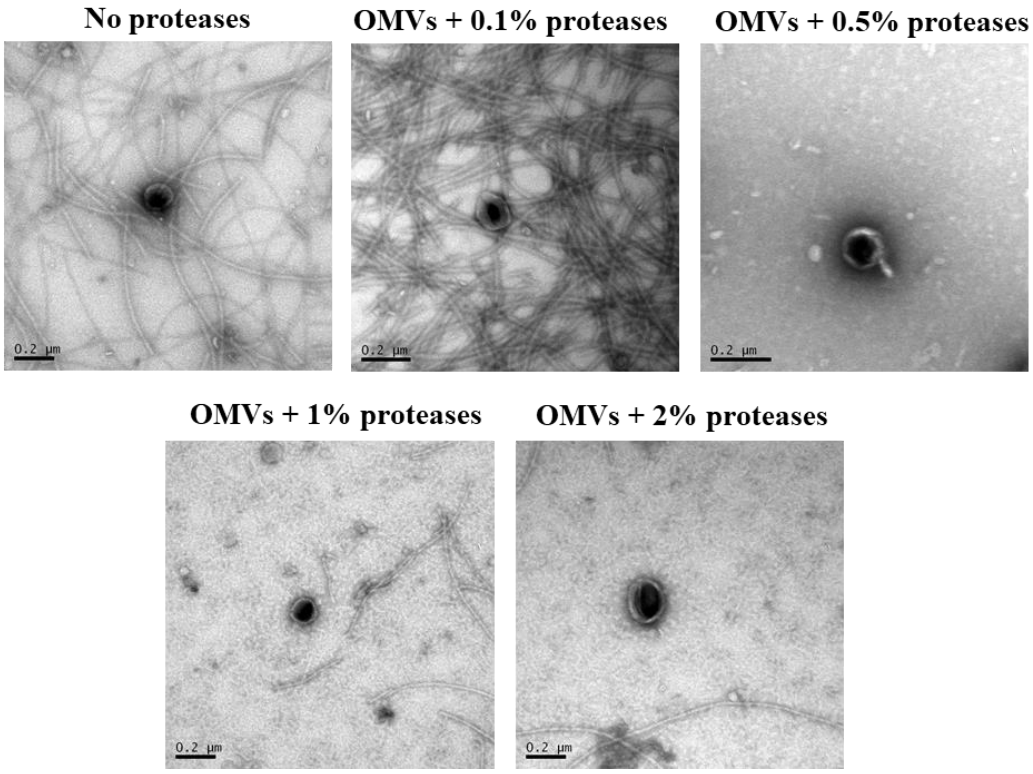


Figure 3.14 Using a mix of *Streptomyces griseus* proteases to remove the co-purified flagella in *E. coli* K-12 OMV samples
A protease stock of 10% (w/v) was prepared in HEPES/CaCl2 buffer which was then diluted to give the other protease concentrations. OMVs resuspended in HEPES were incubated with proteases of varying concentrations at 37ºC for 30 mins. The samples (450 µL) were centrifuged at 4ºC for 30 mins, 13,200 RPM (14,220 x *g*) and the pellet was resuspended in 20 µL 10 mM HEPES buffer in preparation for TEM

The addition of this mix of proteases appeared to be successful at the removal of most flagella at a concentration of 0.5% (w/v) proteases. However, this mix of proteases produced many prominent bands on the SDS-PAGE gel ranging from approximately 10 kDa to 42 kDa. Bands at these molecular weights can obscure the protein profile of the OMVs on the SDS-PAGE gels. Additionally, the addition of 0.5% (w/v) proteases appeared to lyse the OMVs and degrade the OMV-associated proteins (see Appendix B.4 for Figures).

3.2.1.7 Can ultracentrifugation be used to remove Type 1 fimbriae from purified *E. coli* OMV samples?

OMVs were purified from the *E. coli* strain with fimbriae production locked on. This strain overexpresses fimbriae and produces flagella too. The OMV pellet was resuspended in 45% (v/v) OptiPrep then subject to ultracentrifugation to remove fimbriae (Figure 3.15).

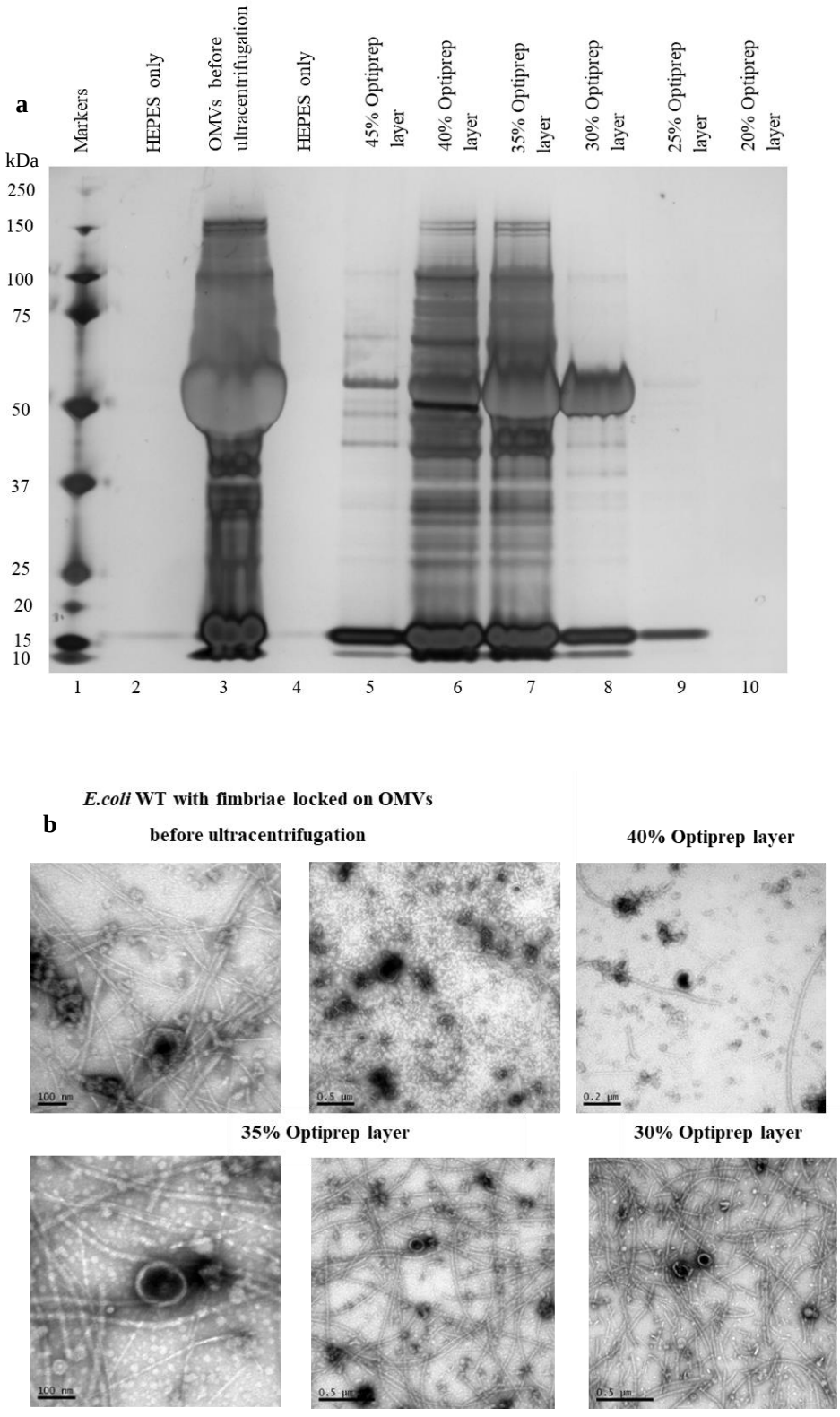


Figure 3.15 Comparing the purity of OMV samples before and after buoyant density ultracentrifugation

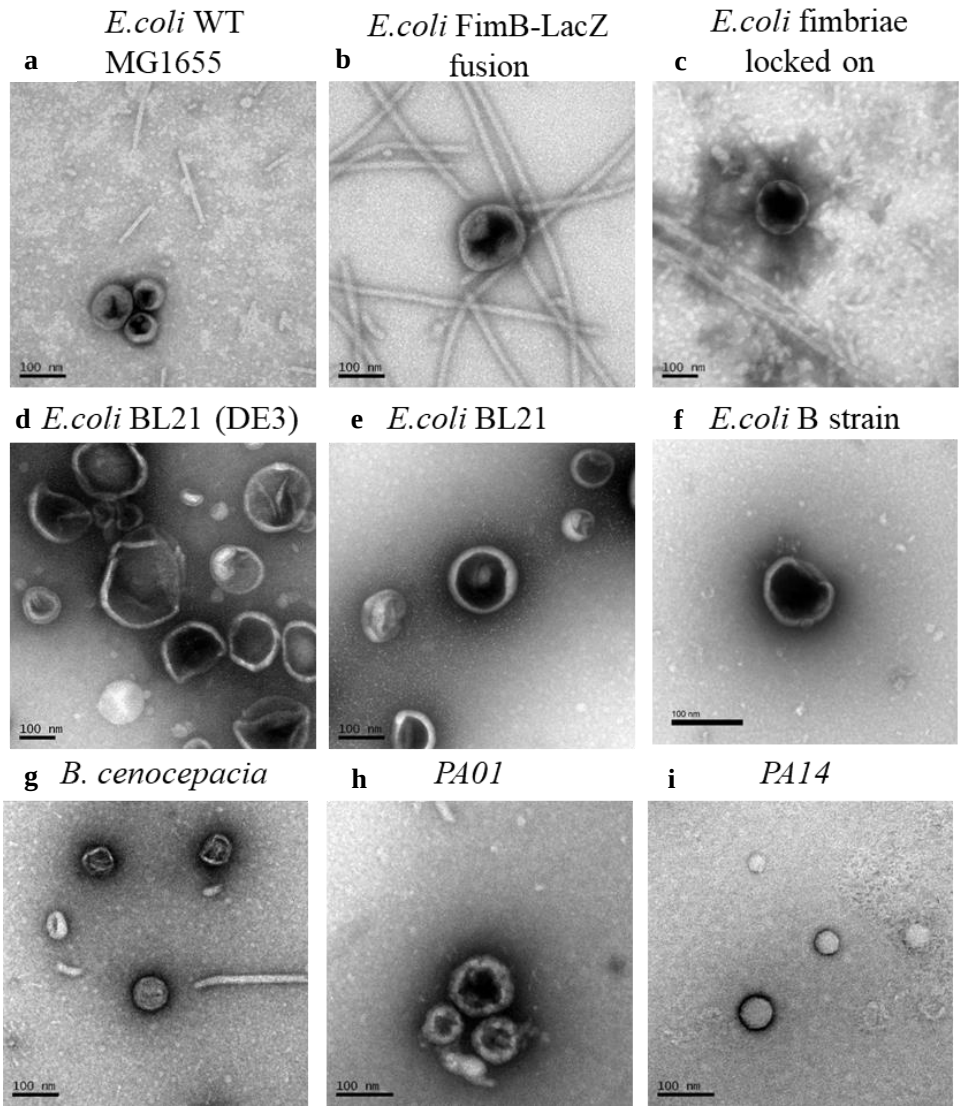
OMVs purified from *E. coli* with fimbriae production locked on strain were subject to buoyant density ultracentrifugation using OptiPrep. All six layers of OptiPrep were extracted and a portion of the sample was TCA precipitated. The concentrated samples were run on an SDS-PAGE gel and silver stained to identify which layer contained the OMVs (**a**). The OptiPrep layers of interest were then visualised by TEM and the purity of the sample was compared before and after ultracentrifugation (**b**).

The SDS-PAGE gel in Figure 3.15**a** shows that the purified OMVs are spread between five of the OptiPrep layers (Lanes 5-9) instead of settling into one layer. The TEM images (Figure 3.15**b**) show that the contaminating fimbriae and flagella are still present and have not been separated from the OMVs.

Both the SDS-PAGE gel and TEM images suggested that ultracentrifugation causes *E. coli* OMVs to spread between all six of the OptiPrep layers rather than settling within one layer. The fimbriae and flagella are also not separated from the OMVs in any sample tested. For this reason, *E. coli* OMVs were resuspended in 10 mM HEPES buffer (details in Section 2.15) for analysis in subsequent experiments rather than using ultracentrifugation so that no OMVs were lost.

3.2.2 Visualisation of OMVs using Transmission Electron Microscopy (TEM)

In this study, OMVs have been purified from a range of Gram-negative bacteria and visualised using TEM. OMVs are approximately 50 nm to 200 nm in diameter and are very similar to those found in the literature. Although there are small variations in size and staining intensity of the OMVs, OMVs from each bacterial strain generally appear the same (Figures 3.16-3.17).



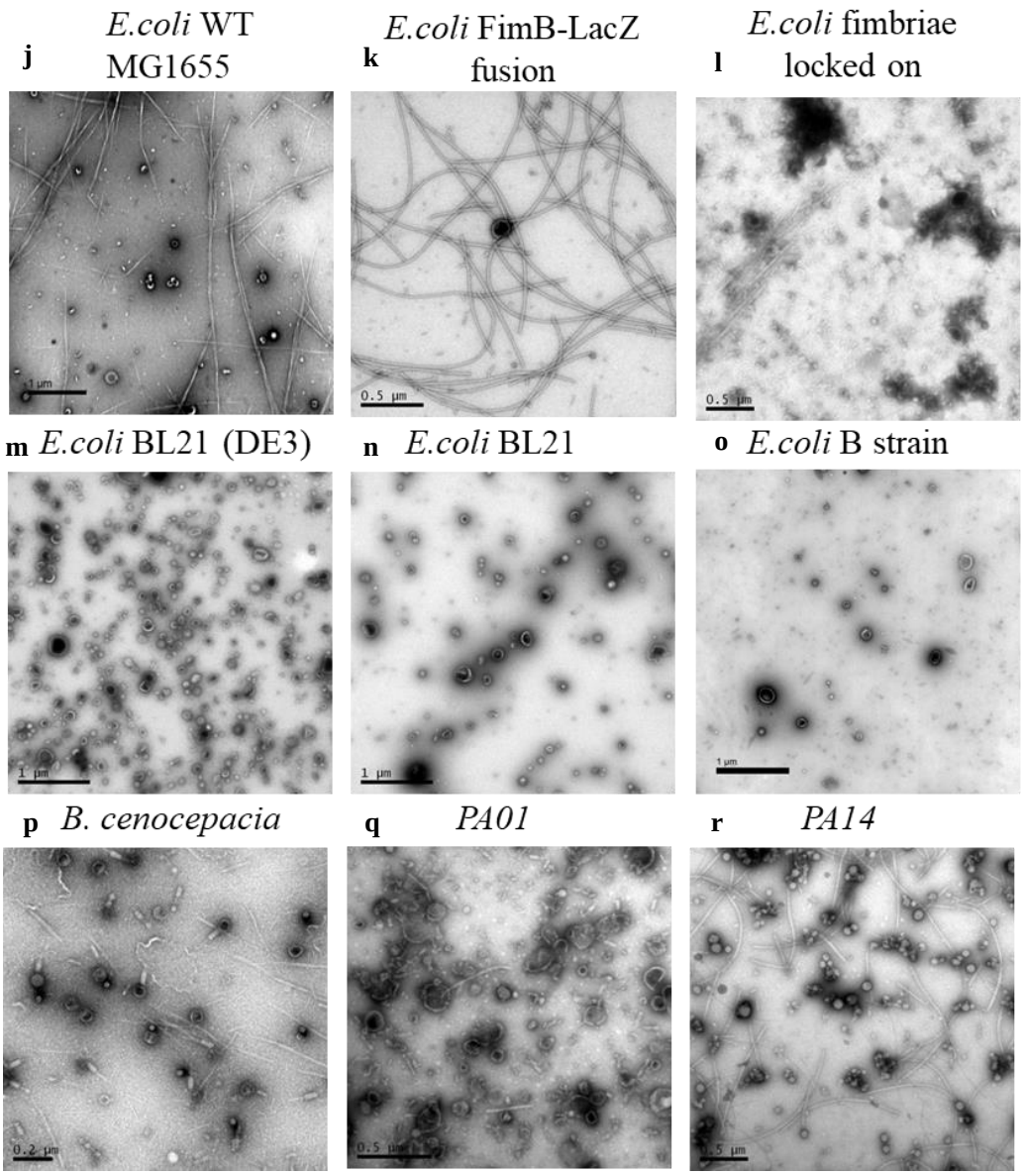


Figure 3.16 Comparison of OMVs purified from a range of Gram-negative bacteria
TEM analysis of purified OMVs from the following strains: *E. coli* WT MG1655 (**a, j**) *E. coli* FimB-LacZ fusion (**b, k**) *E. coli* with fimbriae locked on (**c, l**) *E. coli* BL21 (DE3) (**d, m**) *E. coli* BL21 **(e, n)** *E. coli* B strain (**f, o**) *Burkholderia cenocepacia* (**g, p**) *Pseudomonas aeruginosa* PA01 **(h, q)** *Pseudomonas aeruginosa* PA14 **(i, r).**

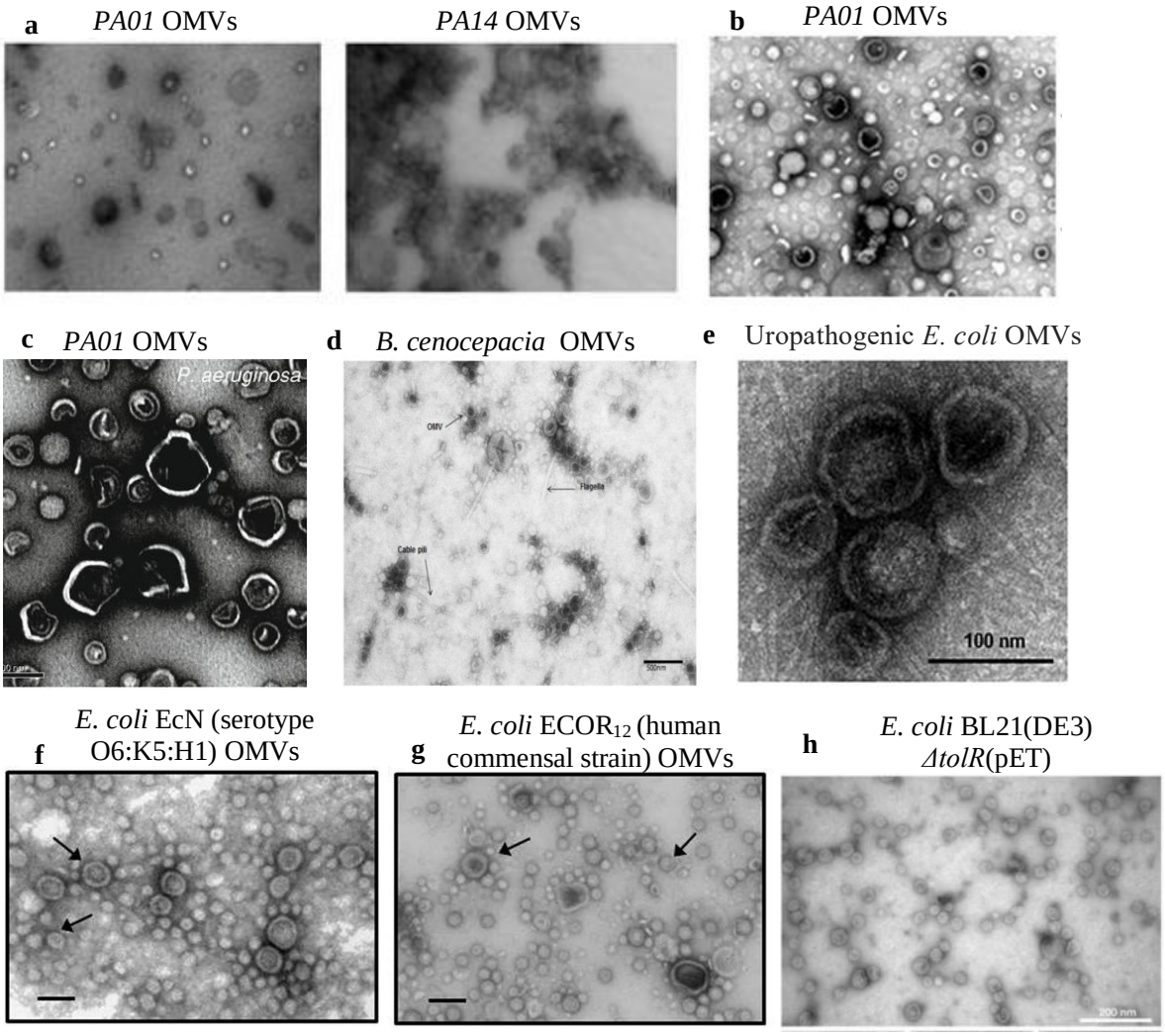


Figure 3.17 Comparison of OMVs purified from a range of Gram-negative bacteria from the literature

TEM images of OMVs from *Pseudomonas aeruginosa* strains *PA01* and *PA14* sourced from Shan *et al.* 2014 (**a**). Scale bars missing in the published images. TEM photo of *P. aeruginosa PA01* biofilm OMVs sourced from Couto *et al.* 2015 (**b**). Scale bar missing in the published image. TEM image of *P. aeruginosa PA01* OMVs sourced from Chutkan *et al.* 2013 (**c**). TEM image of *B. cenocepacia* OMVs from Martins *et al.* 2016 (**d**). TEM image of Uropathogenic *E. coli* OMVs sourced from Svennerholm *et al.* 2017 (**e**). TEM images of OMVs purified from the probiotic *E. coli* strain EcN, serotype O6:K5:H1 (**f**) and *E. coli* $ECOR_{12}$ which is a human commensal strain isolated from a stool sample (**g**). Images sourced from Fábrega *et al.* 2016. The final TEM image is of OMVs from a BL21 (DE3) *ΔtolR*(pET) strain and the image is sourced from (Bartolini *et al.* 2013) (**h**). This is referred to as the 'empty OMV' in the paper and is the negative control for expression of their recombinant protein of interest which is HtrA from *Chlamydia muridarum.*

The purified OMVs from each strain were consistent, reproducible and are the correct size and appearance when compared to those in the literature.

3.2.2.1 Immunogold labelling of *E. coli* BL21 (DE3) OMVs using an anti-OmpA antibody

OmpA is an outer membrane protein in *E. coli* that is known to be present on the surface of OMVs (Kim *et al.* 2009). Detecting OmpA on the surface of purified OMVs is evidence that OMVs have been purified successfully. Purified *E. coli* BL21 (DE3) OMVs were immunogold labelled using an anti-OmpA antibody (Figure 3.18). These immunogold labels are indicated by the blue arrows below.

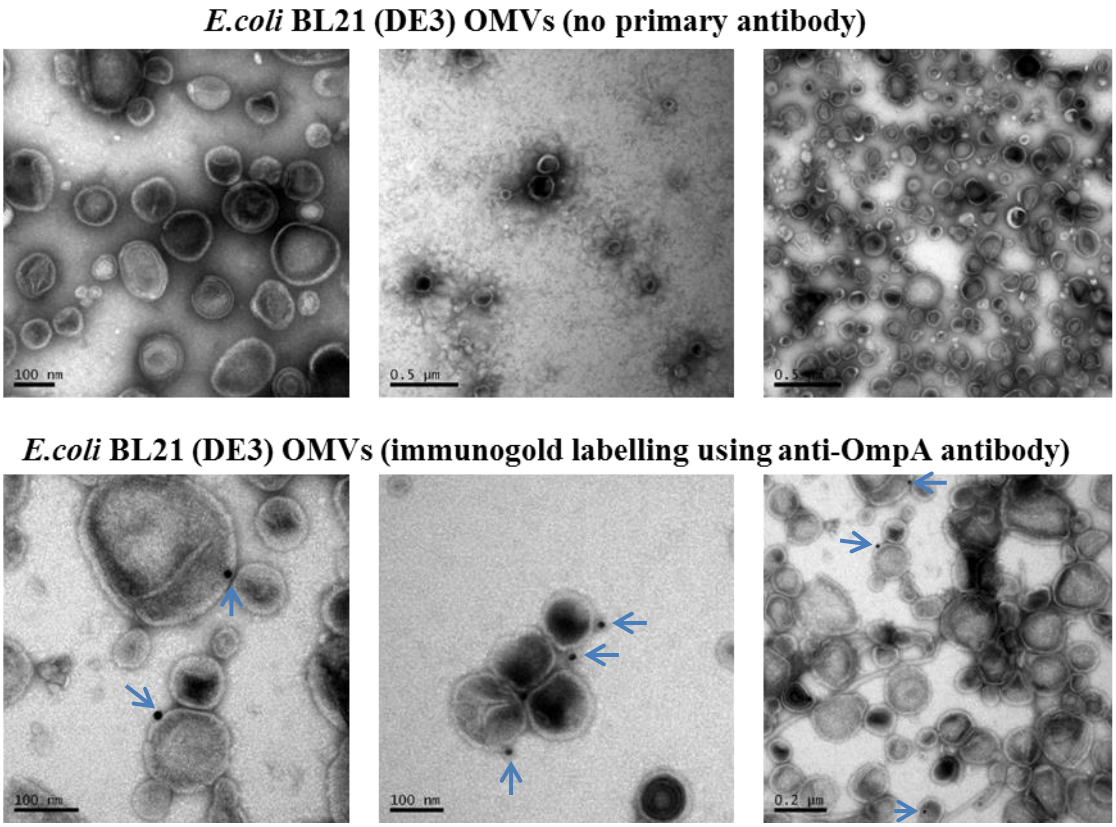


Figure 3.18 Immunolabelling *E. coli* BL21 (DE3) OMVs using anti-OmpA antibody.
Purified *E. coli* BL21 (DE3) OMVs were concentrated 10x. 10 µL OMVs were added to each grid and grids were fixed by washes in 2% (w/v) formaldehyde + 0.5% (v/v) glutaraldehyde in 100 mM CAB pH 7.2. Grids were then washed in 100 mM CAB followed by washes in Tris-buffered saline with 0.05% (v/v) Tween 20 (TBST). Grids were blocked in 2% (w/v) BSA then incubated with anti-OmpA antibody (diluted 1 in 50 in TBST). An identical grid was used as a negative control and that was incubated in TBST only. Grids were left in primary antibody or TBST only at 4ºC overnight. The next day, grids were washed in TBST then incubated in immunogold-conjugated anti-rabbit antibody (which was diluted 1 in 50 in TBST). Grids were incubated in secondary antibody for 1 min then left for 30 mins. Grids were subject to washes in TBST then MQ water. Lastly, grids were negatively stained using 2% (w/v) uranyl acetate in PBS then air dried for 20 mins. Grids were analysed by TEM. The immunogold labels (15 nm in diameter) are indicated by the blue arrows above.

3.2.2.2 Visualising OMV biogenesis from *E. coli* BL21 (DE3) and *Pseudomonas aeruginosa* (*PA14*) cells

In order to gain evidence of OMV budding from bacterial cells, *E. coli* BL21 (DE3) cells and *PA14* cells, (resuspended in LB), were grown on gold EM grids and fixed at various time points over 24 hours. The cells at each time point were examined using EM and studied for evidence of OMV biogenesis (Figure 3.19).

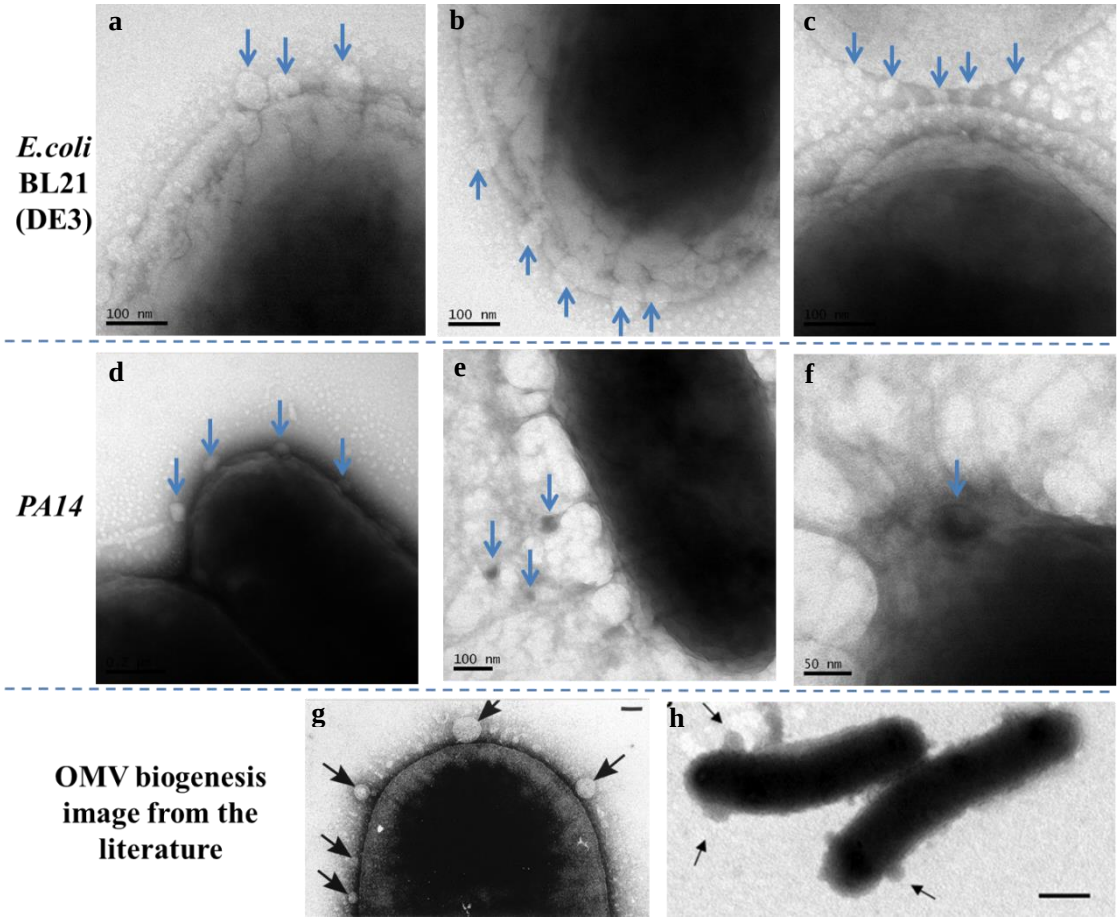


Figure 3.19 Visualisation of OMV biogenesis from *E. coli* BL21 (DE3) and *PA14* cells using EM *E. coli* BL21 (DE3) and *PA14* cells were diluted to an OD600 of 0.1 in LB and added to a gold EM grid. The grids were incubated at 37ºC for 22 hrs and grids were fixed at various time points. Grids were negatively stained using 2% (w/v) uranyl acetate then visualised using EM at various magnifications. Figure 3.19 **a-b** shows *E. coli* BL21 (DE3) cells after 30 mins since addition to the gold EM grid. Figure 3.19 **c** shows *E. coli* BL21 (DE3) cells 22 hrs after addition to the gold grid. Figure 3.19 **d** shows *PA14* cells 6 hrs after addition to the gold grid. Figure 3.19 **e-f** shows *PA14* cells after 22 hrs after addition to the gold grid. Blue arrows indicate budding and released OMVs from cells. Figure 3.19 **g** shows OMV biogenesis from *S. marcescens* and is sourced from Li *et al.* 1998. Figure 3.19 **h** shows OMV biogenesis from *H. pylori* and is sourced from Parker *et al.* 2012.

The OMV biogenesis images produced were similar to those found in the literature and appear to successfully show OMV biogenesis from the *E. coli* BL21 (DE3) and *PA14* cells.

3.2.3 How does the OMV protein profile compare across bacterial strains and species

The protein profiles of OMVs purified from various bacterial strains were visualised using SDS-PAGE and silver staining (Figure 3.20).

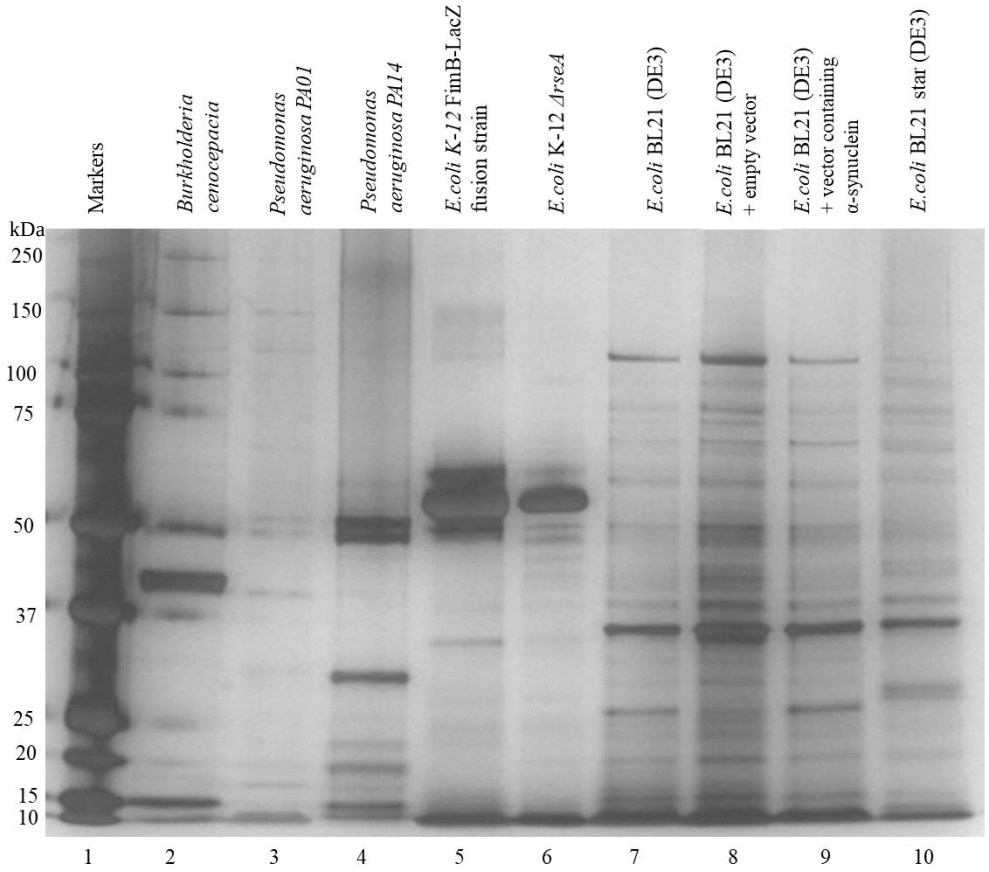


Figure 3.20 – Comparison of protein profiles of OMVs from various bacterial strains
OMVs were purified from a range of *B. cenocepacia, P. aeruginosa* and *E. coli* K-12 and B strains. A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. Proteins were separated using SDS-PAGE and silver stained to visualise OMV protein profiles. Brightness on the original gel images was increased by 15% (no adjustment on contrast).

OMVs from wild type *E. coli* K-12 strains (lanes 5-6) have a very different protein composition to OMVs from recombinant/engineered strains (lanes 7-10). OMVs from the wild type *E. coli* K-12 strains appear to be enriched with specific proteins (for example the prominent band at approximately 55 kDa in lanes 5-6). OMVs from *E. coli* B strains, however, appear to have many more proteins and at different molecular weights (lanes 7-10). Certain proteins also appear to be selectively included and excluded from *P. aeruginosa* and *B. cenocepacia* wild type OMVs compared with the engineered *E. coli* B strains (lanes 2-6 compared with lanes 7-10). The difference in OMVs produced from *E. coli* K-12 and B strains was explored further (see Chapter 4, section 4.2.1). For further information on the *E. coli* BL21 strains in Lanes 8-10 see Appendix B.5.

3.2.3.1 Comparison of the protein profile of OMVs and whole cells

The protein profile of the OMVs was compared to the protein profile of the whole cell (Figure 3.21) to see which proteins are enriched and excluded from the OMVs. The protein profile of OMVs from *E. coli* FimB-LacZ fusion strain, *PA01* and *PA14* show that certain proteins are concentrated within the OMVs when compared to the cells. The prominent bands within OMVs from each strain were extracted and identified by mass spectrometry.

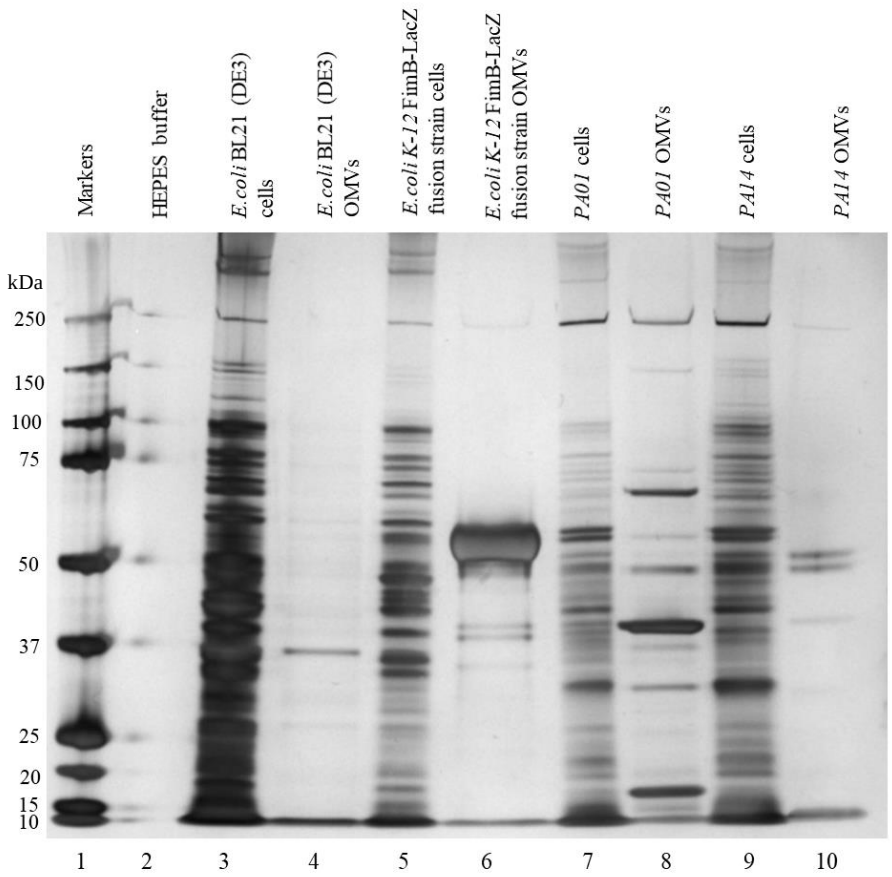


Figure 3.21 Comparison of protein profiles of OMVs compared with the whole bacterial cell. OMVs were purified from *E. coli* BL21 (DE3), *PA14, PA01* and *E. coli* FimB-LacZ fusion strain. 1 mL of each overnight culture was saved from each strain to be used in the 'cell' lanes. All samples were standardised to the same protein concentration then TCA-precipitated prior to loading. Proteins were separated using SDS-PAGE and silver stained to visualise OMV protein profiles.

3.2.3.2 Identification of proteins of interest by mass spectrometry

Bands of interest were extracted from SDS-PAGE gels and identified by mass spectrometry (Table 3.2 and Appendix B.6 for further detail).

Table 3.2 Identification of OMV proteins by mass spectrometry
Bands of interest were excised from silver stained SDS-PAGE gels then identified by mass spectrometry. Proteins were identified by a Peptide Mass Fingerprint (PMF) search in the Mascot database. A match is significant if it has a score greater than 70.

Organism protein originated from	Approx. MW on gel (kDa)	Protein detected (using Peptide Mass Fingerprinting and SwissProt database unless otherwise stated)	Score (score needed to be significant)	Protein MW (Da)	UniProt accession number
E.coli BL21 DE3	35	OmpF	125 (70)	39309	OMPF_ECOLI
E.coli FimB-LacZ fusion	38	Flagellar hook protein	100 (70)	42019	FLGE_ECOLI
	52	Flagellin OS=Escherichia coli (strain K12)	255 (70)	51265	FLIC_ECOLI
E.coli WT fimbriae locked on	55	Flagellin	257 (70)	51265	FLIC_ECOLI
	17	FimA	71 (70)	18214	FIMA1_ECOLI
E.coli WT MG1655	16	Type-1 fimbrial protein, A chain OS=Escherichia coli (strain K12)	71 (70)	18214	FIMA1_ECOLI
	37	Outer membrane protein A OS=Escherichia coli O157:H7	93 (70)	37292	OMPA_ECO57
	55	Antigen 43	120 (70)	106818	AG43_ECOLI
E.coli WT parental BW25113	55	Antigen 43	148 (70)	106818	AG43_ECOLI
	17	FimA	71 (70)	18214	FIMA1_ECOLI
P. aeruginosa PA01	49	B-type flagellin	241 (70)	49213	FLICB_PSEAE
	40	Putative prophage major tail sheath protein	209 (70)	41339	Y807_PSEAB
	32	Elastase	135 (70)	53882	ELAS_PSEAE
P. aeruginosa PA14	52	Aminopeptidase	108 (70)	57818	LAP_PSEAB or LAP_PSEAE
	50	B-type flagellin	281 (70)	49213	FLICB_PSEAE
	31	Elastase	76 (70)	53796 53882	ELAS_PSEAB or ELAS_PSEAE
	31	Chitin-binding protein	71 (70)	42390 42347	CBPD_PSEA or CBPD_PSEAE
	42	Putative prophage major tail sheath protein	135 (70)	41339	Y807_PSEAB

3.2.3.3 Identification of OmpA in *E. coli* OMV samples

A range of *E. coli* OMV samples were probed with an anti-OmpA antibody (Figure 3.22**a**). A band is expected at 37 kDa according to the manufacturer's guidelines. The whole *E. coli* cells were run alongside the OMVs as a positive control. The Western blot was also repeated using cell and OMV samples from *E. coli* BL21 (DE3), *E. coli* FimB-LacZ fusion protein, *PA01* and *PA14* (Figure 3.22**b**). Bands appeared at 37 kDa and 25 kDa for the *E. coli* strains but not for *P. aeruginosa.* This indicates that there was no non-specific binding of the anti-OmpA antibody to *P. aeruginosa* proteins.

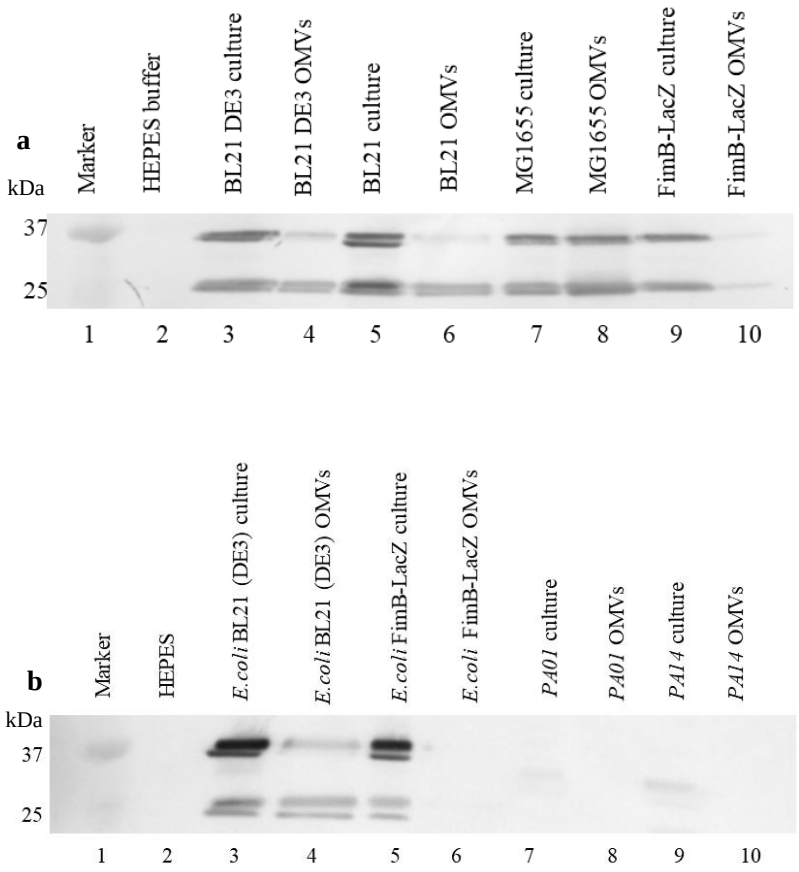
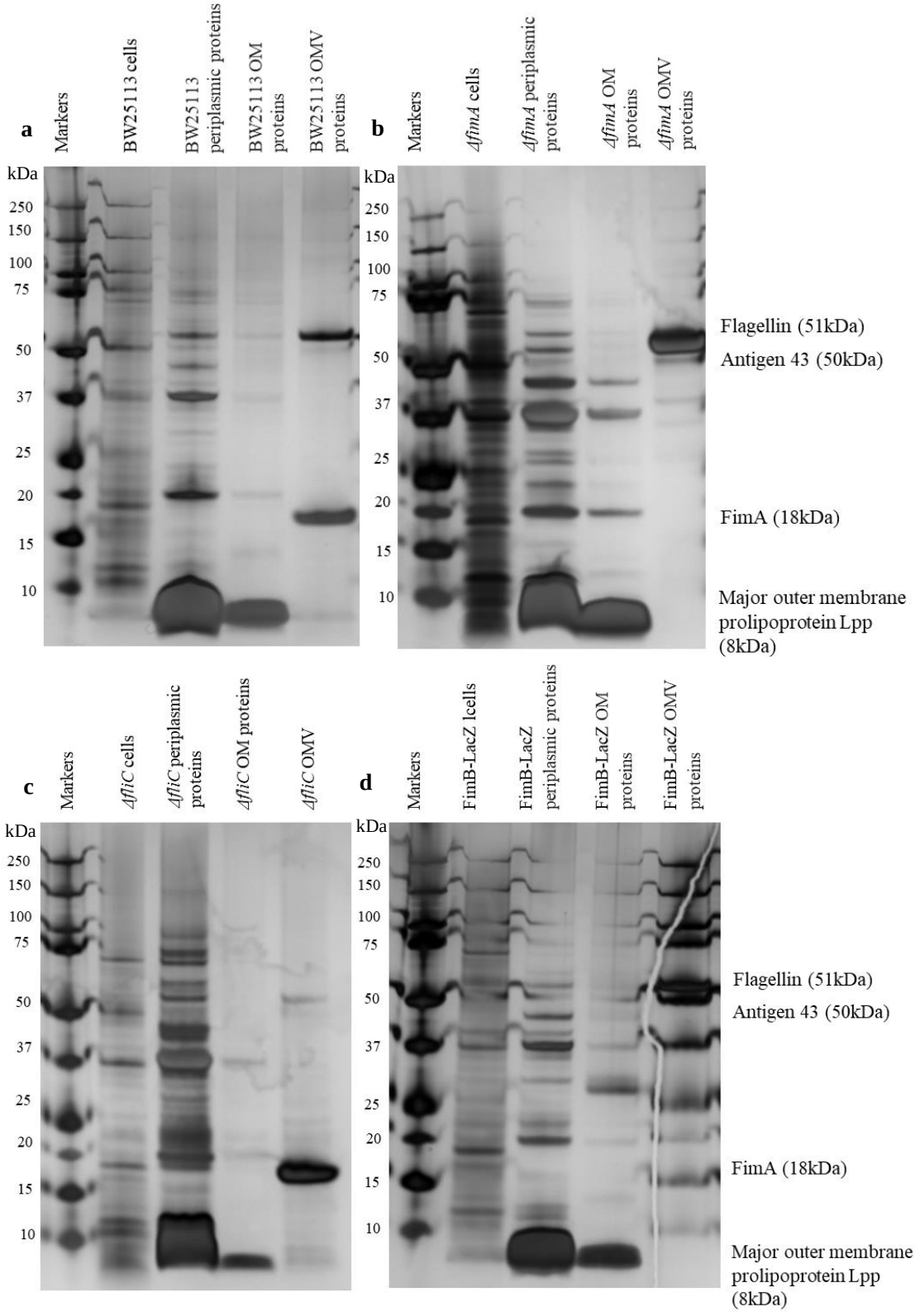


Figure 3.22. Western blotting to detect OmpA in purified OMV and whole cell samples A Bradford assay was performed and all samples were standardised to be the same protein concentration. *E. coli* and *P. aeruginosa* whole cells and OMV samples from each strain of interest used to run an SDS-PAGE gel. Proteins were separated using SDS-PAGE and then transferred to a PVDF membrane for Western blotting. The membrane was probed with an anti-OmpA antibody (Antibody research corporation #111120). The original Western blot images can be found in Appendix B.7.

Bands at approximately 37 kDa were found indicating OmpA in all *E. coli* whole cells and OMVs (Figure 3.22**a**). A band was also present at approximately 25 kDa and there appears to be double banding at each of these molecular weights which could be due to the presence/absence of a signal peptide (see Section 3.3.2.3 for further discussion). OprF is the *P. aeruginosa* equivalent of OmpA in *E. coli* (Confer, Ayalew. 2013). The molecular weight of OprF is 37.6 kDa but has only 37.5% identity to OmpA in *E. coli* (see Appendix B.8). As expected, there were no bands at 25 kDa and 37 kDa in the *P. aeruginosa* cell or OMV samples (**b**).

3.2.3.4 Enrichment of certain proteins within *E. coli* OMVs compared to the whole cell, outer membrane and the periplasm

Previous SDS-PAGE gels and mass spectrometry results (Table 3.2) showed that OMVs from the *E. coli* K-12 strains appear to be heavily enriched with FimA (18 kDa) and/or Flagellin (51 kDa). In order to see if this cargo was enriched in other parts of the cell, six different strains of *E. coli* were subject to a periplasmic and outer membrane extraction protocol. The SDS-PAGE gels in Figure 3.23 indicated that the levels of FimA and Flagellin in the whole cell, periplasm and outer membrane are relatively low and that these proteins are specifically selected to be packaged in OMVs.



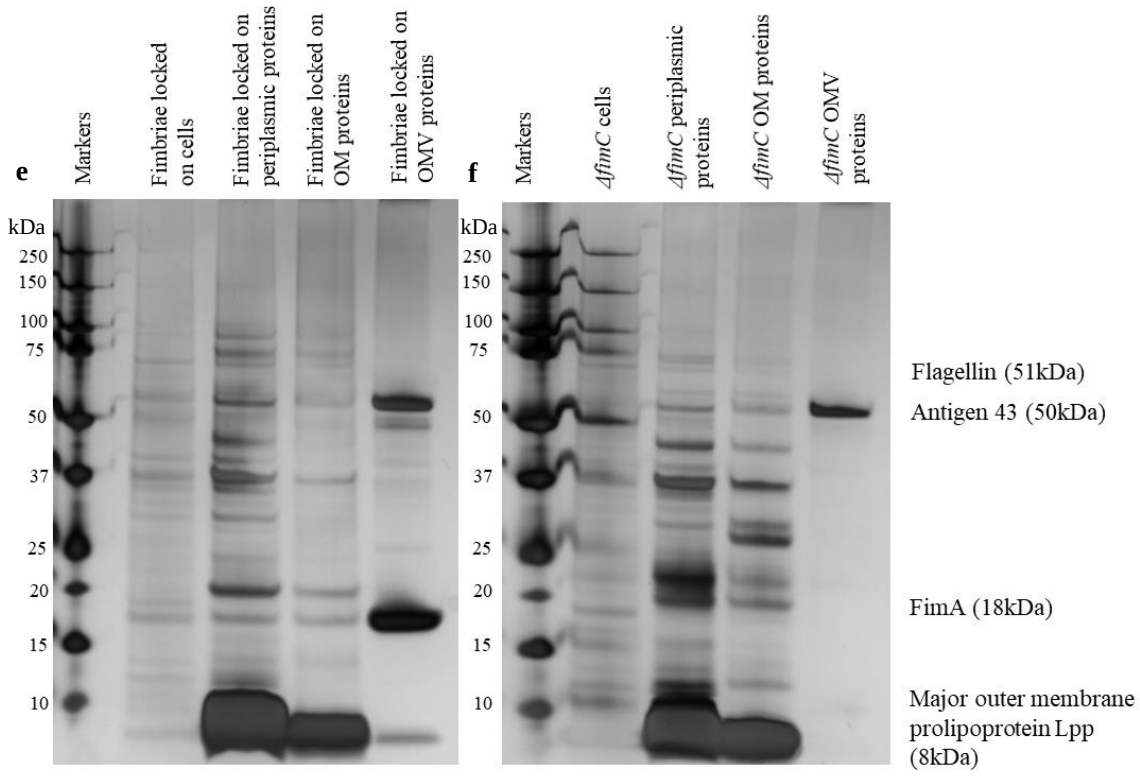


Figure 3.23 FimA and Flagellin are enriched in *E. coli* K-12 OMVs compared to levels in the periplasm and whole cell

A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run and silver stained to visualise protein profiles of *E. coli* OMVs were compared to the whole cell, OM and periplasm. Samples were purified from the following *E. coli* strains: BW25113 Parental (**a**), *ΔfimA* (**b**), *ΔfliC* (**c**), FimB-LacZ (**d**), Fimbriae locked on strain (**e**), and *ΔfimC* strain (**f**).

The OMV protein profiles were found to be very different to the lysed cells, periplasmic proteins and OM proteins. It also appeared that certain proteins were enriched and/or excluded from the OMVs. For example, *E. coli* K-12 OMVs were enriched with (or excluded) the following proteins: FimA (18 kDa), Flagellin (51 kDa) and Antigen 43 α-chain (50 kDa). Interestingly, the presence of FimA and Flagellin appeared to be mutually exclusive in the OMVs except for the *E. coli* strain where fimbriae production is locked on (this is discussed further in Section 4.2.2).

Lastly, the major outer membrane prolipoprotein Lpp was detected in high levels in the periplasmic and OM samples. This was expected as the Lpp protein is located in the periplasm and links the OM with the peptidoglycan layer (Schwechheimer, Kuehn. 2015). OMVs have been found to bud in locations where the Lpp link is absent. The major outer membrane prolipoprotein Lpp is not present in OMVs and so is absent in the OMV samples above (Schwechheimer, Kuehn. 2015).

3.2.4 Are the purified OMVs whole and intact?

3.2.4.1 Proteinase K test: *E. coli* BL21 (DE3) OMVs

The Proteinase K test is used to confirm that isolated OMVs are intact and to distinguish which proteins are outside the OMVs or within the lumen (see Section 3.1.2 for further information). *E. coli* BL21 and BL21 (DE3) OMVs were incubated with Proteinase K in the presence and absence of SDS (Figure 3.24 and Appendix B.9).

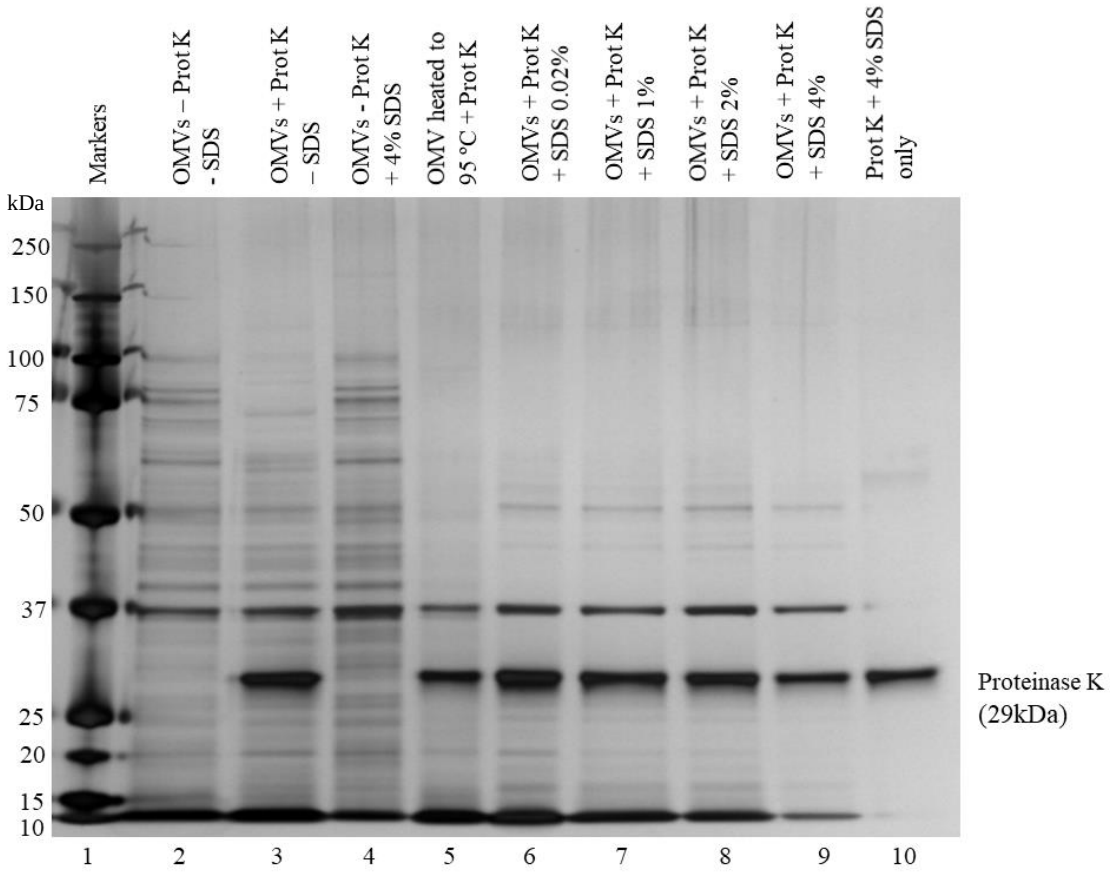


Figure 3.24 Proteinase K test on OMVs from *E. coli* BL21 (DE3) strain

OMVs were incubated in the presence and absence of 10 µg/mL Proteinase K and various concentrations of SDS for 30 mins at 37ºC. 5 mM PMSF was added to inhibit Proteinase K and samples were incubated for another 30 mins at 37ºC. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile.

The sample in Lane 3 contains OMVs that have been treated with Proteinase K but no SDS. The bands in Lane 3 indicate which proteins are present within the lumen of the OMVs and are therefore protected from Proteinase K degradation (compared to Lanes 2 and 4). However, when the OMVs were treated with both SDS and Proteinase K, the majority of the bands disappeared (lanes 6-9). SDS disrupts OMV membranes and allows Proteinase K access to the proteins within the OMVs. Figure 3.24 therefore provides evidence that the OMVs were present and intact.

3.2.4.2 Proteinase K test: *P. aeruginosa PA14* OMVs

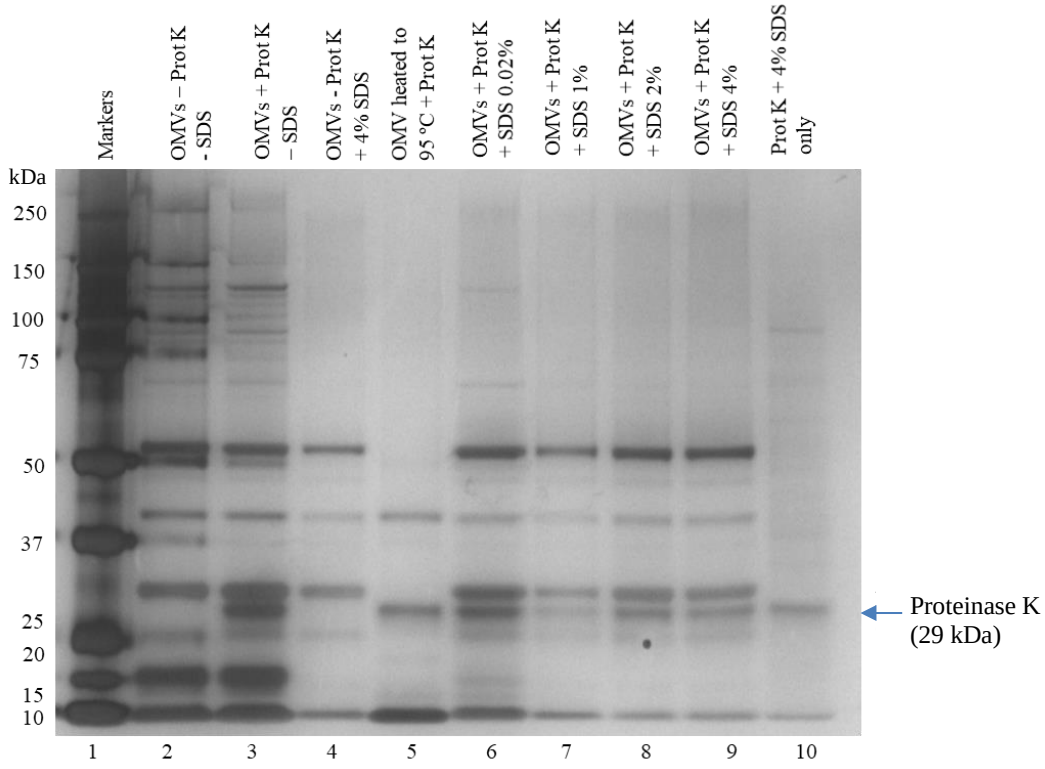


Figure 3.25 Proteinase K test on OMVs from *PA14*

OMVs were incubated in the presence and absence of 10 µg/mL Proteinase K and various concentrations of SDS for 30 mins at 37ºC. 5 mM PMSF was added to inhibit Proteinase K and samples were incubated for another 30 mins at 37ºC. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile.

As above, the sample in Lane 3 contains OMVs that have been treated with Proteinase K but no SDS. The bands in Lane 3 indicate which proteins are protected from Proteinase K within the lumen of the OMVs when compared to Lanes 2 and 4. When *E. coli* BL21 (DE3) OMVs were treated with both SDS and Proteinase K, the majority of the bands disappeared (Figure 3.25). In this case, the protein profile of Lanes 6-9 remains almost unchanged. However, when the OMVs are heated to 95°C before Proteinase K addition, many of the bands disappear. This evidence suggests that *PA14* OMVs are resistant to disruption by SDS detergent. This was also found in the *PA01* strain (see Appendix B.10).

3.2.4.3 Proteinase K test: *E. coli* FimB-LacZ fusion strain OMVs

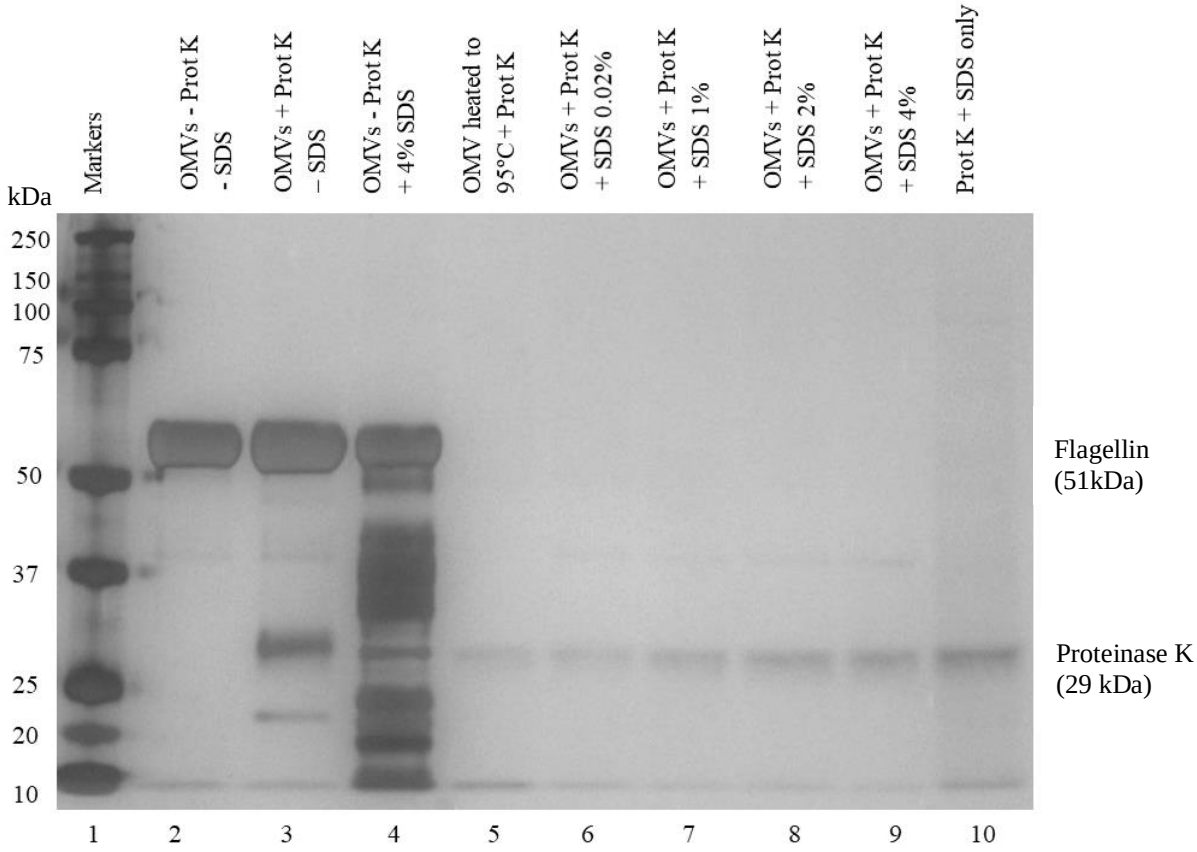


Figure 3.26 Proteinase K test on OMVs from *E. coli* FimB-LacZ fusion

OMVs were incubated in the presence and absence of 10 µg/mL Proteinase K and various concentrations of SDS for 30 mins at 37ºC. 5 mM PMSF was added to inhibit Proteinase K and samples were incubated for another 30 mins at 37ºC. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile. The brightness of the photo was increased by 10% for clarity.

The band in Lane 3 indicates that the main enriched protein in the OMVs (later confirmed as Flagellin) is protected from Proteinase K degradation within the lumen of the OMV. When SDS was added alone to the OMVs (with no Proteinase K), many new bands appeared (Lane 4). The hypothesis was that when the structure of the OMVs was disrupted by SDS, active proteases (that were originally contained within the OMVs) were now released extracellularly. These proteases could then degrade OMV proteins to produce the extra bands seen on the SDS-PAGE gel. This was explored further in Section 3.2.5.

3.2.5 Do *E. coli* OMVs contain active proteases?

E. coli FimB-LacZ OMVs were treated with various concentrations of SDS to determine the minimal concentration needed to disrupt the OMVs and release the proteases. OMVs were incubated with various concentrations of SDS at 37°C for 60 minutes (Figure 3.27**a**). The OMV samples before and after incubation with 1% SDS were visualised by TEM (Figure 3.27**b**).

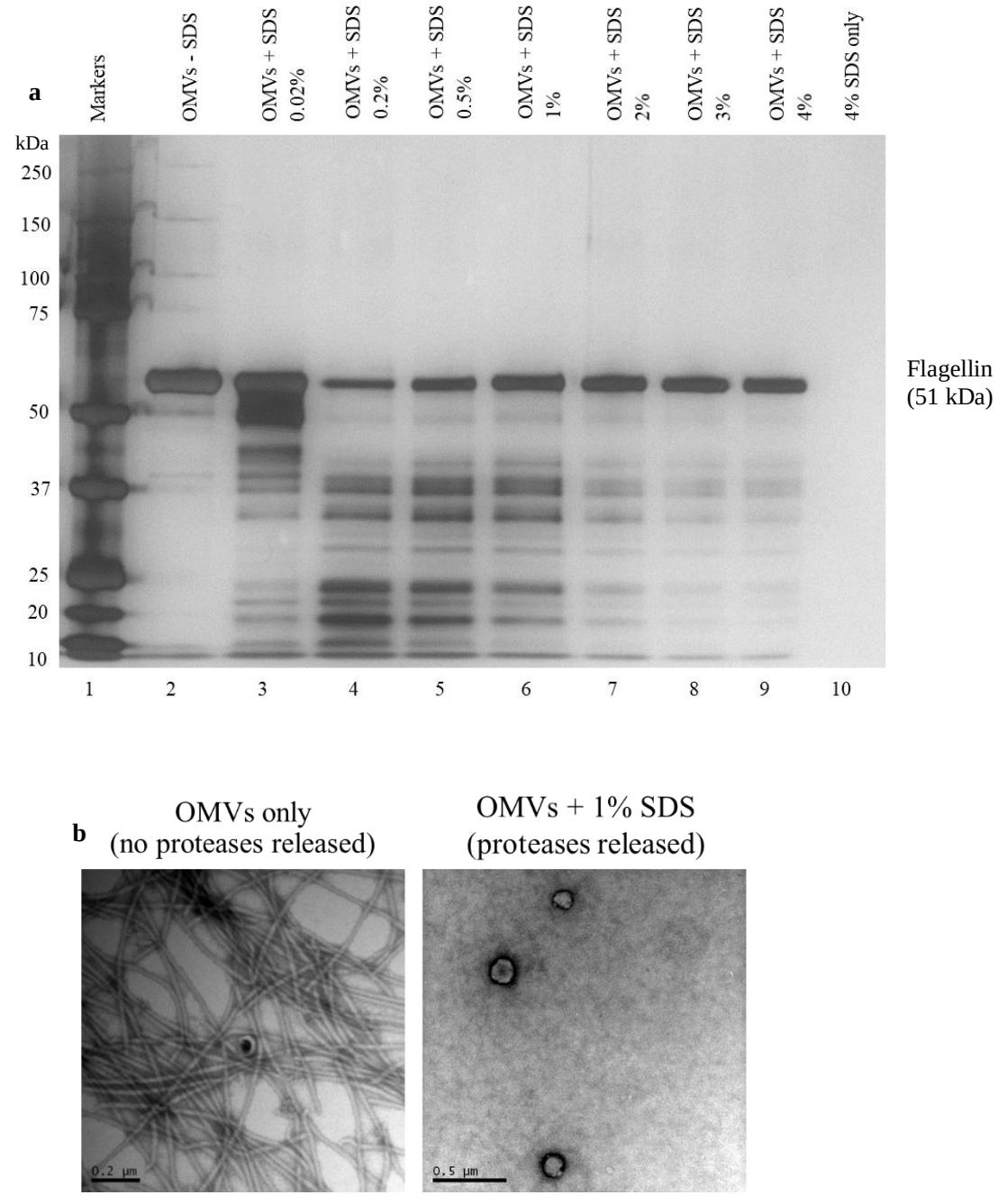


Figure 3.27 The effect of SDS on OMVs from *E. coli* FimB-LacZ fusion

OMVs were incubated in the presence and absence of SDS of various concentrations for 60 mins at 37ºC. 30 µl sample was mixed with 10 µl 4x Reducing sample buffer and heated at 95°C for 5 mins before loading on to an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile (**a**). The brightness of the photo was increased by 10% for clarity. OMVs untreated with SDS (Lane 2) and OMVs treated with 1% SDS (Lane 6) were concentrated 3x by centrifugation at 13,200 RPM. 10µL of sample was visualised by TEM.

As found in Figure 3.26, the addition of SDS causes the extra bands to appear in each sample (Lanes 3-9 of Figure 3.27**a**). Interestingly, the main Flagellin band in the OMV is still present and is not degraded by any released proteases. OMVs with no SDS treatment and OMVs treated with 1% (w/v) SDS were visualised by TEM and compared Figure 3.27**b**. The images indicate that once SDS was added to the OMVs, proteases were released that degraded the co-purified flagella. It seemed possible that the extra banding produced could be flagella-associated proteins, which were degraded when the OMV proteases are released.

3.2.5.1 Evidence that *E. coli* FimB-LacZ fusion strain OMVs contain active proteases

To test this theory further, OMVs were treated with SDS in the presence and absence of protease inhibitors (Figure 3.28). Lastly, a range of the bands produced by the incubation of OMVs with SDS were extracted and identified by mass spectrometry (bands indicated by the blue arrows below).

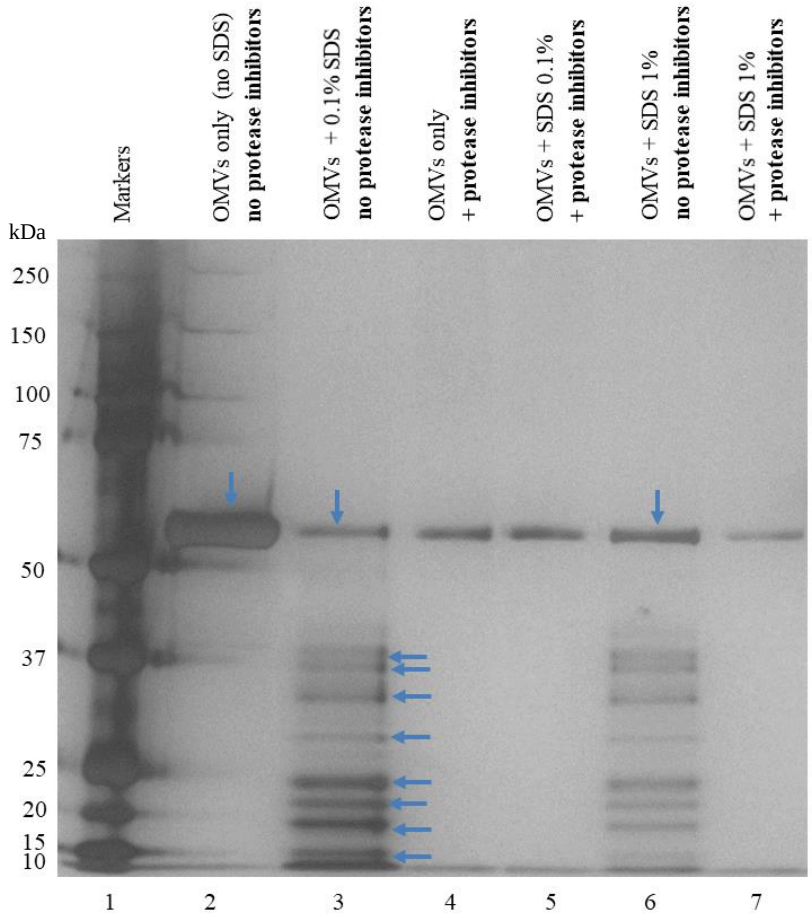


Figure 3.28 The effect of SDS on OMVs from *E. coli* FimB-LacZ fusion in the presence and absence of protease inhibitors

OMVs were incubated in the presence and absence of 0.1% and 1% SDS for 60 mins at 37ºC. 10 mM HEPES buffer was prepared containing a Protease Inhibitor Cocktail (Roche) and this was used in the samples of Lanes 4, 5 and 7. 30 µl sample was mixed with 10 µl 4x Reducing sample buffer and heated at 95°C for 5 mins before loading on to an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile (**a**). The brightness of the photo was increased by 15% for clarity.

All 12 bands above were detected as Flagellin by mass spectrometry (see Appendix B.11). Figure 5.28 shows that the addition of the protease inhibitor prevented the appearance of the additional Flagellin bands (Lanes 4, 5 and 7). This indicates that there are proteases within the *E. coli* OMVs, which are released when SDS is added and the membrane is disrupted. It appears that the proteases degrade the co-purified flagella around the OMVs which causes the appearance of the extra Flagellin bands (as Flagellin is the main structural subunit in flagella). The Flagellin monomer within the OMVs (at 51 kDa) is still protected, however, even after SDS addition. This is evidence that the Flagellin is protected somehow within the OMV separate to the proteases (discussed further in Section 3.3).

3.2.5.2 4-Nitrophenyl acetate substrate

The chromogenic esterase substrate 4-nitrophenyl acetate was used to quantify protease activity from purified OMVs. OMVs were incubated with the substrate in the presence and absence of 0.1% SDS at 37°C for 60 minutes. As an additional negative control, OMVs were heated to 95ºC to inhibit any protease activity in the sample. Protease activity is detected by a colour change from clear to yellow which is measured by the increase in absorbance at 405 nm (Figure 3.29).

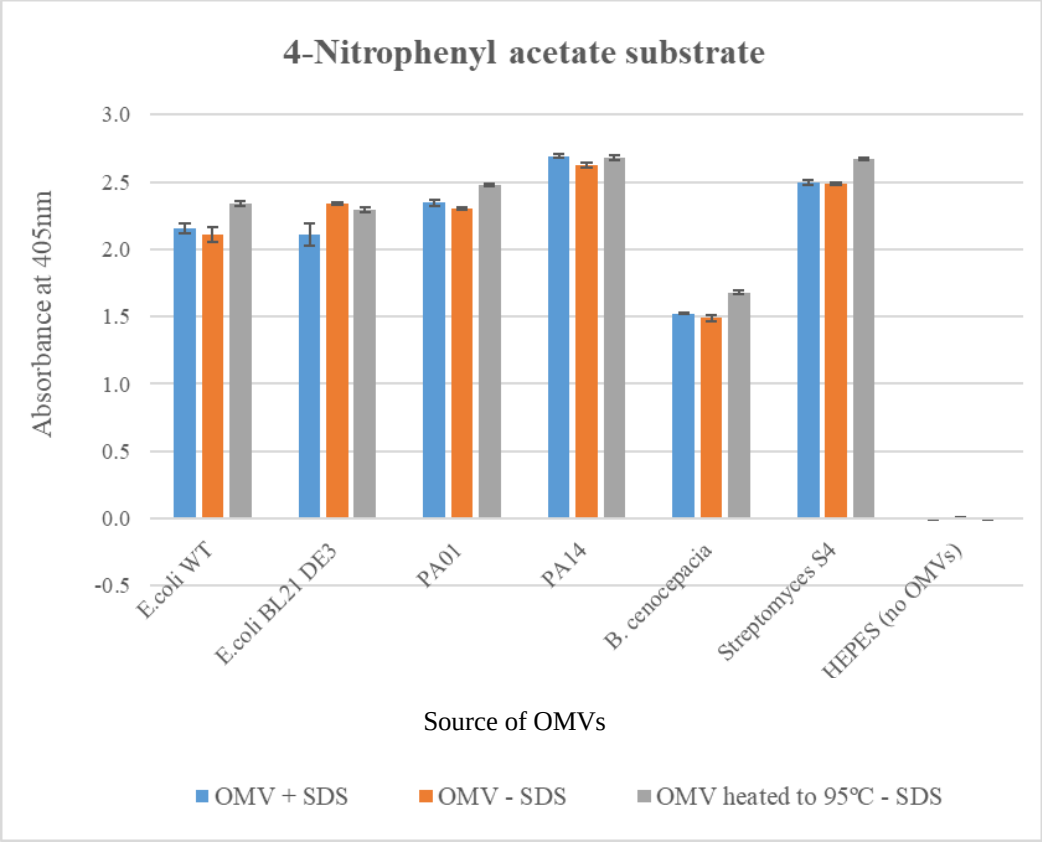


Figure 3.29 Detection of OMV proteases using 4-Nitrophenyl acetate substrate
OMVs were incubated with the chromogenic esterase substrate (4-nitrophenyl acetate) in the presence and absence of 0.1% SDS at 37°C for 60 mins. As an additional negative control, OMVs were heated to 95ºC for 60 mins prior to the assay to inhibit any protease activity in the sample. Protease activity was detected by a colour change from clear to yellow which is measured by the increase in absorbance at 405 nm. The error bars represent 1 Standard Deviation from the mean.

There was a large increase in absorbance at 405 nm when OMVs (resuspended in HEPES) were incubated with the 4-nitrophenyl acetate substrate. There was also no reaction at all with 10 mM HEPES buffer only (negative control). However, it was found that heating the OMVs to 95ºC for 1 hour did not have any effect on the absorbance detected which was unexpected. To explore this further, 1 mL samples were extracted from 3 different points in the OMV purification protocol (see Figure 3.30**a**) and used in the assay. Samples extracted from all points of the OMV purification protocol reacted with the substrate in the same way. After further investigation, it was found that that 4-nitrophenyl acetate reacted with both ammonium sulphate and LB (see Figure 3.30**b)**.

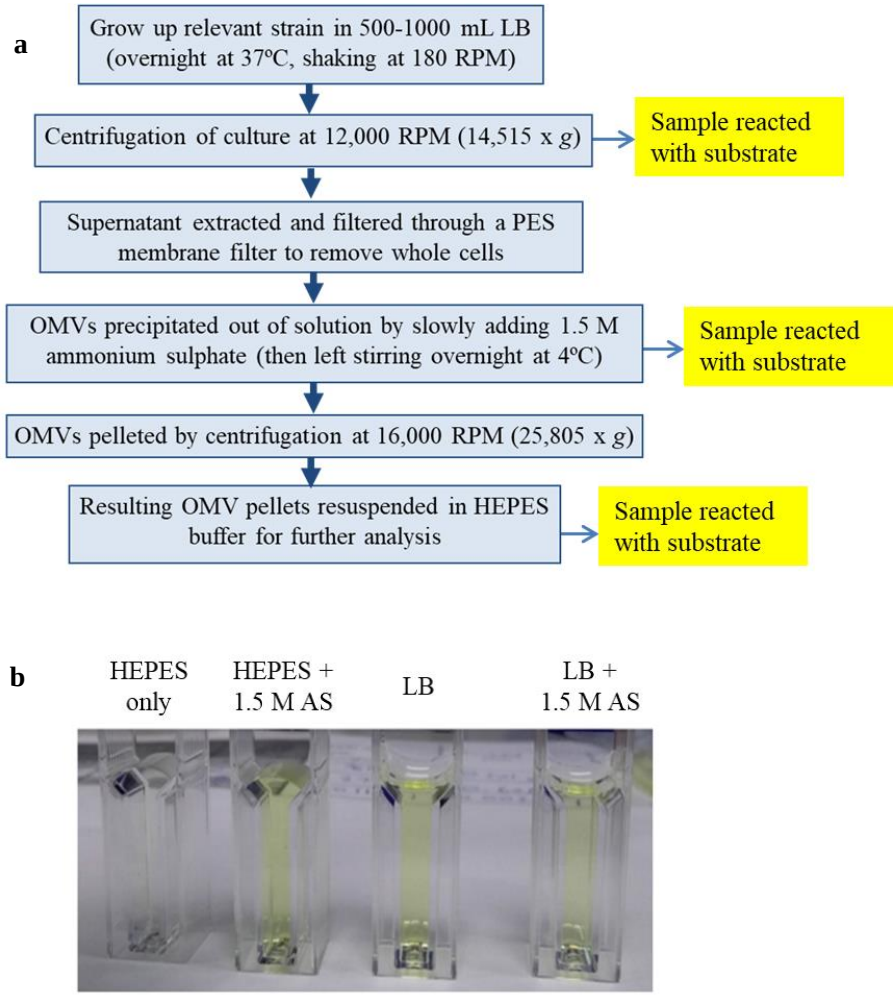


Figure 3.30 4-Nitrophenyl acetate substrate reacts with LB and ammonium sulphate
1 mL samples were extracted from 3 different points in the OMV purification protocol and used for the assay (**a**). OMVs were incubated with the chromogenic esterase substrate (4-nitrophenyl acetate) at 37°C for 60 mins. Protease activity was detected by a colour change from clear to yellow which is measured by the increase in absorbance at 405 nm. The assay was repeated with the following 4 samples: 10 mM HEPES buffer only, 10 mM HEPES buffer with 1.5 M ammonium sulphate added, LB media only and LB with 1.5 M ammonium sulphate added. Photos were taken of the cuvettes to show the colour change found (**b**).

The evidence showed that the protease activity of the OMVs could not be correlated to increase in absorbance using this method.

3.2.6 What is the best method to quantify and compare the number of purified OMVs?

The best method to quantify OMVs is widely debated (Wieser *et al*. 2014). In this project, Bradford assays were used to determine protein concentrations in OMV samples. This was used to standardise samples for loading on to SDS-PAGE gels and as a method to quantify OMVs isolated from different strains. Alternative methods of OMV quantification techniques were also trialled and are outlined below.

3.2.6.1 Quantification of OMVs using a NanoPhotometer 50 (Implen)

An alternative method of determining protein concentration was to use the NanoPhotometer 50 (Implen) using the Protein UV Bradford Assay setting. Known protein concentrations of BSA and lysed *E. coli* OMVs in HEPES were trialled on the NanoPhotometer. However, it was concluded that the NanoPhotometer N50 could not accurately detect low protein concentrations of 75 µg/mL and under (see Appendix B.12). Purified OMV samples can range between 20 µg/mL to 200 µg/mL protein. For this reason, the Bradford assay was used for determining all protein concentrations as it was more accurate.

3.2.6.2 Characterisation and Quantification of OMVs using Dynamic Light Scattering (DLS)

The Litesizer 500 was used to characterise OMVs using the DLS mode. The DLS was optimised to be used as an alternative method to quantify isolated OMVs and compare OMV production between various strains. Details of the DLS machine set up and optimisation are given in Appendix B.13.

The Relative Frequency of the OMVs are given in 3 forms:

1. **Intensity weighted:** this represents the size at which most light is scattered
2. **Volume weighted:** this indicates the size where most of the vesicles are by volume
3. **Number weighted:** this indicates how many vesicles there are at specific sizes

Samples from the following *E. coli* strains were trialled on the DLS and the Relative Frequency of the OMVs was determined:

1. *E. coli* B Parental strain
2. *E. coli* BL21 strain
3. *E. coli* BL21 (DE3) strain
4. *E. coli* K-12 BW25113 Parental strain
5. *E. coli ΔfimA*
6. *E. coli ΔfliC*

The DLS results for all three OMV samples purified from *E. coli* B strains showed very distinct peaks in the 100-300 nm region (Figure 3.31**a**). The average particle size for the *E. coli* B strains appeared to be correct as the TEM images showed that the OMVs can range from approximately 50-400 nm in diameter (Figure 3.31**b**). See Appendix B.14 for individual Figures for each strain.

Comparison of *E. coli* B strain OMV particle size using DLS

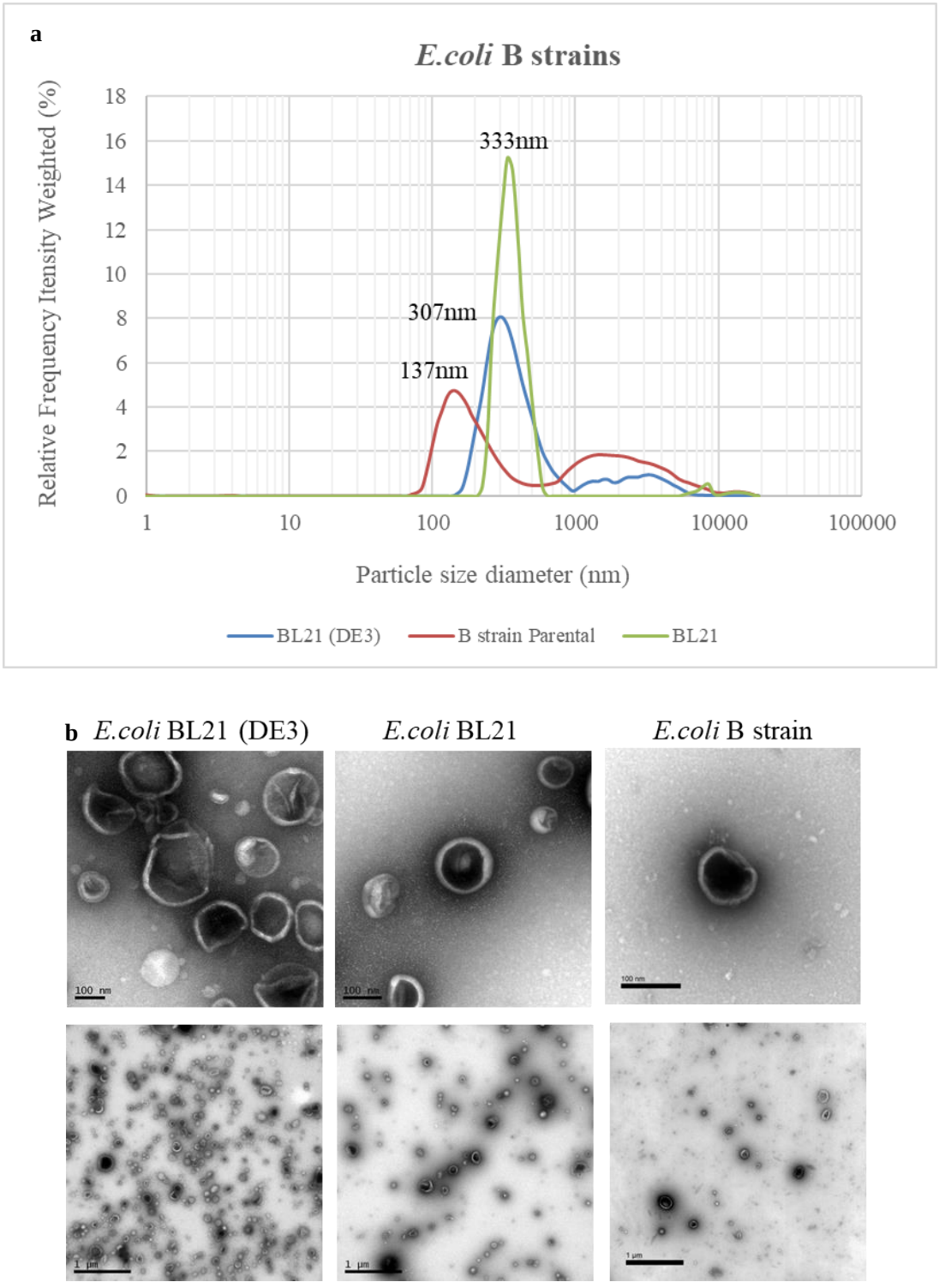


Figure 3.31 Dynamic light scattering to analyse particle size of *E. coli* B strain OMVs

The Litesizer 500 was used to calculate the Relative Frequency of OMVs (Intensity Weighted). OMVs resuspended in 10 mM HEPES buffer were purified from *E. coli* B strains: BL21 (DE3), BL21 and B strain parental and 1 mL samples were run on the DLS. An average of all repeats was taken to generate a graph using Microsoft Excel for each strain showing the average size of the OMVs in nm (**a**). The particle size diameter was compared to the TEM images of OMVs from each strain (**b**).

Comparison of *E. coli* K-12 strain OMV particle size using DLS

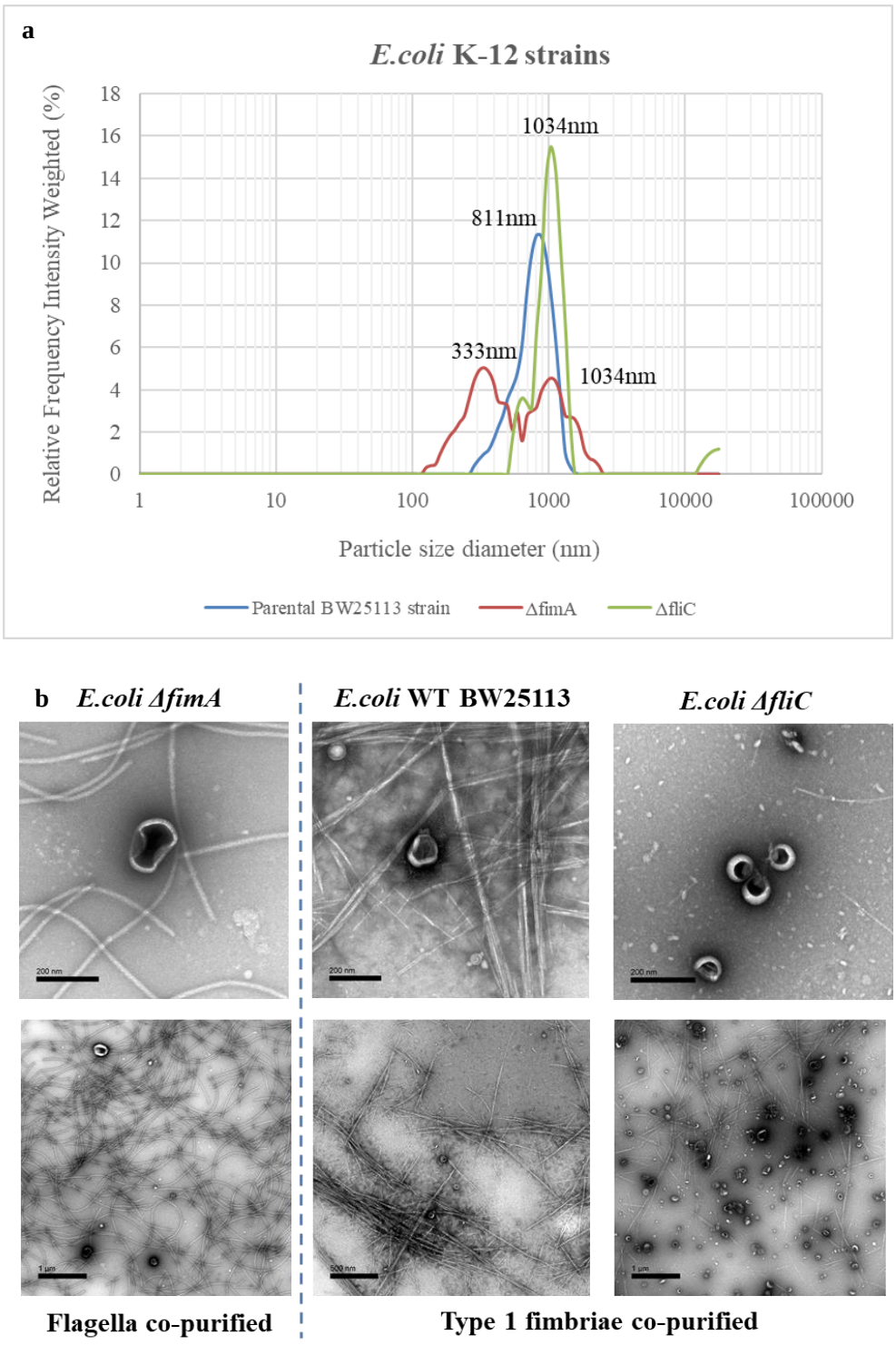


Figure 3.32 Dynamic light scattering to analyse particle size of *E. coli* K-12 strain OMVs

The Litesizer 500 was used to calculate the Relative Frequency of OMVs (Intensity Weighted). OMVs resuspended in 10 mM HEPES buffer were purified from *E. coli* K-12 strains: *ΔfimA*, BW25113 and *ΔfliC* and 1 mL samples were run on the DLS. An average of all repeats was taken to generate a graph on Microsoft Excel for each strain showing the average size of the OMVs in nm (**a**). The particle size diameter was compared to the TEM images of OMVs from each strain (**b**).

The OMVs from the *E. coli* K-12 strains were found to be approximately 100-200 nm in diameter in the TEM images (Figure 3.32**b**). However, OMV samples from the *E. coli* WT BW25113 strain and the *ΔfliC* strain show no peak at 100-200 nm (Figure 3.32**a**). Instead, peaks of 811 nm and 1034 nm were detected which is most likely to represent the co-purified Type 1 fimbriae. OMVs from the *ΔfimA* strain gave a peak of 333 nm and 1034 nm. The first of these peaks could represent the OMVs, however, this value seems high when compared to the TEM images. It is likely that flagella are being detected rather than the OMVs.

3.2.6.3 Quantification of OMVs using DLS

An attempt was made to quantify *E. coli* OMVs using the average mean intensity value of each sample. This has previously been used to compare light scattering in the literature (Simpanya *et al.* 2008). Unfortunately, the mean intensity value for each replicate varied hugely which is reflected in the error bars of 1 Standard Deviation (Figure 3.33). The co-purification of flagella and fimbriae will also cause this value to be mis-representative of the OMV concentration. For this reason, the DLS was no longer used for OMV quantification.

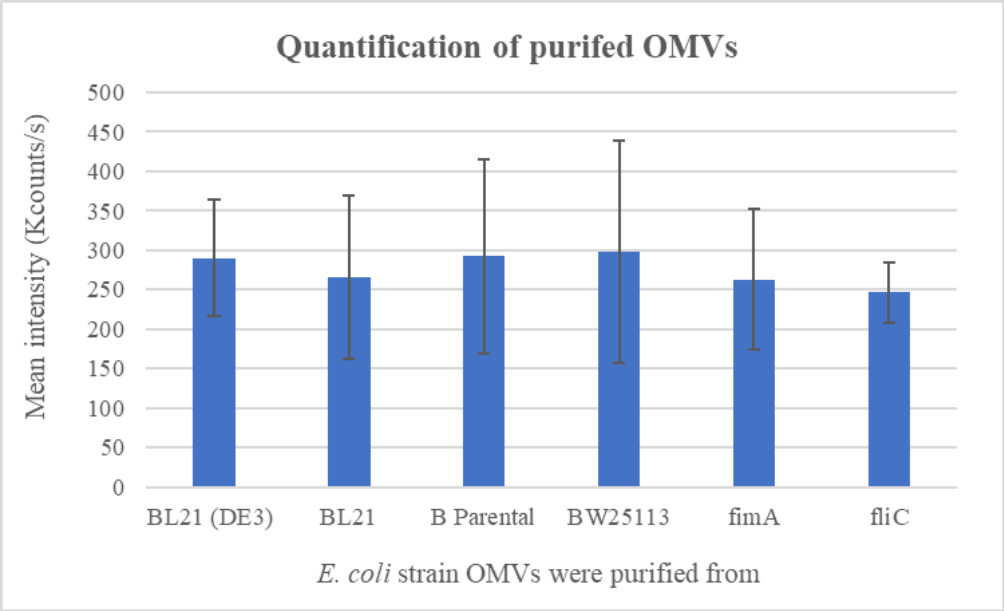


Figure 3.33 Dynamic light scattering to quantify purified *E. coli* OMVs

OMVs resuspended in 10 mM HEPES buffer were purified from *E. coli* strains: BL21 (DE3), BL21, B strain Parental, *ΔfimA*, BW25113 and *ΔfliC* and 1 mL samples were run on the DLS. For each repeat of an individual OMV sample, a Mean Intensity is calculated. An average of all repeats was taken and displayed as a bar chart with error bars representing 1 Standard Deviation.

3.3 Discussion

3.3.1 Optimisation of the OMV purification protocol

3.3.1.1 The OMV purification protocol developed is reproducible (Section 3.2.1)

OMVs purified from *E. coli* BL21 (DE3) on five different dates were compared to confirm that the OMV purification protocol developed was reproducible. The TEM images and the protein profile densitometry plots of the purified OMVs were compared. The protein profile and TEM images were almost identical each time the OMVs were purified from *E. coli* BL21 (DE3). OMV yield was quantified by using the Bradford assay. The average protein yield was 26 µg/mL protein and all OMV samples were between 21 and 35 µg/mL protein. The protein profile of *E. coli* OMVs shows many proteins of all sizes. This agrees with other SDS-PAGE gels in the literature (Figure 1, Fantappiè *et al*. 2014).

3.3.1.2 Live bacterial cells are not purified using this OMV purification protocol (Section 3.2.1)

During each OMV purification, 1 mL of PES membrane filtered supernatant was spread on to a LB agar plate and incubated at 37 °C for 24-48 hours to check that there was no bacterial growth. During the OMV purification protocol, 1.5 M ammonium sulphate was then slowly added to the supernatant to precipitate the OMVs. The addition of 1.5 M ammonium sulphate to LB was shown to fully inhibit the growth of five strains of *E. coli* (Figure 3.7).

3.3.1.3 The choice of PES or SFCA membrane type does not affect the OMV sample purity, yield or proteome (Section 3.2.1)

During OMV purification, there is a filtration of the bacterial supernatant through a membrane filter of pore size 0.2 µm or 0.45 µm to remove any remaining live cells. A 0.22 µm pore size was chosen to filter *P. aeruginosa* OMVs as the majority appear to be 200 nm or less. *E. coli* OMVs, however, can range up to 400 nm in diameter. A study in 2005 found that filtration of *Neisseria lactamica* OMVs through a 0.22 µm pore caused loss of approximately 50% of the OMVs (Gorringe *et al.* 2005). Care was taken to gain the highest yield of *E. coli* OMVs as possible so a 0.45 µm pore membrane filter was used.

To test the type of membrane filter to use, *PA14* and *PA01* were filtered using PES and SFCA membranes to see if the OMV purification was enhanced or affected. The TEM images showed no difference in the purity of the OMVs as there was flagella was co-purified when using both filters. There was also no difference in the protein profile when using either filter. PES membranes were chosen for future OMV purifications as they are most cost effective and have a faster flow rate than SFCA membrane filters.

3.3.1.4 Purified *P. aeruginosa* OMVs were separated from flagella by ultracentrifugation but this was not the case for *E. coli* OMVs (Section 3.2.1)

The ultimate aim of the ultracentrifugation step was to purify OMVs without any contaminants from the cell. Ultracentrifugation of *PA14* and *PA01* OMVs was successful at removing contaminating flagella from the sample. However, this technique was not successful at separating *E. coli* K-12 OMVs from contaminating flagella. Both the SDS-PAGE gels and EM images suggested that the OMVs were spread between all six of the OptiPrep layers rather than settling within one layer. Furthermore, in every layer both OMVs and flagella were present so there was no benefit to ultracentrifugation of the OMVs. Further attempts to remove flagella and fimbriae from the OMV sample were unsuccessful. This included extra filtration steps, extra centrifugation steps and addition of proteases. For this reason, the *E. coli* OMVs pellets were resuspended in 10 mM HEPES buffer for analysis in subsequent experiments rather than using ultracentrifugation so that no OMVs were lost. It also appears that others in the literature struggle to separate OMVs from contaminating flagella and fimbriae even after ultracentrifugation (Figure 3.34).

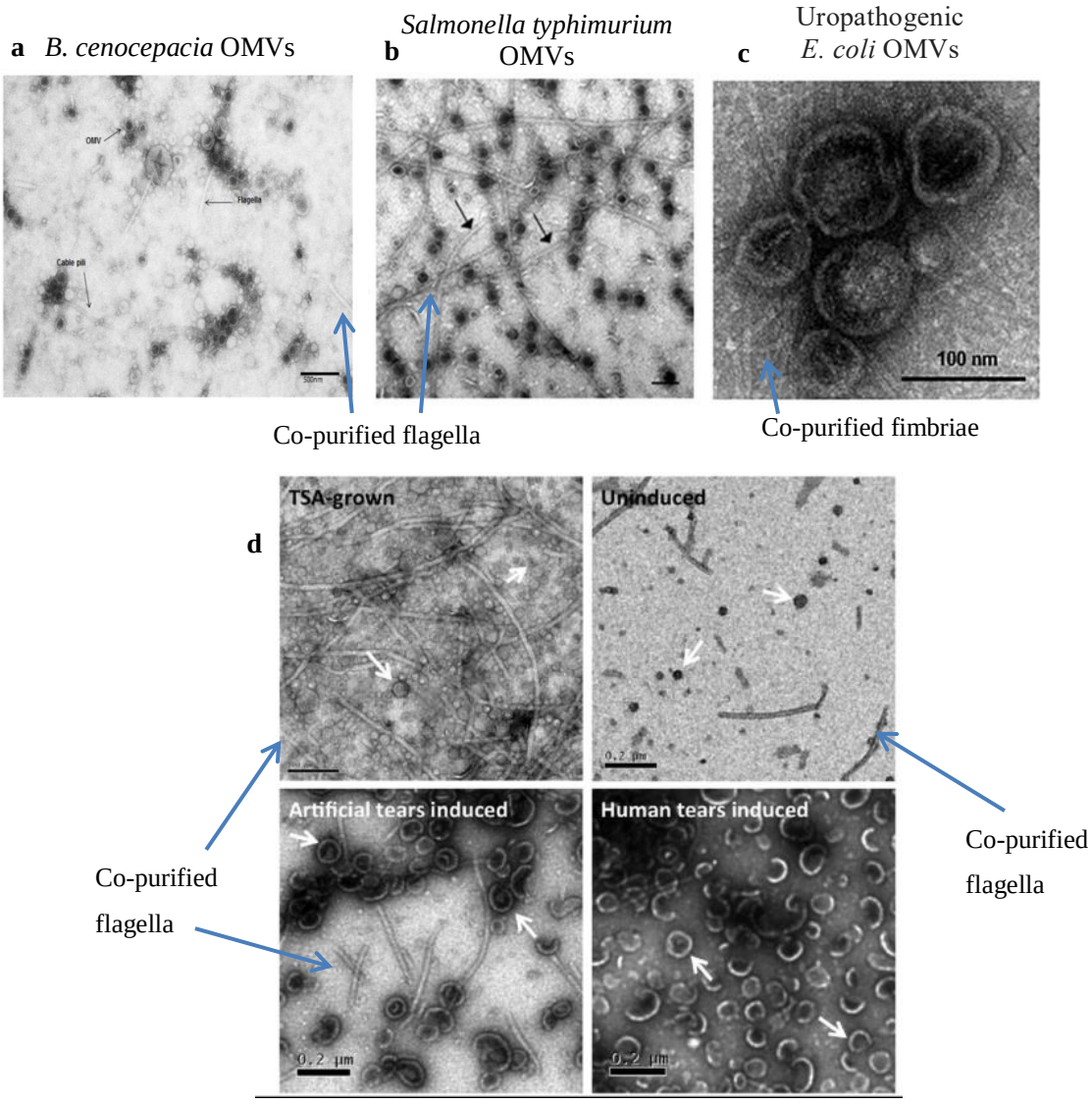


Figure 3.34 Comparison of OMVs purified from a range of Gram-negative bacteria from the literature

TEM images of OMVs from: *B. cenocepacia* OMVs sourced from Martins *et al.* 2016 (**a**). *Salmonella typhimurium* OMVs sourced from Bitto *et al.* 2016 (**b**). Uropathogenic *E. coli* OMVs sourced from Svennerholm *et al.* 2017 (**c**) *P. aeruginosa* (*PA01*) OMVs sourced from Metruccio *et al.* 2016 (**d**).

3.3.2 Success and limitations of OMV characterisation techniques

Table 3.3 Characterisation methods used on purified OMVs (Section 3.2.2-3.2.6)

OMV characterisation techniques used	Figure number
Visualisation of OMVs by transmission electron microscopy	3.10, 3.16
Immunogold labelling of *E. coli* OMVs using an anti-OmpA antibody	3.18
Visualisation of OMV biogenesis by growing *E. coli* and *PA14* on EM grids	3.19
Visualisation of OMV protein profiles using SDS-PAGE	3.20
Comparison of the protein profiles of OMVs compared with whole cells, periplasmic proteins and OM proteins	3.21, 3.23
Mass spectrometry identification of OMV proteins	Table 3.2
Western blotting to detect OmpA in *E. coli* OMVs	3.22
Evidence that purified OMVs are whole and intact (Proteinase K test)	3.24, 3.25, 3.26
Evidence that purified *E. coli* OMVs contain active proteases	3.27, 3.28
Quantification of OMVs by dynamic light scattering	3.31, 3.32, 3.33

3.3.2.1 Purified OMVs were visualised by TEM and compared to those in the literature (Section 3.2.2)

All evidence indicates that the purification protocol has successfully isolated OMVs from all bacterial strains tested. The EM images show OMVs of the expected size, shape and appearance when compared with those in the literature (see Figure 3.17). OMV biogenesis was observed by growing *E. coli* BL21 (DE3) cells and *Pseudomonas aeruginosa* (*PA14*) cells on gold EM grids and fixing at various time points over 24 hours. When comparing the EM images to OMV biogenesis images in the literature, it appears that the experiment was successful (see Figure 3.19). Lastly, immunolabelling using an anti-OmpA antibody was applied to OMVs purified from *E. coli* BL21 (DE3). OmpA was found on the surface/membrane of the OMVs as expected. However, this could be optimised further as there were too few immunogold labels. The OMV biogenesis experiment using *E. coli* BL21 (DE3) cells could also be repeated using immunogold labelling of OmpA.

3.3.2.2 Analysis of the OMV proteomes from different bacterial strains (Section 3.2.3)

The SDS-PAGE gels of the OMV protein profiles indicated that OMVs from the recombinant *E. coli* B strains contained a greater range of proteins than OMVs from the *E. coli* K-12, *PA01* and *PA14* strains. The non-recombinant (or 'wild type') strains produced OMVs which consistently contained large amounts of particular proteins and less of others. This suggests that certain proteins are selectively targeted to the OMVs in large concentrations and others are excluded. If a protein was to be targeted to OMVs for therapeutic purposes, it could be fused with one of the dominant proteins within these OMVs which are very prominent on the SDS-PAGE gels and consistently found in OMVs of that strain.

Understanding of the natural packaging of cargo into the OMVs of wild type strains is essential to maximise the selectivity and yield of cargo for therapeutic purposes. Similarly, engineered strains such as *E. coli* BL21 (DE3) produce higher yields of OMVs than wild type strains but have more diversity in the OMV proteome. *E. coli* B strains also have no extracellular appendages (ie. fimbriae or flagella) and

are not pathogenic in nature. All of these factors should be taken into consideration when choosing bacterial strains for drug delivery or vaccines. Table 3.4 outlines the main proteins found in *P. aeruginosa* OMVs and how the findings relate to the literature. The proteome of *E. coli* OMVs are discussed further in Chapter 4.

Table 3.4 Proteins identified within *P. aeruginosa* OMVs

Protein found in *P. aeruginosa* OMVs	Bacterial strain OMV originated from	Further information about the protein identified	References
B-type flagellin	*PA01, PA14*	Main structural component of flagella, used for motility. Virulence factor	Previously identified in *PA01* biofilms and OMVs (Couto *et al.* 2015)
Putative prophage major tail sheath protein	*PA01, PA14*	OMVs are thought to be used as a 'decoy' to sequester bacteriophages so that they cannot lyse the bacterial cell from which the OMVs were secreted.	Probable bacteriophage components identified in *PA01* biofilms and OMVs (Couto *et al.* 2015)
Elastase	*PA01, PA14*	"Seven secreted factors are known to exist extracellularly [in *PA01* biofilms] : **elastase** LasB, esterase EstA, PasP, **chitin binding domain protein** CbpD, chitinase ChiC, lactonizing lipase LipA, and an **aminopeptidase** (PA2939) of the M28 family of metalloproteases."	Known to be excreted extracellularly in *PA01* biofilms (Couto *et al.* 2015)
Aminopeptidase	*PA01, PA14*		
Chitin-binding protein	*PA01, PA14*		

One limitation of identifying bands of interest by mass spectrometry (MS) is that the proteins identified may not be truly OMV-associated and may have been co-purified along with the OMVs and still present within the sample. To ensure that proteins of interest are OMV-associated, Proteinase K tests were also performed on OMV samples to determine which proteins are protected within the OMVs. Flagellin and FimA became proteins of interest in *E. coli* OMVs (Chapter 4) so antibodies to each protein were purchased. These antibodies were used for Western blotting and immunogold labelling OMVs as extra evidence that these proteins are OMV associated along with the mass spectrometry data. Lastly, although MS is very sensitive, it does not show the relative abundance of each protein found which is another limitation.

3.3.2.3 OmpA was detected in *E. coli* OMV samples by Western blotting (Section 3.2.3)

Western blotting of *E. coli* OMV samples gave rise to two bands at 37 kDa and 25 kDa. OmpA in *E. coli* is known to have a molecular weight of approximately 37 kDa. The protein also contains a signal peptide which is 21 amino acids long and could give OmpA a 2-3 kDa difference when run on an SDS-PAGE gel depending on if the signal peptide is present or absent. In the literature, 25 kDa and 27 kDa

proteins have also been found to be products of the *ompA* gene (Crowlesmith *et al.* 1980). However, the 37 kDa band was the main focus when analysing the Western blots.

3.3.2.4 Purified OMVs were whole and intact: Proteinase K test (Section 3.2.4)

The bands remaining when Proteinase K is added to the OMVs (in the absence of SDS) indicated which proteins are within the lumen of the OMV. This is because these proteins were protected from degradation by Proteinase K within the OMVs. However, when the OMVs were treated with both SDS and Proteinase K, the majority of the bands disappeared. This test provided evidence that the purified *E. coli* OMVs were present and intact before SDS addition. It also showed which proteins are OMV-associated and which proteins to select for mass spectrometry. *P. aeruginosa* OMVs, however, appeared to be resistant to membrane disruption by SDS. Treatment with 5% SDS had no effect on the protein profile when Proteinase K was added compared with *E. coli* OMVs which are disrupted with as little as 0.02% SDS. This could be investigated further using a range of detergents and comparing the lipid composition of *E. coli* and *P. aeruginosa* OMVs.

3.3.2.5 Purified *E. coli* OMVs contained active proteases (Section 3.2.5)

An initial Proteinase K test on the *E. coli* FimB-LacZ fusion strain OMVs indicated that active proteases were present within the OMVs (Figures 3.25). When these OMVs were incubated with SDS, it appeared that proteases were released which caused degradation of proteins to form additional bands on the SDS-PAGE gel. TEM images suggest that SDS caused lysis of the OMVs and release of active proteases which then degraded the surrounding flagella. All of the extra bands formed on the SDS-PAGE gel were identified to be Flagellin by mass spectrometry which is the main structural subunit of flagella (Figures 3.2.7-3.2.8).

Next, OMVs from a range of bacterial strains were incubated for 1 hour with a chromogenic esterase substrate (4-nitrophenyl acetate) (Figure 3.29-3.30). Unfortunately, it was discovered that the substrate was unexpectedly reacting with both LB and 1.5 M ammonium sulphate. For this reason, the colour change could not clearly be correlated with OMV protease activity. In order to characterise OMV proteases further, kits such as Sigma Protease Fluorescent Detection Kit PF0100 or Zymogram gels (Novex) could be used. If OMV protease activity directly correlates to OMV production, a colorimetric assay could potentially be developed to quantify OMVs and learn more about factors that trigger OMV release.

3.3.2.6 There are limitations to the quantification of OMVs (Section 3.2.6)

Bradford assay

The best method to quantify OMVs is widely debated (Wieser et al. 2014). OMVs can be quantified using protein concentration or lipid concentration. Bradford assays are a reliable way to determine protein concentrations in OMV samples. This was used to standardise samples for loading on to SDS-PAGE gels and as a method to quantify OMVs isolated from different strains.

Quantification of OMVs using a NanoPhotometer 50 (Implen)

The NanoPhotometer 50 (Implen) was trialled to quantify protein in OMVs. However, it was concluded that the NanoPhotometer could not accurately detect below 75 µg/mL of protein. As purified OMV samples can range between 20 µg/mL to 200 µg/mL, this method of quantification was not appropriate.

Characterisation and Quantification of OMVs using DLS

Lastly, The Litesizer 500 was used to characterise the OMVs using the DLS function. Although the DLS gave reproducible results for *E. coli* B strains, the DLS detected the co-purified fimbriae and flagella rather than the OMVs in *E. coli* K-12 strains. For this reason, the *E. coli* K-12 OMVs could not successfully be quantified and compared. A limitation of the DLS is that often values can be slightly larger than expected. The Litesizer assumes that all particles are spherical and so if there is movement of the OMVs during measurement or aggregation of OMVs, it may detect particles to be larger than their true size.

3.3.3 Comparison of OMVs between different bacterial strains

3.3.3.1 OMVs from wild type and recombinant strains have different protein profiles (Section 3.2.3)

The number of OMVs released from the engineered and proprietary BL21 strains was higher than *E. coli* WT strains when comparing numbers on the TEM images. Unfortunately, the quantification of *E. coli* K-12 and B strain OMVs using the Bradford assay are not directly comparable. This is because purified K-12 strain OMV samples also contain flagella and/or fimbriae so not all proteins quantified are OMV-associated.

The SDS-PAGE gels of the OMV protein profiles indicated that OMVs from the recombinant *E. coli* B strains contained a greater range of proteins than OMVs, from the wildtype *E. coli* K-12, *PA01* and *PA14* strains. The non-recombinant strains produced OMVs which consistently contained large amounts of particular proteins and less of others. This suggests that certain proteins are selectively targeted to the OMVs in large concentrations for release and others are excluded. If a protein was to be targeted to OMVs, it could be fused with one of the dominant proteins within the OMVs of *PA01/PA14/E. coli* WT, which are very clear on SDS-PAGE gels and consistently found in OMVs of that strain.

The OMV purification protocol and characterisation techniques developed in this Chapter were used further in Chapters 4 and 5 to study vesicle production in both Gram-negative and Gram-positive bacteria.

Chapter 4

E. coli K-12 strains package FimA and Flagellin into OMVs in a mutually exclusive way

4.1 Introduction

4.1.1 Brief comparison of *E. coli* K-12 and B strains

E. coli K-12 strains and *E. coli* B strains are very commonly used as model organisms in the scientific community. *E. coli* is widely used in biotechnology due to its rapid doubling time, ease of manipulation and our extensive knowledge of the genome. One of the main differences between *E. coli* B strains and K-12 strains is that B strains are deficient in producing fimbriae and flagella. B strains are also deficient in certain proteases including Lon and OmpT (Bachmann, B.J. *et al.* 1972). The ultimate aim is to manipulate *E. coli* strains into producing OMVs with specific cargo for therapeutic use.

4.1.2 *E. coli* pathogenicity and extracellular appendages

As discussed previously in Section 1.6, *E. coli* cells express fimbriae and flagella during infection. Type 1 fimbriae and flagella are crucial for colonisation of the urinary tract, but mediate opposing virulence objectives, as flagella are used for motility and fimbriae are used for adhesion to sites to cause infection. Previous studies in the literature indicate that pathogenic *E. coli* cells reciprocally regulate the expression of flagella and fimbriae (Cooper *et al.* 2012). However, this finding has never been applied to OMVs. In this study, FimA and Flagellin monomers were found in *E. coli* K-12 OMVs packaged in a mutually exclusive way which, has not yet been addressed in the literature. Polymerised FimA monomer forms the main structural subunit of Type 1 fimbriae and polymerised Flagellin monomer forms the main structural subunit of flagella.

4.1.3 Coli Genetic Stock Center (CGSC): Keio collection series

The Keio collection is a series of *E. coli* strains where individual genes were systematically deleted. The parent strain (from which all the knockout strains were made) is named ‘BW25113’ and is very closely related to *E. coli* K-12 MG1655 (Baba *et al.* 2006). To study the mutually exclusive packaging of FimA and Flagellin monomers into *E. coli* K-12 OMVs, a series of Keio collection knockout strains were used. Table 4.1 below briefly describes the role of each protein in the *E. coli* cell which can be used as a reference. Diagrams of flagella and fimbriae biosynthesis can be found in Section 1.6.

Table 4.1 Description of the Keio collection knockout strains used to study the mutually exclusive packaging of FimA and Flagellin. Note: T1F refers to Type 1 fimbriae.

Strain name	Function of the protein knocked-out	Reference
E.coli Δ*fimA* (JW4277-1)	"FimA monomers comprise the bulk of the type 1 pilus structure"	Schwan *et al.* 2011*
E.coli Δ*fimB* (JW4275-1)	"The site-specific recombination that allows phase variation to occur requires two trans-acting factors located proximally upstream of *fimS*, encoded by *fimB* and *fimE*." "FimB can bind to the *fimS* element to either switch from Phase-ON to Phase-OFF or vice versa, with a slight bias towards the Phase-OFF over the Phase-ON orientation."	
E.coli Δ*fimC* (JW4279-1)	FimC is a periplasmic chaperone protein that helps translocate the fimbrial proteins through the periplasm until the FimC-Fim protein complex reaches the FimD usher.	
E.coli Δ*fimD* (JW5780-1)	"FimD is an integral outer membrane protein that serves as an usher, allowing surface localization of the nascently forming T1F"	
E.coli Δ*fimE* (JW4276-1)	"The site-specific recombination that allows phase variation to occur requires two trans-acting factors located proximally upstream of *fimS*, encoded by *fimB* and *fimE*". "FimE binds to switch *fimS* from Phase-ON to Phase-OFF."	
E.coli Δ*fimF* (JW4281-1)	"FimF and FimG are associated with FimH adhesin, forming a fibrillum structure that anchors the adhesin to the pilus shaft and controls the length of the T1F"	
E.coli Δ*fimG* (JW4282-2)		
E.coli Δ*fimH* (JW4283-3)	"FimA does not mediate binding to the mannose containing receptor. An adhesin, encoded by the *fimH* gene, is responsible for this binding"	
E.coli Δ*fimI* (JW5779-1)	"The *fimI* gene was the last gene within the fim operon to be characterized. FimI's function is not known."	
E.coli Δ*fimZ* (JW5073-1)	"Our results indicate that FimY and FimZ independently activate the PfimA promoter which controls the expression of the fim structural genes. FimY and FimZ were also found to strongly activate each other's expression and weakly activate their own expression."	Saini *et al.* 2009
E.coli Δ*fliC* (JW1908-1)	"Each filament may comprise as many as ˜30000 flagellin subunits and can grow up to ˜15 μm"	Vonderviszt *et al.* 2000-2013
E.coli ΔlrhA (JW2284-6)	"It is suggested that LrhA is a key regulator controlling the transcription of flagellar, motility and chemotaxis genes by regulating the synthesis and concentration of FlhD"	Lehnen *et al.* 2002
E.coli ΔfliD (JW1909-1)	"Flagella are crucial for bacterial motility and pathogenesis. The flagellar capping protein (FliD) regulates filament assembly by chaperoning and sorting flagellin (FliC) proteins after they traverse the hollow filament and exit the growing flagellum tip. In the absence of FliD, flagella are not formed"	Postel *et al.* 2016
E.coli ΔfliS (JW1910-1)	"To prevent premature polymerization of newly synthesized flagellin molecules, FliS, the flagellin-specific chaperone, binds flagellin and facilitates its export"	Galeva *et al.* 2014
E.coli ΔflhA (JW1868-1)	"The major filament protein (flagellin) and the filament-cap protein (FliD) bind to the FlhA cytoplasmic domain (FlhA-C) only in complex with their cognate chaperones (FliS and FliT)." "Deletion of flhA caused severely defective biofilm formation."	Bange *et al.* 2010 Svensson *et al.* 2014

4.1.4 Main chapter aims:

1. To compare OMV cargo and composition from a variety of both *E. coli* B strains and K-12 strains
2. To gain insight into how specific proteins are enriched and/or excluded from *E. coli* OMVs
3. To gain insight into the function of any cargo discovered (i.e. why would this be beneficial *in vivo*)
4. To identify any target proteins of interest in OMVs, which could be used for therapeutic purposes e.g. drug delivery and vaccines.

4.2 Results

4.2.1 Comparison of OMVs purified from *E. coli* B strains and K-12 strains

4.2.1.1 TEM images of purified OMVs

OMVs were purified from two recombinant *E. coli* B strains: BL21 and BL21 (DE3) and two *E. coli* K-12 WT strains: *E. coli* WT MG1655 and *E. coli* FimB-LacZ fusion strain (see Table 3.1 in Section 3.1.3 for strain introduction). OMVs were purified from the four strains above concurrently for a direct comparison. The TEM images (Figure 4.1), indicate that *E. coli* B strains (**a, c**) hypervesiculate compared to WT K-12 strains (**b, d**). Furthermore, purified OMVs from *E. coli* K-12 strains are co-purified with flagella and/or fimbriae whereas *E. coli* B strain cells are deficient in flagella and fimbriae. This results in a purer OMV sample with no contaminants. For attempts at quantification of these OMV samples, see Section 3.2.6.

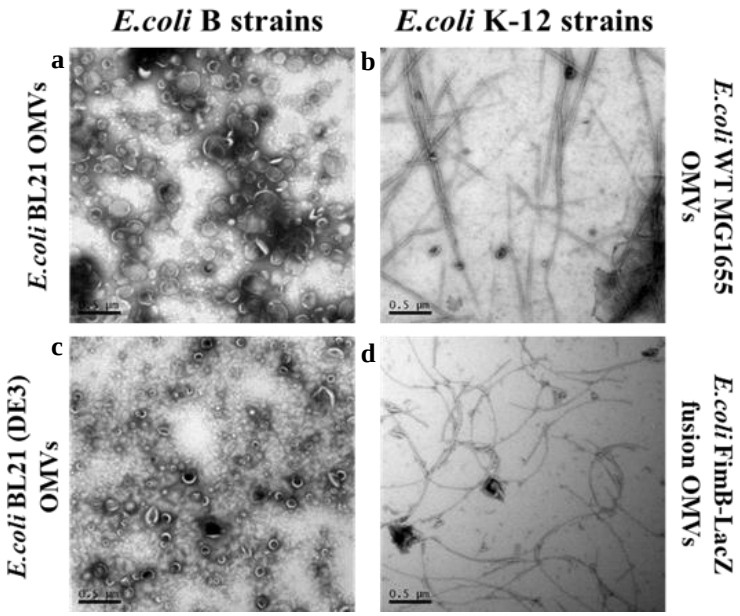


Figure 4.1 Comparison of OMVs purified from *E. coli* B strain and K-12 strains
TEM analysis of purified OMVs from four different *E. coli* strains: *E. coli* BL21 (**a**), *E. coli* WT MG1655 (**b**), *E. coli* BL21 (DE3) (**c**) and *E. coli* FimB-LacZ fusion (**d**)

4.2.1.2 Protein profile of *E. coli* OMVs (SDS-PAGE gel)

In Section 3.2.5, *E. coli* OMVs were incubated with SDS which appeared to cause disruption to the membranes and allow release of proteases from the OMVs. These proteases degraded some of the OMV-associated proteins and changed the OMV protein profile. Although in this case the addition of SDS had no effect on the OMV protein profile, there was a clear difference in the banding profile of the recombinant (B strain) and WT (K-12 strain) OMVs (see Figure 4.2 and Appendix C.1 for the original sample labelling).

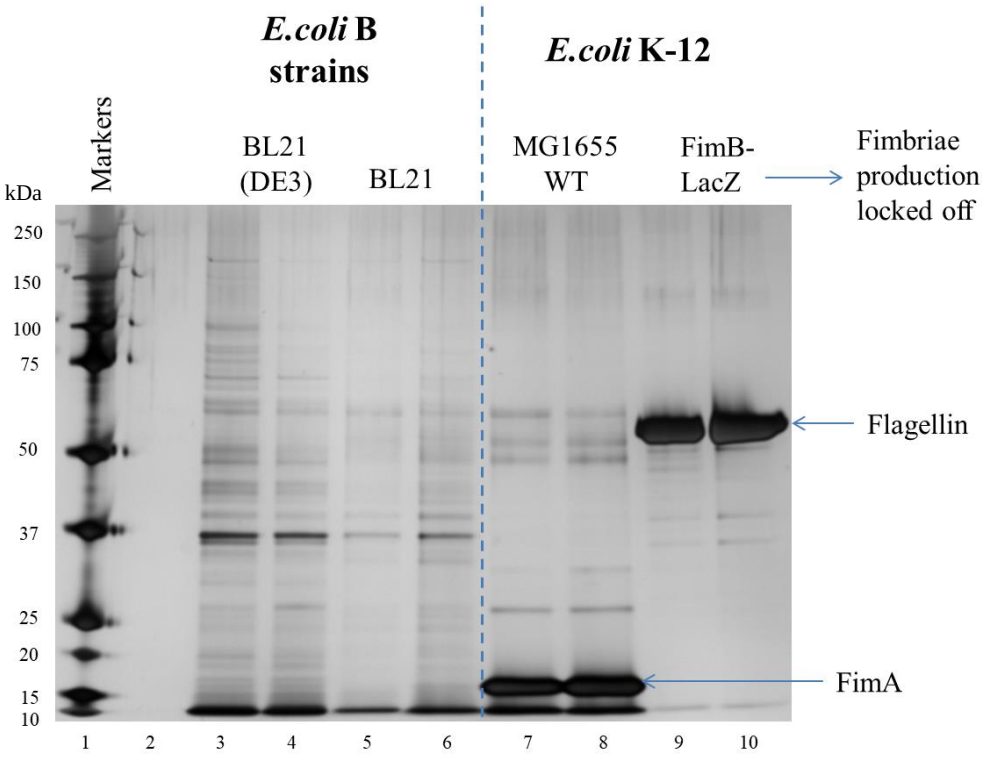


Figure 4.2 Comparison of the protein profile of OMVs purified from *E. coli* B strain and K-12 strains.

A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile. FimA and Flagellin bands labelled were extracted and identified by mass spectrometry (see Appendix C.1).

OMVs from *E. coli* B strains (lanes 3-6) have many proteins of all sizes with no particular protein band dominating the lane profile. However, OMVs from K-12 strains (in lanes 7-10) appear to have 1 main protein enriched in the OMVs and very few other proteins. One unexpected result was the difference in the banding pattern between *E. coli* WT MG1655 and *E. coli* FimB-LacZ fusion strain OMVs. In *E. coli* WT MG1655 OMVs, the prominent band at approximately 18 kDa was identified as FimA by mass spectrometry and the band at approximately 51 kDa was identified as Flagellin (see Appendix C.1). This finding was repeated three times for each band from three different gels. *E. coli* WT MG1655 OMVs (lanes 7-8) are enriched with the protein FimA (18 kDa) but the Flagellin band (51 kDa) is missing entirely. In the *E. coli* FimB-LacZ fusion strain OMVs (lanes 9-10), Flagellin is present but FimA is absent. It appears that FimA and Flagellin are packaged in a mutually exclusive way when comparing OMVs from these two strains.

4.2.1.3 What causes hypervesiculation in *E. coli* B strains compared to K-12 strains?

E. coli B strains have a deletion of the *ompT* and *lon* gene which are present in the K-12 strains. *E. coli* *Δlon* and *ΔompT* strains were purchased from the Keio mutant collection at the Coli Genetic Stock Center (CGSC). The OMVs from these two mutant strains were compared to the Keio collection

parental strain BW25113. OMVs were also isolated from *E. coli* BL21, BL21 (DE3) and the parent of the *E. coli* B strains (see Section 2.4 for further information on strains used).

As previously found, there appeared to be a higher number of OMVs purified from B strains compared to K-12 strains (see Figure 4.3 and Appendix C.2 for additional EM images). An overview of how to identify fimbriae and flagella by eye from EM images was developed in Section 3.2.1.5. *E. coli* B strains did not have any co-purified fimbriae or flagella. *E. coli* parental BW25113 strain OMVs were co-purified with fimbriae (**g**), *E. coli ΔfimA* OMVs were co-purified with flagella (**f**) and *E. coli ΔfliC* OMVs were co-purified with fimbriae. Interestingly, deletion of either *ompT* or *lon* resulted in the co-purification of flagella instead of fimbriae (**d, e**). When either protease (OmpT or Lon) is absent, the cell switches from production of fimbriae to flagella.

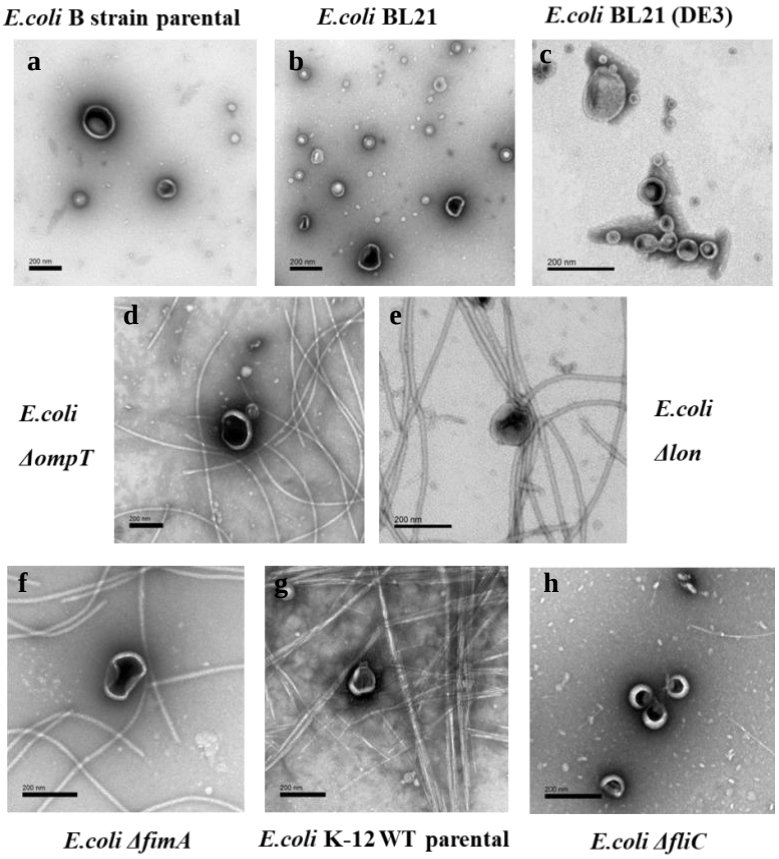


Figure 4.3 Comparison of OMVs purified from *E. coli* B strain and K-12 strains including *Δlon and ΔompT*
TEM analysis of purified OMVs from a range of *E. coli* B and K-12 strains. **B strains:** *E. coli* B strain parental (**a**), *E. coli* BL21 (**b**), *E. coli* BL21 (DE3) (**c**). **K-12 strains:** *E. coli ΔompT* (**d**), *E. coli Δlon* (**e**), *E. coli ΔfimA* (**f**), *E. coli* WT Parental BW25113 (**g**), *E. coli ΔfliC* (**h**).

The protein profiles of the OMVs from all 8 strains were compared by SDS-PAGE and Western blotting (Figure 4.4).

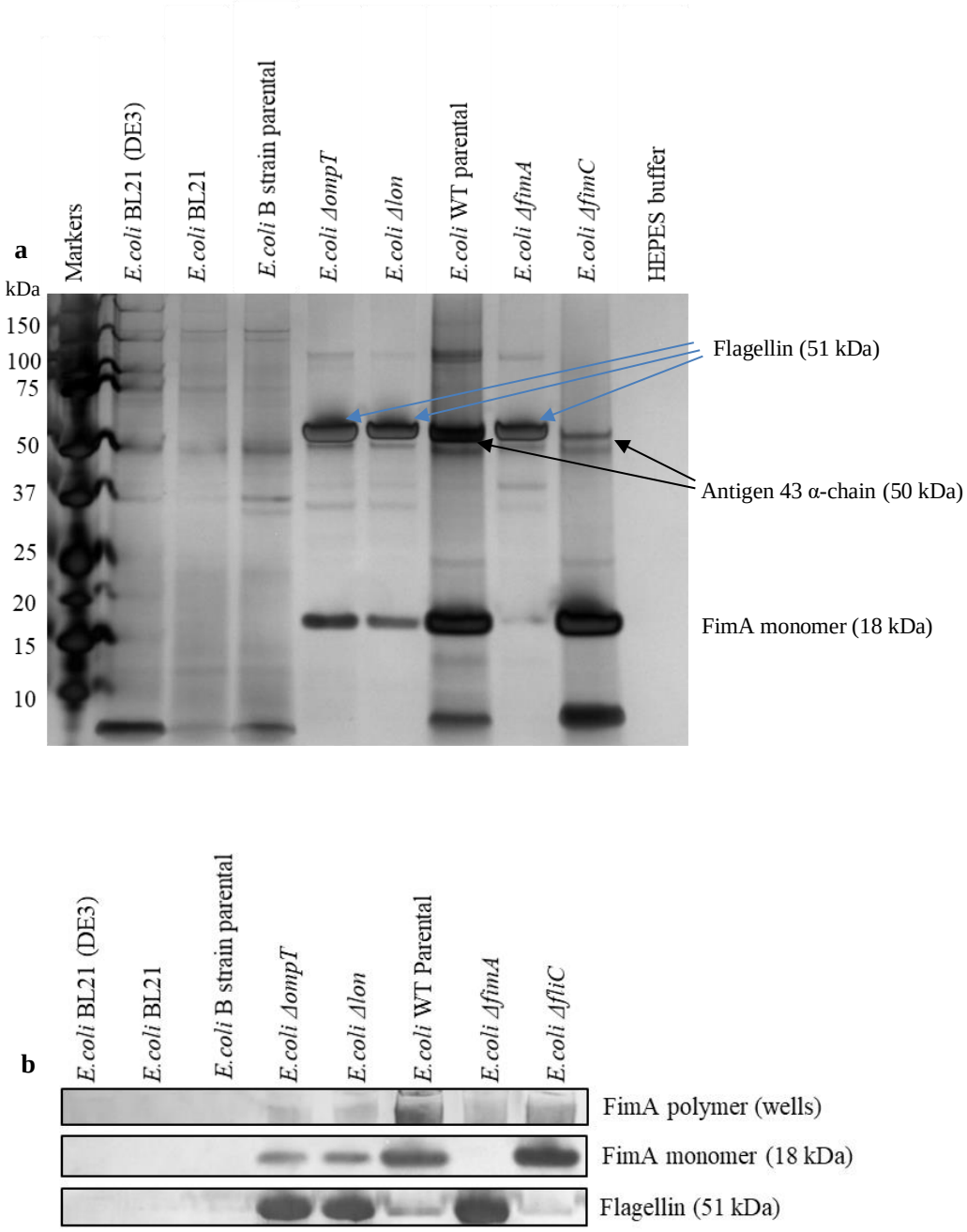


Figure 4.4 Comparison of the protein profile of OMVs purified from *E. coli* B strain and K-12 strains.

OMVs were purified from a range of *E. coli* K-12 and B strains including *ΔompT* and *Δlon*. A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. Proteins were separated using SDS-PAGE and silver stained to visualise OMV protein profiles (**a**). Purified OMV samples were probed using the following antibodies anti-FimA monomer, anti-FimA polymer and anti-Flagellin (**b**). See Appendix C.3 for original Western blot images.

OMVs purified from *E. coli* B strains have many proteins with no particular one enriched in the OMVs. Interestingly, deletion of either the *ompT* or *lon* gene caused Flagellin to be packaged within the OMVs, while still packaging FimA monomer too (compare lanes 5-6 to lane 7). It also caused the *E. coli* cell to produce flagella instead of fimbriae and package Flagellin along with FimA monomer in OMVs.

4.2.2 Evidence for Enrichment of FimA and/or Flagellin monomer into *E. coli* K-12 OMVs

4.2.2.1 OMVs were purified from an *E. coli* K-12 strain with fimbriae production locked on

FimA is the main structural component of Type 1 fimbriae and is not usually found in a monomeric form. During the formation of Type 1 fimbriae, FimA enters the periplasm in an unfolded form and binds to the periplasmic chaperone FimC. FimC catalyses FimA folding then delivers it to the usher protein FimD. Lastly, the FimA monomer is incorporated into the Type 1 fimbriae (Nishiyama.*et al.* 2005). Similarly, Flagellin is believed to bind to the chaperone FliS in the cytosol to prevent premature polymerisation of Flagellin monomers until they are needed for flagella biosynthesis (Muskotál *et al.* 2006). To explore this further, OMVs were purified from an additional *E. coli* K-12 strain with fimbriae production locked on (further details this strain can be found in Section 2.4). Interestingly, OMVs from this strain contain both Flagellin and FimA and the packaging is no longer mutually exclusive (see Figure 4.5).

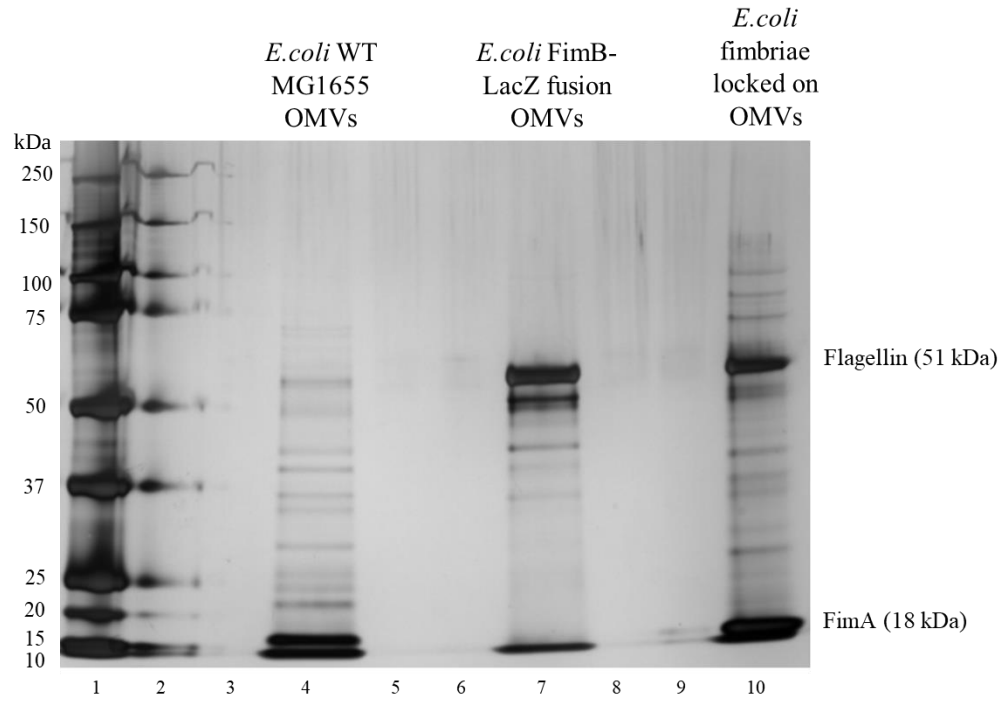


Figure 4.5 Comparison of the protein profile of OMVs purified from *E. coli* MG1655 OMVs, FimB-LacZ fusion and *E. coli* with fimbriae production locked on

A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile. The bands at 18 kDa and 51 kDa have been identified as FimA and Flagellin in previous mass spectrometry of OMVs from *E. coli* with fimbriae locked on.

4.2.2.2 Immunogold labelling of embedded *E. coli* cells and OMVs to detect FimA and Flagellin monomer within the OMVs

Further evidence was needed to confirm if the Flagellin and FimA bands in the SDS-PAGE gel reflected proteins packaged within the OMVs or whether the bands represented subunits of whole flagella or fimbriae which had been co-purified with the OMVs. Cells and OMVs from various *E. coli* strains were embedded in resin and sectioned to give a cross-section of the proteins inside the OMVs. Immunogold labelling was used to detect FimA and Flagellin monomer within the OMVs. Figure 4.6 shows immunogold labelled cells and OMVs purified from the Keio collection parental strain BW25113. Both FimA monomer and Flagellin monomer were detected within the whole cells (**d** and **e**). However, only FimA monomer was detected within the OMVs and not Flagellin (**a** and **b**). This confirms the findings in Figure 4.5. Lastly, it was noted that the immunogold labels present around the OMVs in Figure 4.6**a** were most likely due to the leakage of OMV contents during the OMV fixing and embedding process.

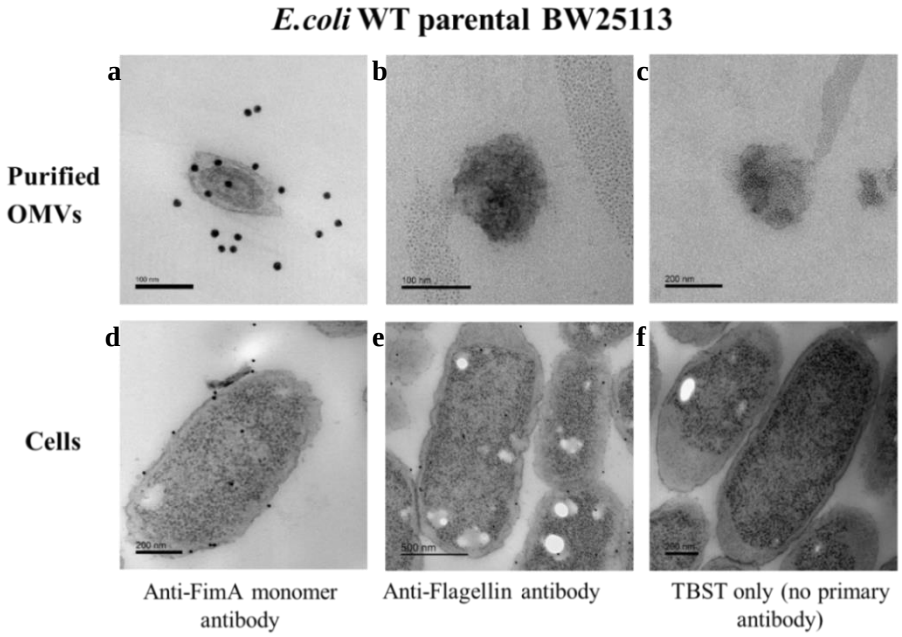


Figure 4.6 Immunogold labelling of embedded *E. coli* parental BW25113 strain cells and OMVs

TEM analysis of thin-sectioned cells and OMVs embedded in resin. The sections were immunogold labelled and probed with anti-FimA monomer antibody (**a** and **d**), anti-Flagellin antibody (**b** and **e**). As a negative control, the embedded OMVs were incubated in TBST only (no primary antibody). The samples were still incubated with the secondary antibody as usual (**c** and **f**).

4.2.2.3 How do the levels of FimA and Flagellin in OMVs compare to FimA/Flagellin levels in the whole cell and periplasm?

Six different strains of *E. coli* were grown overnight in 750 mL LB. 500 mL of this culture was used to purify OMVs and 100 mL was used for periplasmic protein extraction. The 'whole cell' sample is the *E. coli* culture only. The SDS-PAGE gels indicated that the levels of FimA and Flagellin in the whole cell and periplasm are relatively low and that these proteins are specifically selected to be packaged in OMVs (Figure 4.7).

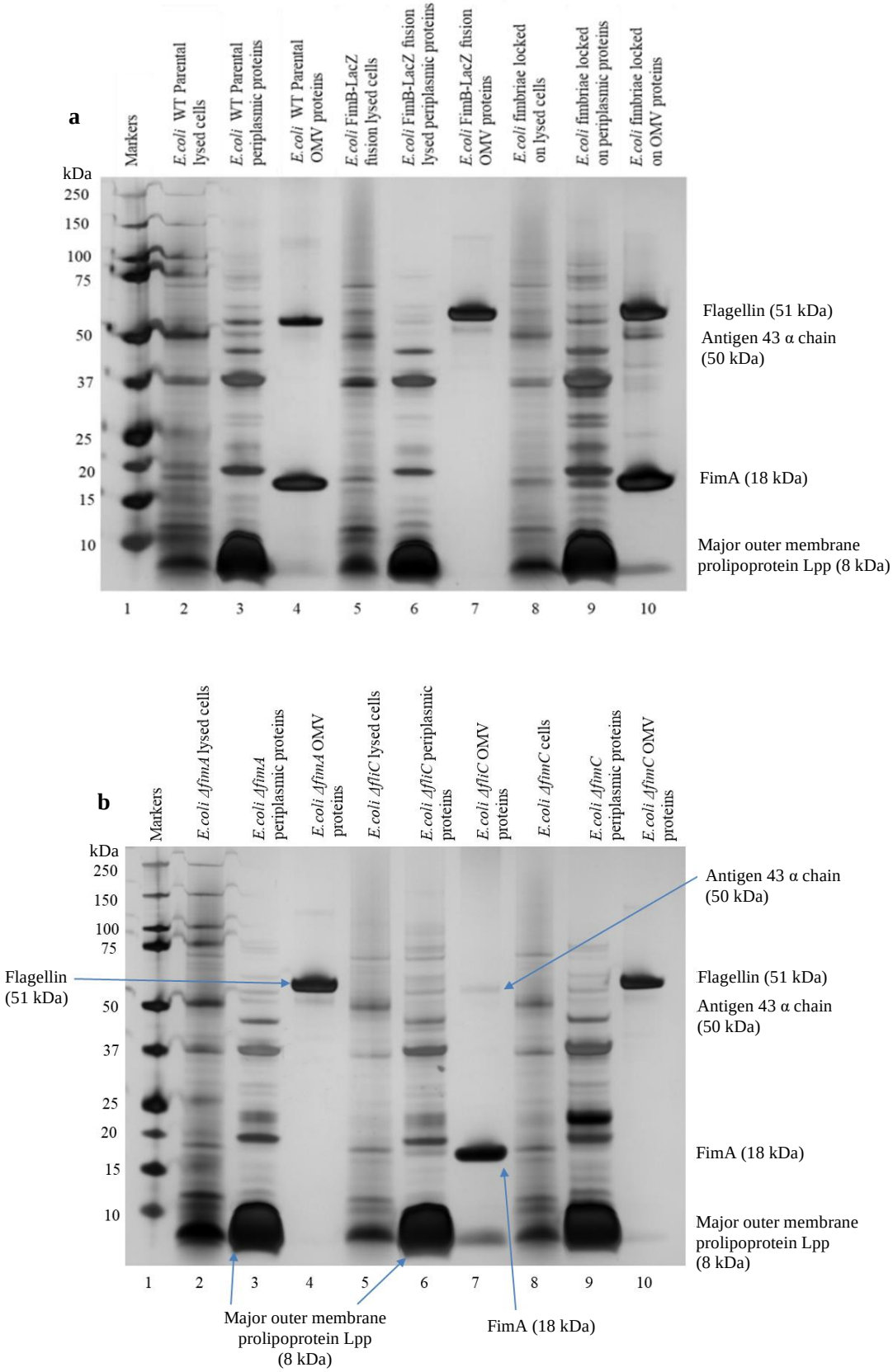


Figure 4.7 FimA and Flagellin are enriched in *E. coli* K-12 OMVs compared to levels in the periplasm and whole cell

A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise protein profiles of *E. coli* OMVs were compared to the whole cell and periplasm. SDS-PAGE gel **a** shows the *E. coli* WT parental strain BW25113, *E. coli* FimB-LacZ strain and the *E. coli* fimbriae locked on strain. Gel **b** shows the *ΔfimA* strain, *ΔfliC* strain and *ΔfimC* strain. The identity of proteins labelled in **b** were confirmed by mass spectrometry (Appendix C.14).

The samples from Figure 4.7 were used for Western blotting to detect Flagellin and FimA monomers in all samples (Figure 4.8).

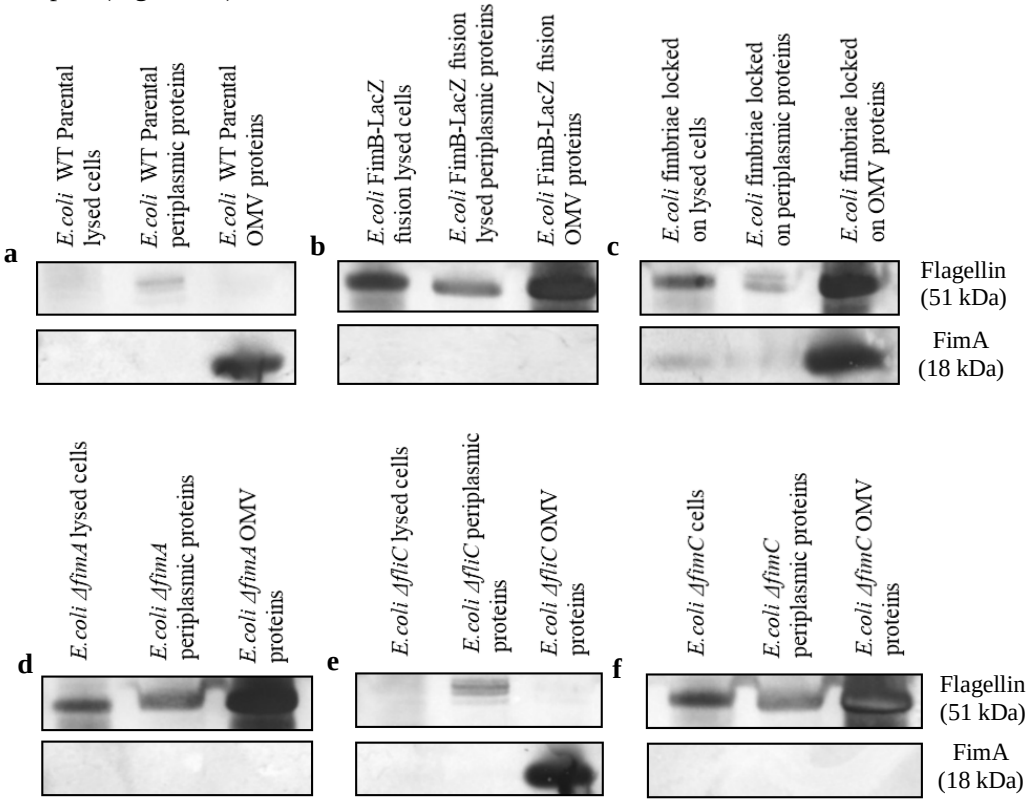


Figure 4.8 FimA and Flagellin are enriched in *E. coli* K-12 OMVs compared to levels in the periplasm and whole cell

A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. Western blots were performed on periplasmic proteins, OMVs and proteins from the whole cell using anti-FimA monomer and anti-Flagellin antibodies. The presence of FimA and Flagellin was compared in the following strains: *E. coli* WT parental BW25113 (**a**), *E. coli* FimB-LacZ fusion protein (**b**), *E. coli* with fimbriae locked on (**c**), *ΔfimA* (**d**), *ΔfliC* (**e**) and *ΔfimC* (**f**). See Appendix C.4 for full photos of the Western blots.

Figure 4.8 indicated that there was a clear enrichment of monomeric FimA and/or Flagellin in OMV purification samples compared with the periplasm and whole cell. The presence of FimA or Flagellin was also found to be mutually exclusive in the OMVs except for the *E. coli* strain where fimbriae production was locked on (**c**). The regulation of which protein was packaged had somehow been disrupted, which was explored further in Chapter 4.2.3.

In the *E. coli* parental strain BW25113 (**a**), a faint band was present that appeared to be Flagellin within the periplasmic fraction. However, previous mass spectrometry results have identified this as an Antigen 43 subunit (50 kDa) rather than Flagellin (51 kDa). This band was also found in the *ΔfliC* periplasmic protein sample (**e**), which has also previously been identified as Antigen 43. Flagellin and Antigen 43 proteins have 41% amino acid identity (see Appendix C.5 for BLAST alignment). Antigen 43 is composed of two protein subunits: α (50 kDa) and β (53 kDa) (Kjærgaard *et al.* 2000). As

Flagellin is 51 kDa, this makes differentiating between Flagellin and Antigen 43 bands difficult and cross-reactivity of the anti-Flagellin antibody with Antigen 43 is possible. For this reason, mass spectrometry was used to differentiate between Flagellin and Antigen 43 bands on SDS-PAGE gels if needed.

4.2.2.4 Proteinase K test: Evidence for Flagellin monomer protection within *E. coli* K-12 OMVs

As discussed in Section 3.2.4, the Proteinase K test confirms which proteins are present within the OMVs. To confirm the presence of FimA and Flagellin within *E. coli* K-12 OMVs, the Proteinase K test was applied. According to ExPASy peptide cutter tool (see Appendix C.6), Proteinase K will cause 251 cleavages in Flagellin (FLIC_ECOLI) and 108 cleavages in FimA (FIMA1_ECOLI). When Proteinase K is added to the OMVs alone (without SDS), proteins within the lumen of the OMVs should be protected from degradation and the bands representing them will still be present on the SDS-PAGE gels. Any proteins outside the OMVs will be degraded and the bands will disappear on the SDS-PAGE gel. Figure 4.9 gives strong evidence that Flagellin is protected within OMVs from *E. coli* FimB-LacZ fusion strain (lane 3 compared to lanes 5-9).

Proteinase K test: OMVs from *E. coli* FimB-LacZ fusion strain

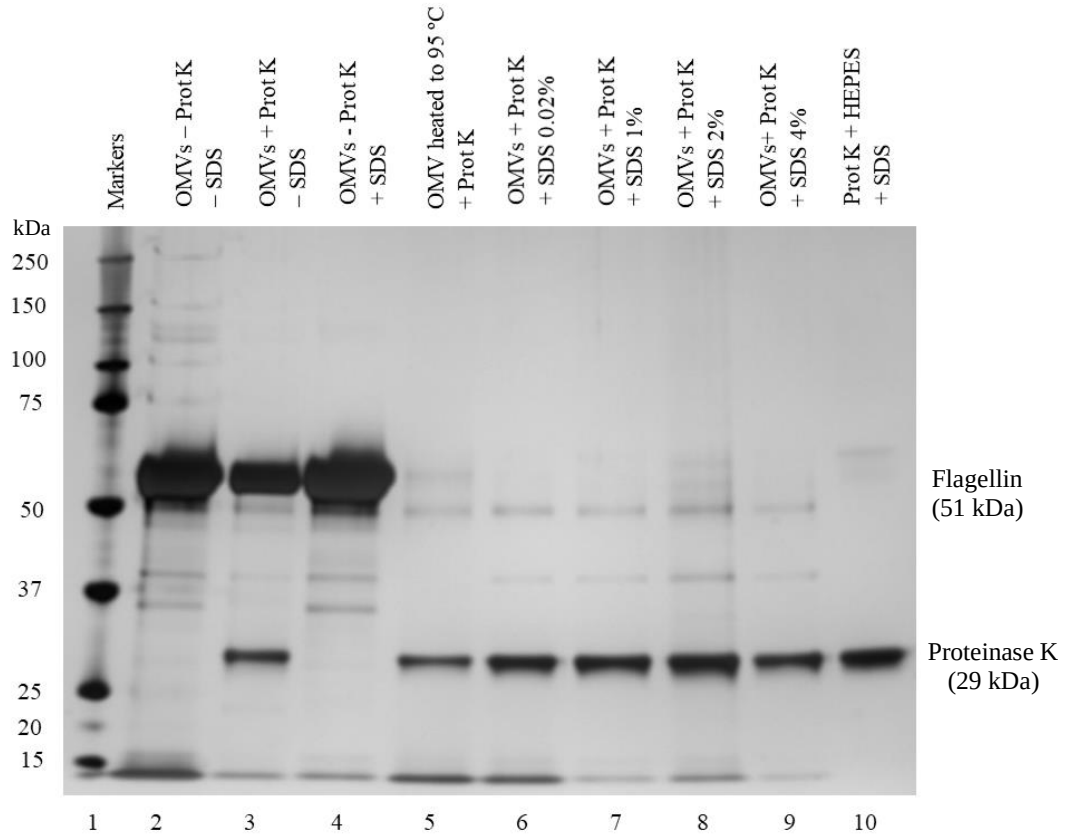


Figure 4.9 Proteinase K test on OMVs from *E. coli* FimB-LacZ fusion strain

OMVs were incubated in the presence and absence of 10 µg/mL Proteinase K and various concentrations of SDS for 30 mins at 37ºC. 5 mM PMSF was added to inhibit Proteinase K and samples were incubated for another 30 mins at 37ºC. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile.

4.2.2.5 Proteinase K test: Evidence for FimA monomer protection within OMVs

Proteinase K test: OMVs from *E. coli* WT MG1655 strain

The Proteinase K test was applied to OMVs from the WT MG1655 strain to confirm the presence of FimA within the OMVs as in Figure 4.9. Unexpectedly, the FimA monomer band remained unchanged in all lanes (Figure 4.10).

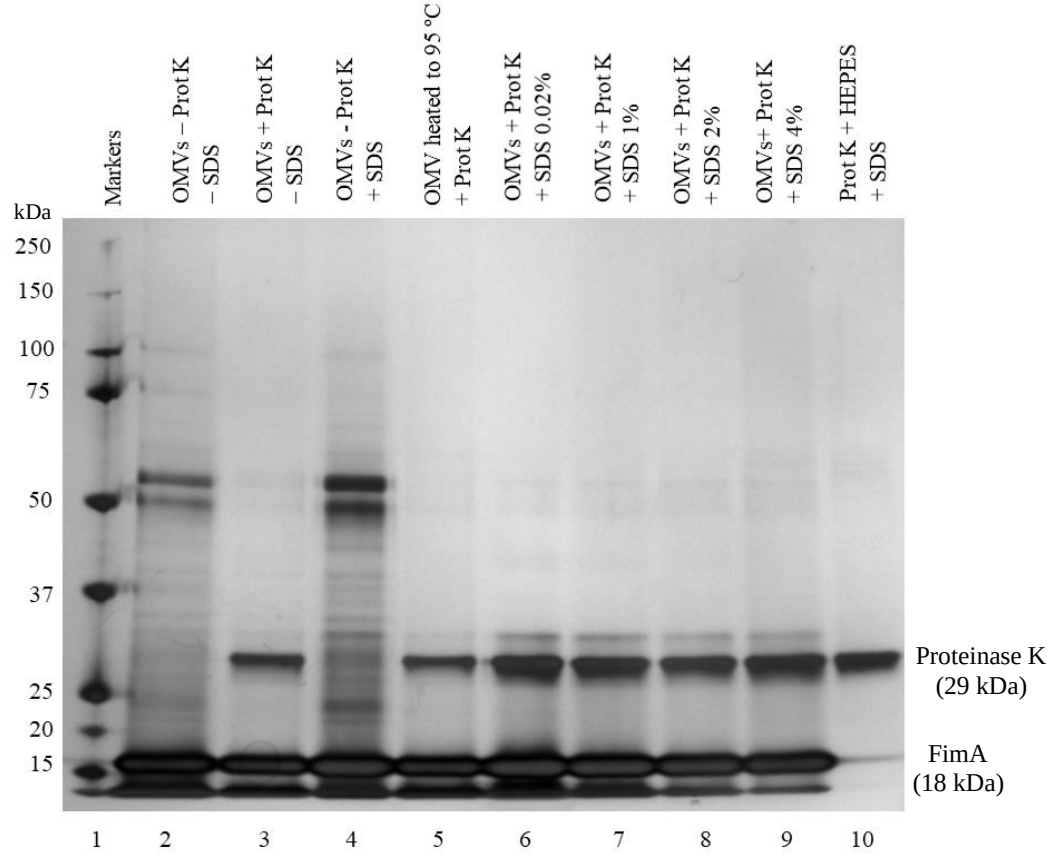


Figure 4.10 Proteinase K test on OMVs from *E. coli* WT MG1655 strain

OMVs were incubated in the presence and absence of 10 µg/mL Proteinase K and various concentrations of SDS for 30 mins at 37ºC. 5 mM PMSF was added to inhibit Proteinase K and samples were incubated for another 30 mins at 37ºC. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile.

This result was unexpected as addition of Proteinase K to FimA (FIMA1_ECOLI) should cause 108 cleavages (see Appendix C.6) and the FimA band should have disappeared. OMVs from *E. coli* with fimbriae production locked on contained Flagellin and FimA so the Proteinase K test was repeated using OMVs from this strain (Figure 4.11). The Proteinase K test successfully showed the protection of Flagellin within the OMVs that were structurally intact in Lane 3. However, the FimA monomer band remained undegraded by Proteinase K which was explored further.

***E. coli* WT MG1655 with fimbriae locked on**

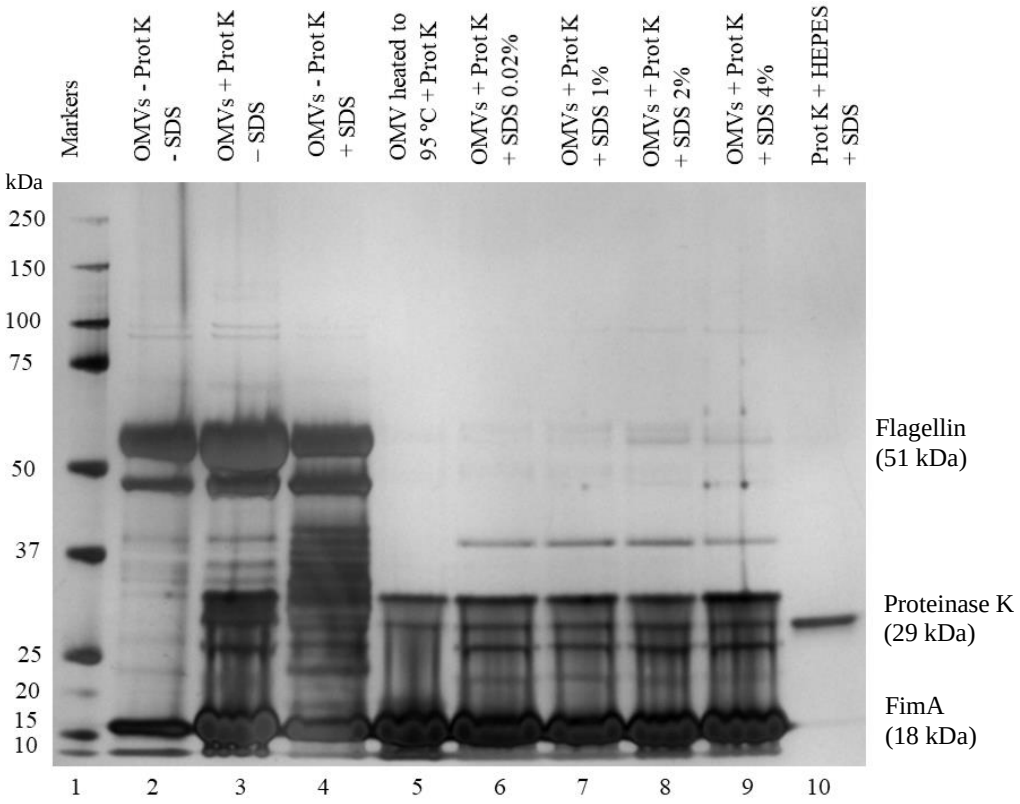


Figure 4.11 Proteinase K test on OMVs from *E. coli* WT with fimbriae locked on

OMVs were incubated in the presence and absence of 10 µg/mL Proteinase K and various concentrations of SDS for 30 mins at 37ºC. 5 mM PMSF was added to inhibit Proteinase K and samples were incubated for another 30 mins at 37ºC. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile.

The conditions were optimised to allow degradation of FimA by Proteinase K. Addition of Proteinase K to FimA (FIMA1_ECOLI) should result in 108 cleavages but in this case the protein remains uncleaved. There were various hypotheses about why the FimA monomer was not degraded in these conditions. It could be that the protein was not in the correct conformation to be digested and that all the cleavage sites were inaccessible and protected within the protein. Alternatively, the FimA monomer could be protected from Proteinase K degradation due to its location within the OMV. For example, it could be compartmentalised within the OMV or FimA could be protected by being part of a compound which makes it inaccessible to proteases. Reference for the structure of FimA monomer can be found here: https://www.uniprot.org/uniprot/P04128.

4.2.2.6 Optimisation of the Proteinase K test conditions in order to apply the test to OMV samples containing FimA monomer

The following methods were trialled to degrade FimA monomer in the OMV samples:

1. Temperature: Performing the Proteinase K test at 60ºC (instead of 37ºC) to aid FimA protein unfolding and allow Proteinase K easier access to the protein cleavage sites.

2. Method of OMV lysis: OMVs were previously lysed by heating at 95ºC for 30 minutes. OMVs were also lysed by sonication to allow proteases full access to the FimA protein. The sonication conditions chosen were those used in a paper in the literature (Metruccio *et al.* 2016).

3. Protease used: OMVs were treated with both Proteinase K and Trypsin alone and together. The concentration of proteases used was increased to be 10x more than those used in the literature for similar experiments (Mugita *et al.* 2017).

4. Assay time: The proteases were incubated with the OMV samples for: 0, 30, 60, 120 and 180 minutes.

The Proteinase K test was repeated on OMVs from *E. coli* with fimbriae locked on with variations in the proteases used, protease concentration, reaction temperature and method of OMV lysis (Figures 4.12 and 4.13). The FimA band remained unchanged in all conditions despite the Flagellin band degradation in all conditions tested.

a

Lane number	OMVs lysed by heat	OMVs lysed by sonication	10µg/mL Proteinase K addition	100µg/mL Proteinase K addition	Reaction at 37°C	Reaction at 60°C
1						
2	✔					✔
3	✔			✔	✔	
4	✔			✔		✔
5		✔				✔
6		✔	✔		✔	
7		✔	✔			✔
8		✔		✔	✔	
9		✔		✔		✔
10				✔		✔

b

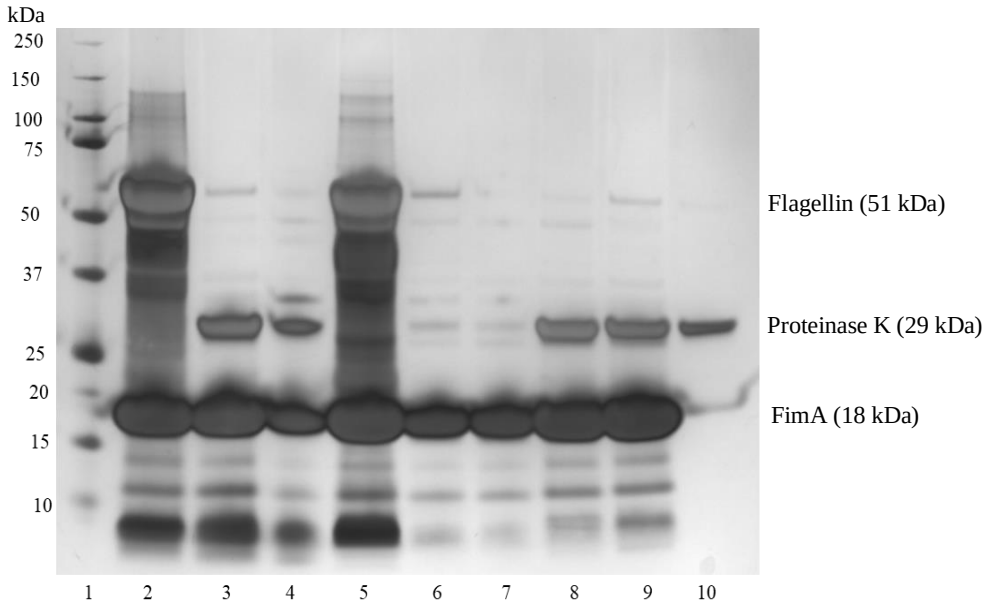


Figure 4.12 Proteinase K test on OMVs from *E. coli* WT with fimbriae locked on

The Proteinase K test was repeated on OMVs from *E. coli* with fimbriae locked on with variations in protease concentration, reaction temperature and method of OMV lysis (**a**). TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile (**b**).

a

Lane number	OMVs lysed by heat	OMVs lysed by sonication	10µg/mL Proteinase K addition	100µg/mL Proteinase K addition	20µg/mL trypsin addition	100µg/mL trypsin addition	Reaction at 37°C	Reaction at 60°C
1								
2	✔							✔
3	✔			✔		✔	✔	
4	✔			✔		✔		✔
5		✔						✔
6		✔	✔		✔		✔	
7		✔	✔		✔			✔
8		✔		✔		✔	✔	
9		✔		✔		✔		✔
10				✔		✔		✔

b

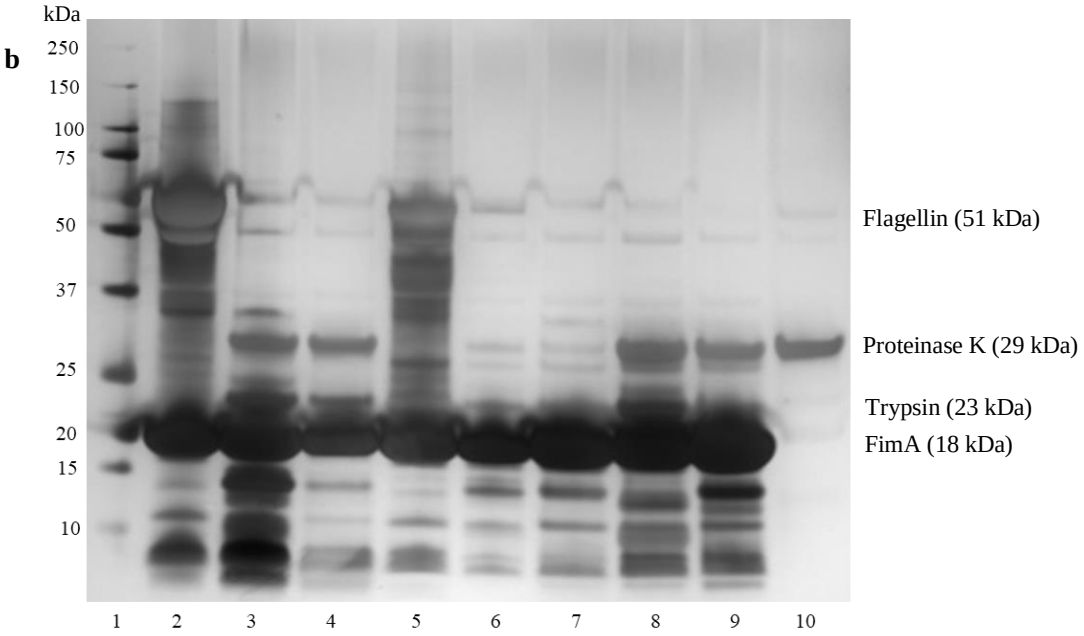


Figure 4.13 Proteinase K test on OMVs from *E. coli* WT with fimbriae locked on

The Proteinase K test was repeated on OMVs from *E. coli* with fimbriae locked on with variations in protease concentration, reaction temperature and method of OMV lysis (**a**). TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile (**b**).

All the methods trialled were unsuccessful at degrading FimA. Previously, the FimA monomer band at 18 kDa had been extracted and successfully verified by mass spectrometry. During the mass spectrometry procedure, the proteins were digested with trypsin and FimA had been successfully digested and identified. During the mass spectrometry procedure, the proteins were treated with 10 mM DTT in 50 mM ammonium bicarbonate and 55 mM iodoacetamide in ammonium bicarbonate. DTT disrupts the structure of proteins by reducing the disulphide bonds. Iodoacetamide then prevents reformation of disulphide bonds in proteins to keep them denatured. DTT and iodoacetamide were added to the OMV samples to denature the FimA monomer and allow Proteinase K access to the cleavage sites (Figure 4.14 and Appendix C.7)

a

Lane number	OMVs	10µg/mL Proteinase K	50mM NH_4HCO_3	10mM DTT	55mM iodoacetamide	HEPES buffer	HEPES/ 20mM $CaCl_2$ buffer	Reaction performed at 37°C	Reaction performed at 60°C
1									
2	✓		✓				✓	✓	
3	✓	✓	✓					✓	
4	✓	✓	✓	✓				✓	
5	✓	✓	✓		✓			✓	
6	✓	✓		✓	✓			✓	
7	✓	✓	✓	✓					✓
8	✓	✓	✓		✓				✓
9	✓	✓		✓	✓				✓
10		✓	✓			✓		✓	

b

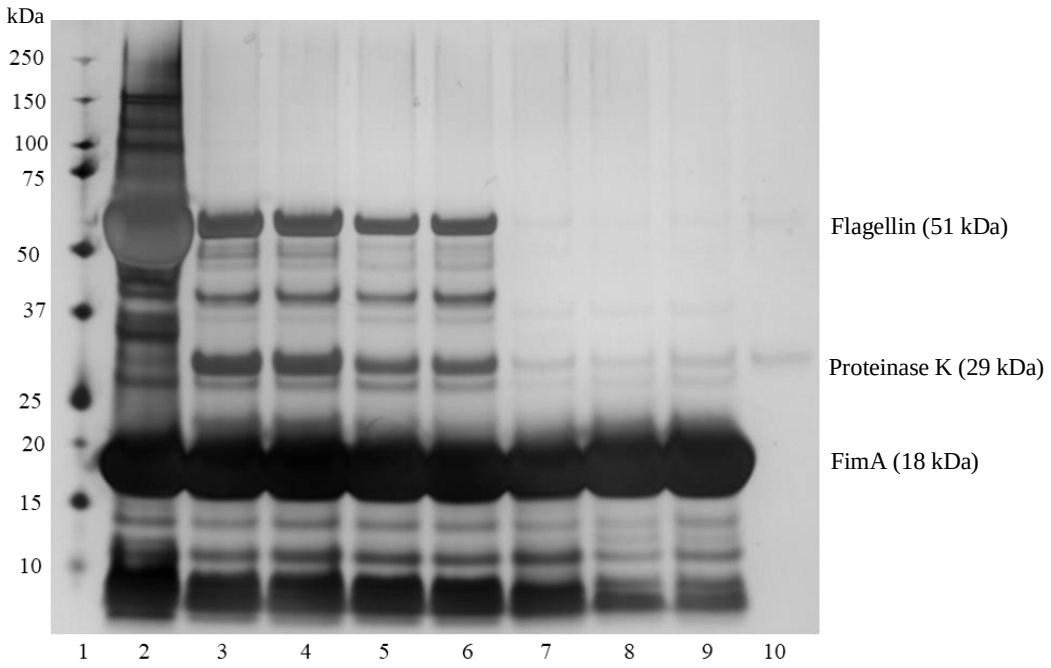


Figure 4.14 Proteinase K test on OMVs from *E. coli* WT with fimbriae locked on

The Proteinase K test was repeated on OMVs from *E. coli* with fimbriae locked on with the addition of DTT, iodoacetamide and ammonium bicarbonate alone (**a**). TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile (**b**).

Flagellin was degraded (at least partially) in all the conditions tested (Figure 4.14). However, the FimA monomer still remained uncleaved by Proteinase K or trypsin (see Appendix C.7). As Flagellin was very easily degraded and FimA monomer was not, the next hypothesis to test was whether FimA monomer was protected by lipids within the OMV in a different location to Flagellin. A lipid extraction was performed on the OMV samples to remove any lipid-protein interactions that could be protecting FimA. This 'lipid-free' OMV sample was then subject to digestion with Proteinase K and trypsin and was still unsuccessful (Figure 4.15).

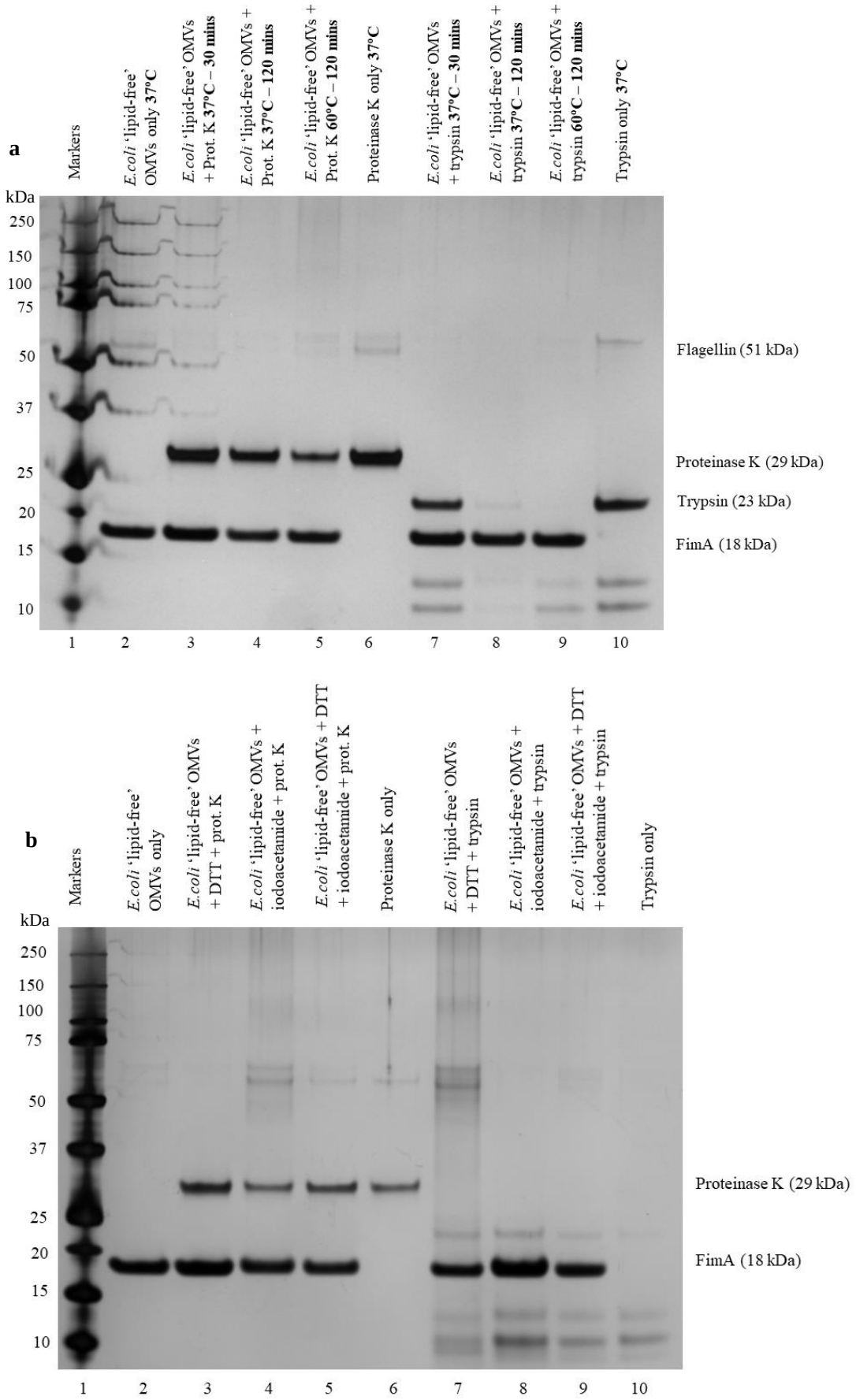


Figure 4.15 Proteinase K test on the proteins of OMVs from *E. coli* WT with fimbriae locked on (all lipids extracted)

The Proteinase K test was repeated on the lipid-free OMV sample from *E. coli* with fimbriae locked on using both Proteinase K (a) and Trypsin (**b**). TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile.

It was known that the FimA band (18 kDa) was cleaved successfully by trypsin during mass spectrometry when the band was extracted from the SDS-PAGE gels. In order to recreate these conditions, OMV samples from *E. coli* with fimbriae locked on were run on an SDS-PAGE gel in duplicate. One lane was silver stained to locate the FimA monomer protein and the next lane was unstained and had not been processed using the silver staining procedure (see Section 2.8.4 for more details). This was to see if it was part of the silver staining procedure that made FimA susceptible to protease degradation. The bands containing FimA were extracted and the FimA protein was purified from the SDS-PAGE gel. This purified FimA was then subject to digestion by Proteinase K and trypsin (see Figure 4.16).

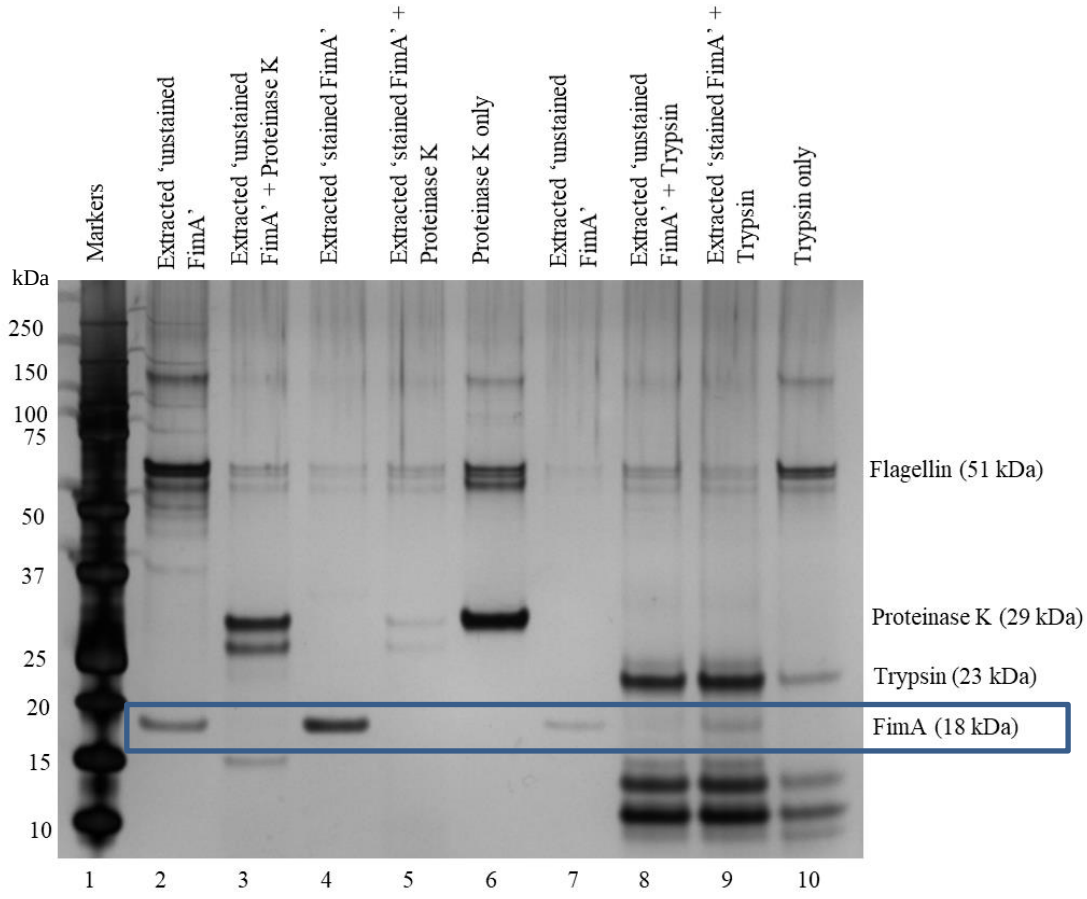


Figure 4.16 Proteinase K test on the proteins of OMVs from *E. coli* WT with fimbriae locked on (all lipids extracted)

The Proteinase K test was repeated on FimA protein that was isolated from the SDS-PAGE gel of OMV sample from *E. coli* with fimbriae locked on. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile.

The FimA monomer protein that had been isolated from the SDS-PAGE gel was now susceptible to digestion by Proteinase K and trypsin. The silver staining process had no effect on whether the protein was digested as the FimA was still degraded in Lanes 3 and 8. In the only cases where digestion of FimA had been successful, the OMV samples have been TCA precipitated first before Proteinase K/ trypsin digestion. The final method trialled was to compare digestion of FimA monomer by Proteinase K before and after TCA precipitation for the SDS-PAGE gel (see Figure 4.17).

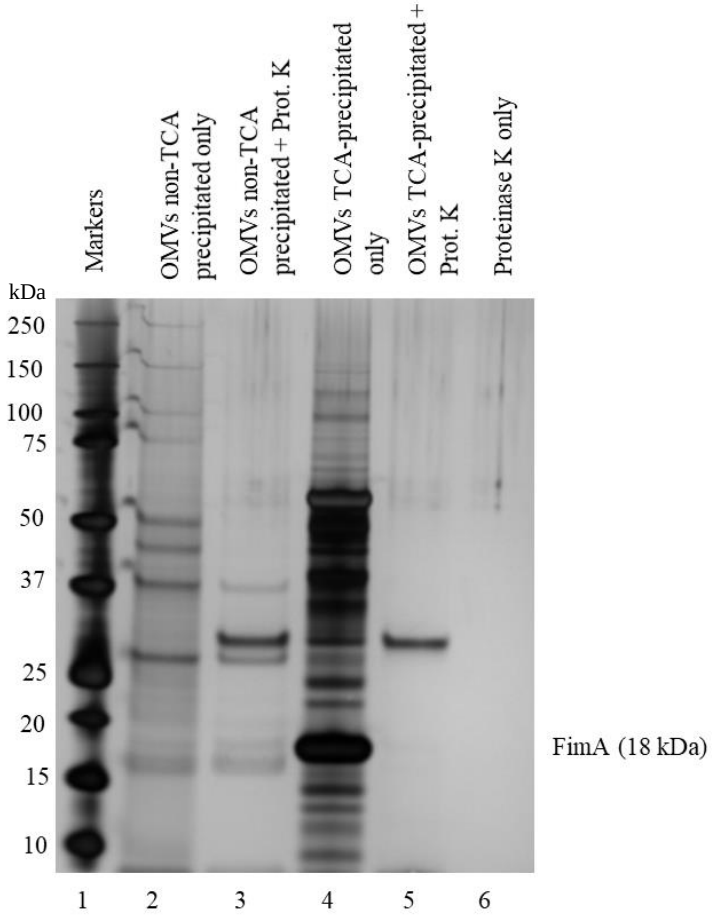


Figure 4.17 Proteinase K test on OMVs from *E. coli* WT with fimbriae locked on before and after TCA precipitation of the OMV proteins

The digestion of FimA was compared before and after TCA precipitation. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile.

It appeared that TCA precipitation of the FimA monomer made it susceptible to Proteinase K degradation. TCA is an acid that disrupts hydrogen bonding and causes the denaturation of proteins as they lose their secondary structure (Koonz *et al.* 2014). This appears to denature FimA in a way that allows Proteinase K access to the cleavage sites that were previously sequestered. Although the FimA monomer could now be digested, this method could not be incorporated into the Proteinase K test because the OMVs would no longer be intact. Evidence that FimA is present within the OMVs by the Proteinase K test was not possible so EM and Western blotting studies with an anti-FimA monomer antibody was favoured.

4.2.3. Is the packaging of FimA and Flagellin into OMVs mutually exclusive?

4.2.3.1 Comparison of OMVs from a variety fimbriae-associated protein knockouts and Flagellin

The aim was to see if deletion of a range of proteins involved in Type 1 fimbriae synthesis affects the presence of FimA and/or Flagellin in the OMVs produced. This was also to gain insight into which proteins play a role in targeting FimA and Flagellin to the OMVs. Figure 4.18 shows TEM images of the OMVs purified from each knockout strain compared to the parent strain of the Keio collection (BW25113). Each *E. coli* cell generally has flagella or fimbriae expressed, which can switch depending on which protein is knocked out. Flagella or fimbriae are co-purified with the OMVs and can also be seen in the EM images. A description of how to distinguish fimbriae or flagella on the TEM images can be found in Section 3.2.1.5.

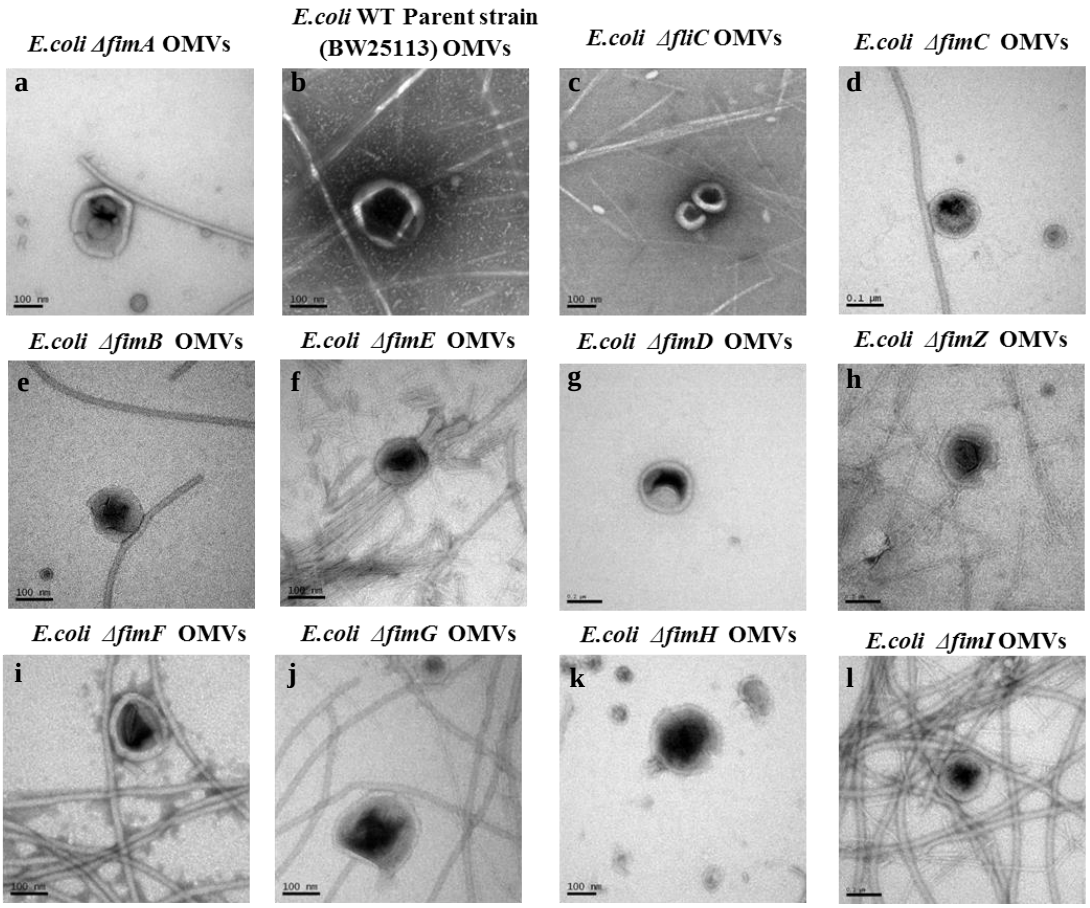


Figure 4.18 Comparison of OMVs from a variety of fimbriae-associated deletion mutants and Flagellin by TEM

TEM analysis of purified OMVs from twelve different *E. coli* strains: **a** *ΔfimA,* **b** Parental BW25113, **c** *ΔfliC,* **d** *ΔfimC,* **e** *ΔfimB,* **f** *ΔfimE,* **g** *ΔfimD,* **h** *ΔfimZ,* **i** *ΔfimF,* **j** *ΔfimG,* **k** *ΔfimH,* **l** *ΔfimI*

The OMV samples were then ran on an SDS-PAGE gel to compare the protein profiles produced after each knockout (Figure 4.19). Bands of interest were identified by mass spectrometry and are labelled below (see Appendix C.8). The whole *E. coli* cells were also run on an SDS-PAGE gel to compare the protein profile to the OMVs (see Appendix C.8). The whole cells have many proteins with no particular band dominating the protein profile. This is opposite to the protein profile of the OMVs which are dominated by bands at 18 kDa and/or 50-51 kDa.

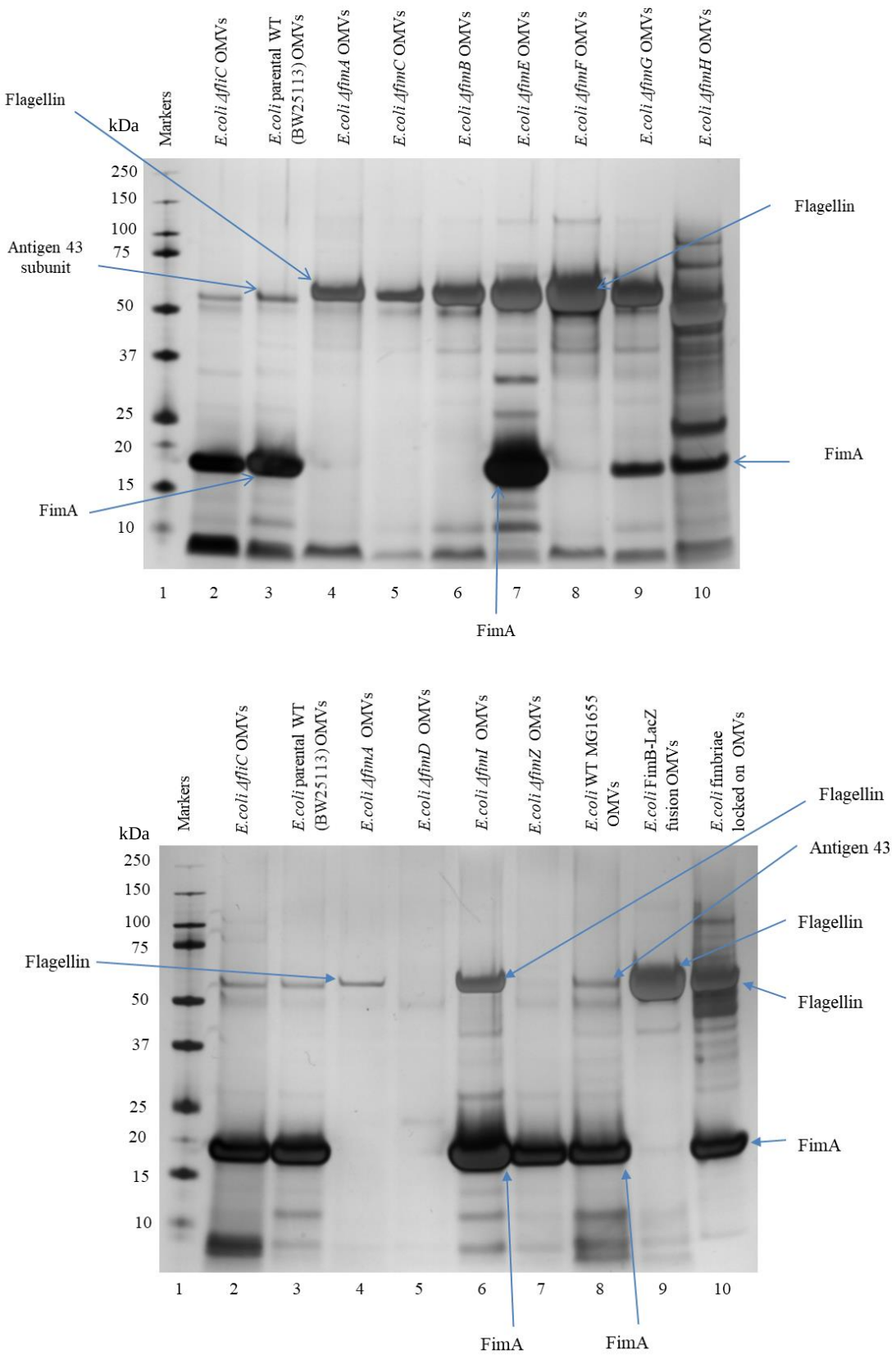


Figure 4.19 Comparison of OMV protein profile from a variety of fimbriae-associated deletion mutants and Flagellin by SDS-PAGE

OMVs were purified from twelve different *E. coli* strains from the Keio collection (CGSC) and three strains which had previously been studied (MG1655, FimB-LacZ fusion and fimbriae locked on strain). A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile. Bands of interest were identified by mass spectrometry and are labelled above.

The purified OMV samples from Figures 4.18 and 4.19 were used to produce Western blots, which were probed with the following antibodies: anti-Flagellin, anti-FimA monomer and anti-polymerised FimA (Figure 4.20). FimA monomer and Flagellin antibodies were used to test the hypothesis that FimA and Flagellin are packaged in a mutually exclusive way in *E. coli* K-12 OMVs.

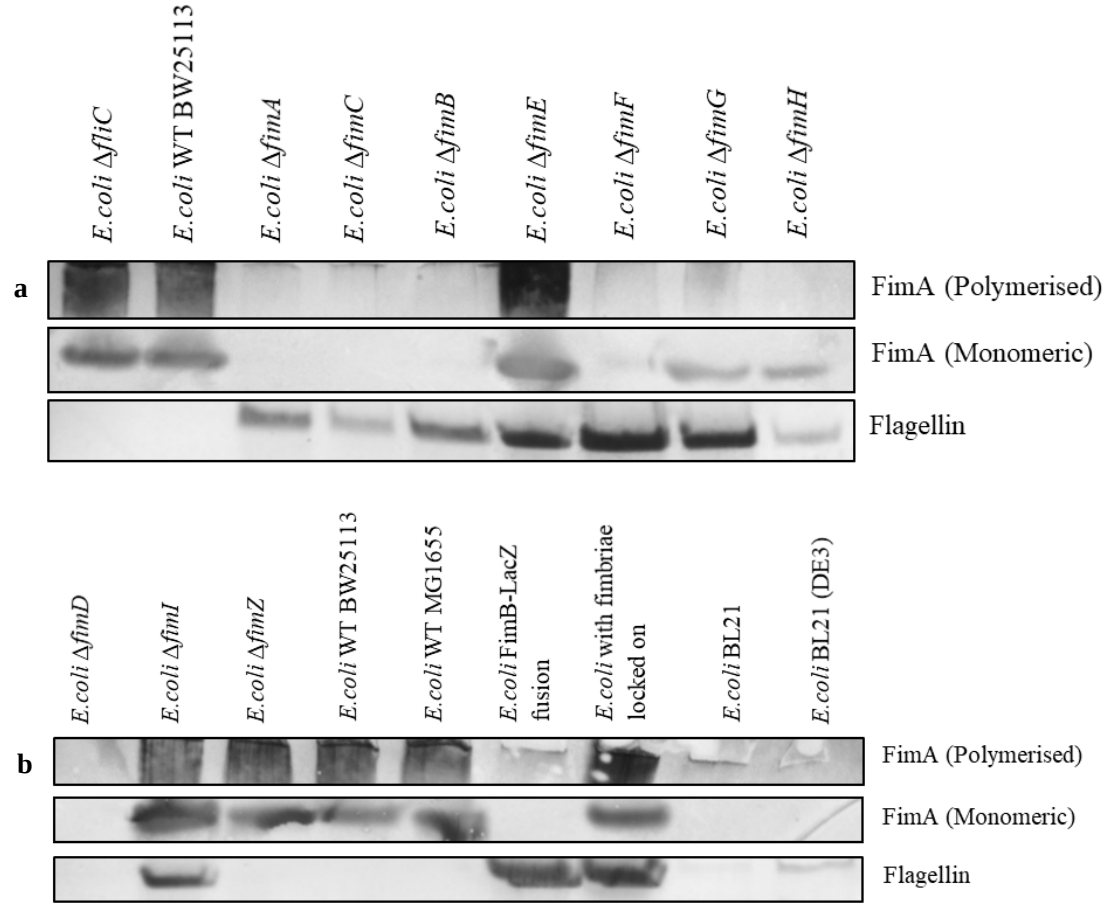


Figure 4.20 Comparison of OMV protein profile from a variety of fimbriae-associated deletion mutants and Flagellin by Western blotting

OMVs were purified from twelve different *E. coli* strains from the Keio collection (CGSC). A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. Purified OMV samples were probed using the following antibodies anti-FimA monomer, anti-FimA polymer and anti-Flagellin. For original Western blot images see Appendix C.9.

The Western blots showed clear enrichment of FimA and/or Flagellin in *E. coli* K-12 OMVs compared with all other proteins. The packaging of FimA and Flagellin also appears to be mutually exclusive unless there's a specific mutation to disrupt the regulation (summarised on Table 4.2). The hypotheses behind each result is discussed in Section 4.3.3.

Table 4.2 Mutual exclusivity of FimA and Flagellin packaging in OMVs from various *E. coli* strains. The introduction to the fimbriae-associated proteins can be found in Section 4.1.3 and the discussion of findings can be found in Section 4.3.3.

E.coli strain name	FimA monomer in OMVs?	Flagellin monomer in OMVs?	Is packaging mutually exclusive?
WT parental BW25113	Yes	No	Yes
ΔfliC	Yes	No	Yes
ΔfimA	No	Yes	Yes
ΔfimC	No	Yes	Yes
ΔfimB	No	Yes	Yes
ΔfimE	Yes	Yes	No
ΔfimF	No	Yes	Yes
ΔfimG	Yes	Yes	No
ΔfimH	Yes	Yes	No
ΔfimD	No	No	Neither present
ΔfimI	Yes	Yes	No
ΔfimZ	Yes	No	Yes
WT MG1655	Yes	No	Yes
FimB-LacZ fusion	No	Yes	Yes
Fimbriae locked on	Yes	Yes	No
BL21	No	No	Neither present
BL21 (DE3)	No	No	Neither present

4.2.3.2 Purification of OMVs from *E. coli* strains containing a range of knock outs of flagella-associated proteins

OMVs were purified from *E. coli* strains containing deletions of various genes associated with flagella biosynthesis. Figure 4.21 shows TEM images of the OMVs purified from each knockout strain compared to the parent strain of the Keio collection (BW25113). Each *E. coli* cell generally has flagella or fimbriae expressed and these are co-purified with the OMVs and can also be seen in the EM images.

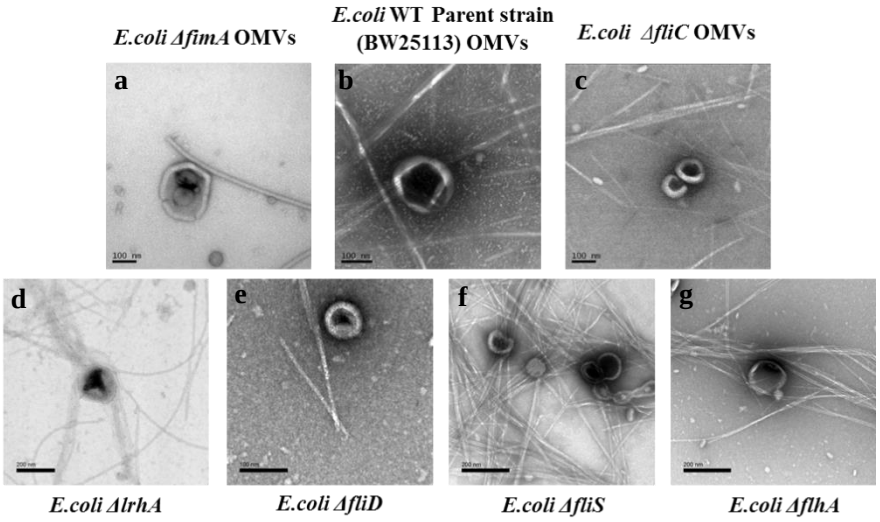


Figure 4.21 Comparison of OMVs from a variety of flagella-associated deletion mutants by TEM TEM analysis of purified OMVs from four extra *E. coli* strains: **a** *ΔfimA*, **b** Parental BW25113, **c** *ΔfliC*, **d** *ΔlrhA*, **e** *ΔfliD*, **f** *ΔfliS*, **g** *ΔflhA*

The OMV samples were then ran on an SDS-PAGE gel to compare the protein profiles produced after each knockout (Figure 4.22**a**). Bands of interest were identified by mass spectrometry and are labelled below. Deletion of *lrhA* appeared to cause dysregulation of the packaging of FimA and Flagellin in OMVs as both were found together (Lane 5). These samples were then used to produce Western blots which were probed with the following antibodies: anti-Flagellin, anti-FimA monomer and anti-polymerised FimA (Figure 4.22**b**).

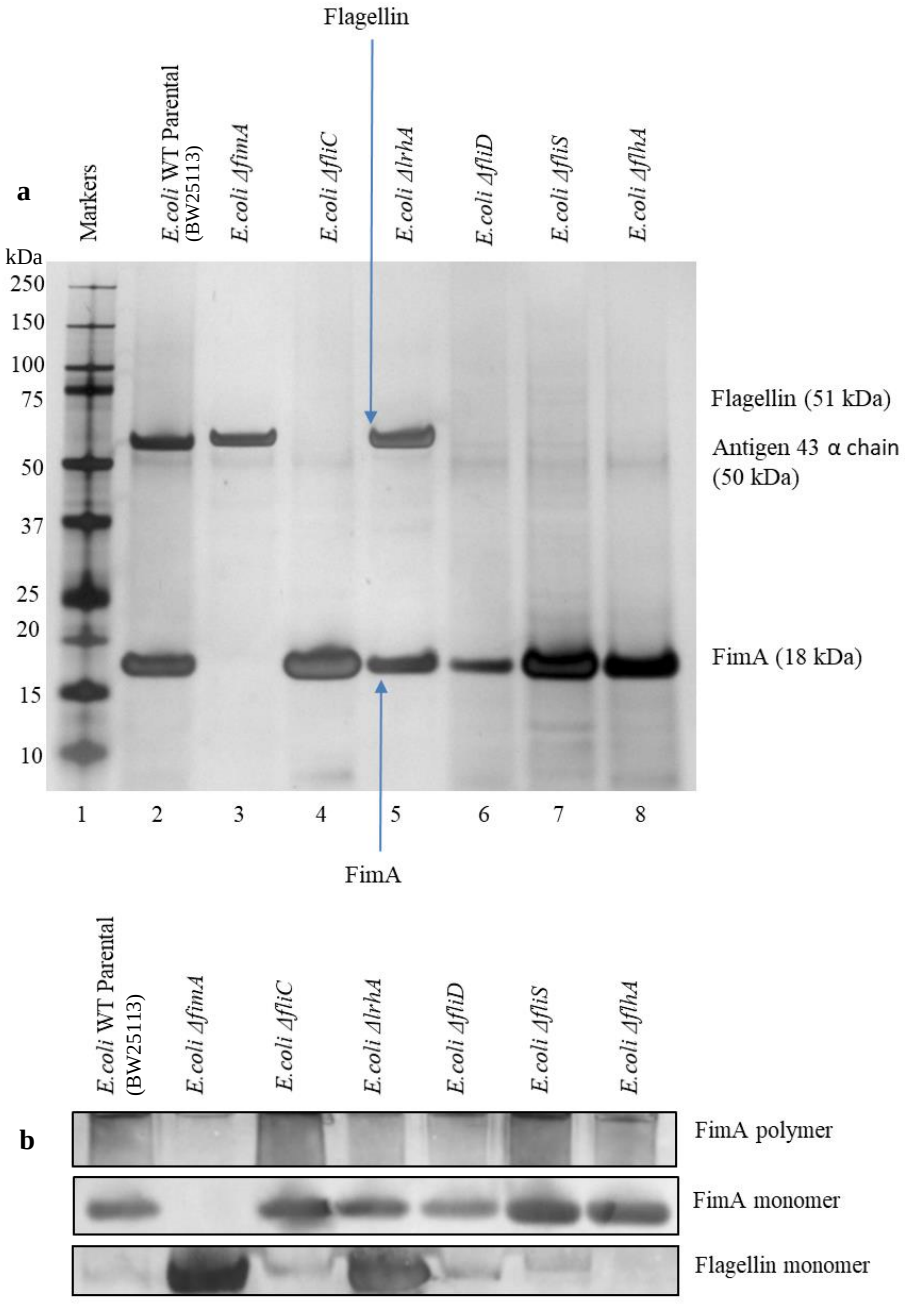


Figure 4.22 Comparison of OMV protein profile from a variety of flagella-associated deletion mutants by SDS-PAGE and Western blotting

OMVs were purified from four additional *E. coli* strains from the Keio collection (CGSC). A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile. Bands of interest were identified by mass spectrometry and are labelled above (**a**). Purified OMV samples were used to perform Western blots which were probed using the following antibodies anti-FimA monomer, anti-FimA polymer and anti-Flagellin (**b**). Original Western blot images and mass spectrometry details are listed in Appendix C.10.

The packaging of FimA and Flagellin also appeared to be mutually exclusive unless there's a specific mutation to disrupt the regulation (summarised in Table 4.3). The introduction to the flagella-associated proteins can be found in Section 4.1.3.

Table 4.3 Mutual exclusivity of FimA and Flagellin packaging in OMVs from various *E. coli* strains

***E.coli* strain name**	**FimA monomer in OMVs?**	**Flagellin monomer in OMVs?**	**Is packaging mutually exclusive?**
WT parental BW25113	Yes	No	Yes
ΔfliC	Yes	No	Yes
ΔfimA	No	Yes	Yes
ΔlrhA	Yes	Yes	No
ΔfliD	Yes	No	Yes
fliS	Yes	No	Yes
flhA	Yes	No	Yes

The reasons for the mutual exclusivity are discussed further in Section 4.3.3.

4.2.4. Which conditions lead to packaging of FimA and Flagellin into *E. coli* K-12 OMVs?

4.2.4.1 Packaging of FimA monomer into OMVs under different conditions

OMVs were purified from *E. coli* WT parental strain (BW25113) at three different temperatures: 18ºC, 25ºC and 37ºC to see if temperature affects the packaging of FimA monomer into OMVs. OMVs were also purified from three different points on the growth curve at 37ºC: early log phase, mid log phase and stationary phase (indicated on Figure 4.23**a**). This was to determine at which stage of bacterial growth FimA is packaged. Firstly, growth curves were produced by growing the *E. coli* parental strain (BW25113) at 25ºC and 37ºC using a microplate reader (Figure 4.23**a**).

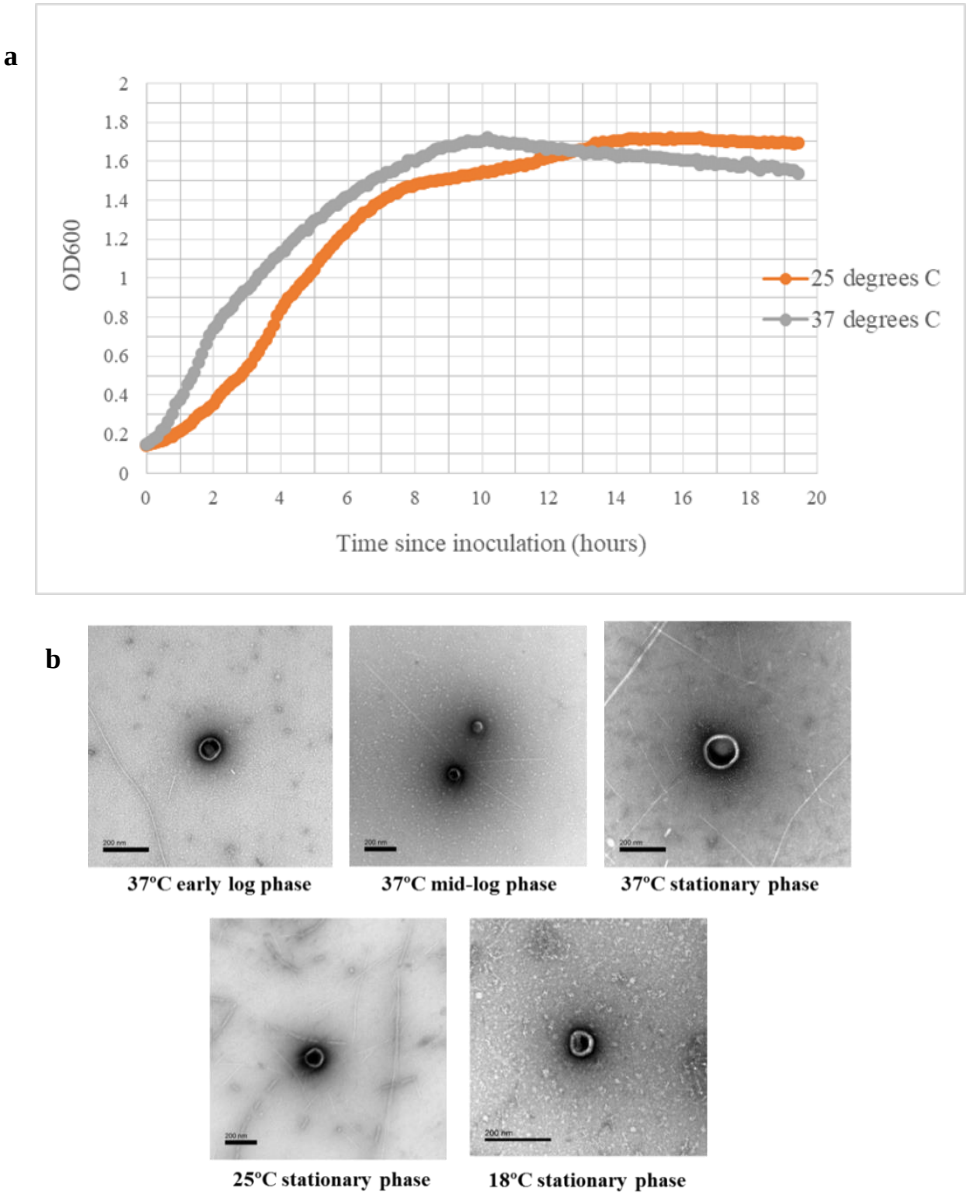


Figure 4.23 Growth of *E. coli* parental (BW25113) strain at 25ºC and 37ºC

Growth curves were produced for *E. coli* parental (BW25113) strain at 25ºC and 37ºC, 180 RPM, overnight. The strain was diluted to OD600 0.1 using LB before addition of 500 µL to each well of the 48 well sterile plates. OD600 readings were taken every 8 mins and the growth curve graphs were generated on Microsoft Excel using the raw data (**a**). The growth curve of each strain was done in triplicate and the average was taken. See Appendix C.11 for graphs with error bars of 1 standard deviation. The arrows on the growth curves indicate the time point that the OMVs were harvested at the following stages: early-log, mid-log and stationary phase. The purified OMVs were visualised by TEM (**b**).

The protein profile of the OMVs was visualised using SDS-PAGE and silver staining (Figure 4.24**a**). The same samples were used for a Western blot, probing with anti-FimA monomer antibody and FimA polymer (Figure 4.24**b**).

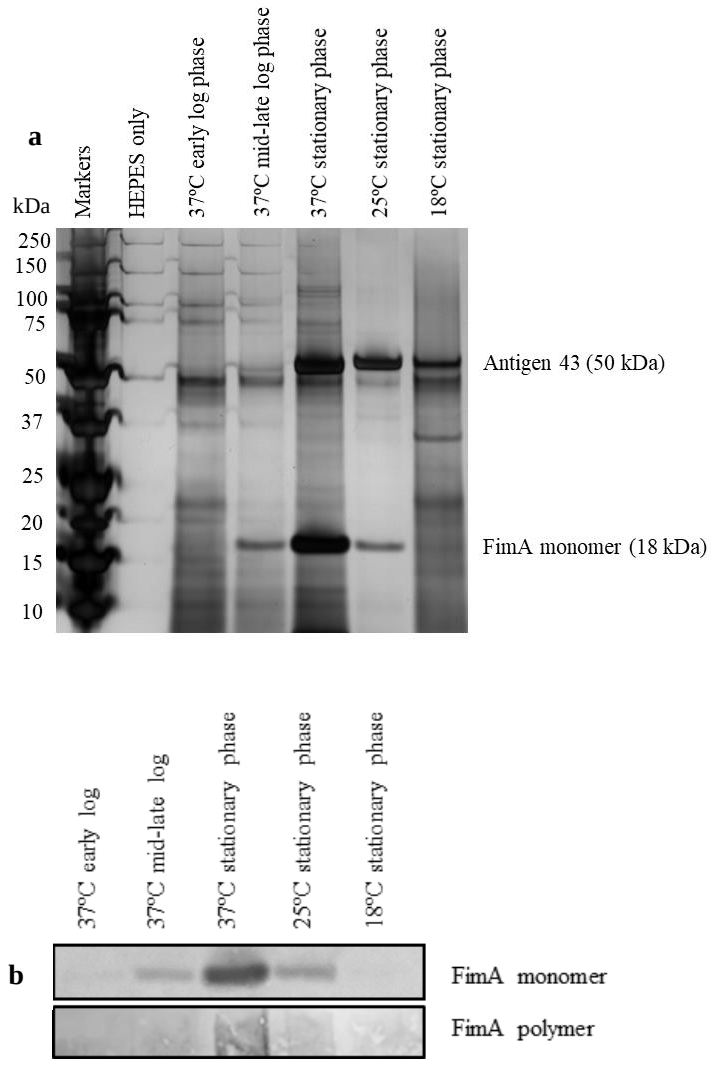


Figure 4.24 What conditions lead to the packaging of FimA into *E. coli* WT BW25113 OMVs?

OMVs were purified from *E. coli* WT BW25113 at three different stages of growth and at 3 different temperatures. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. One SDS-PAGE gel was silver stained to visualise the OMV protein profile (**a**) the others were transferred to a PVDF membrane for a Western blot probing with the anti-FimA monomer and anti-FimA polymer antibodies (**b**). Original Western blot images can be found in Appendix C.12.

Figure 4.24**b** indicates that FimA is packaged into OMVs in mid-late log stage and stationary phase but not early log phase. Also, the Western blot shows that the more fimbriae there are (represented by polymerised FimA), the more FimA is packaged into the OMVs. Lastly, FimA is packaged into OMVs at 37°C and 25°C but not 18°C so is affected by changes in temperature.

4.2.5. FimA and Flagellin are reciprocally regulated in some OMVs from clinical isolates

OMVs were purified from 6 clinical isolates which were either isolated from the urine of patients with UTI infections or the blood from patients with bacteraemia caused by *E. coli*. See Appendix C.13 for the antibiotic resistance information of each clinical isolate strain. The purified OMVs were visualised by TEM and compared to OMVs from the *E. coli* WT Parental BW25113 strain, *ΔfimA* and *ΔfliC* (Figure 4.25).

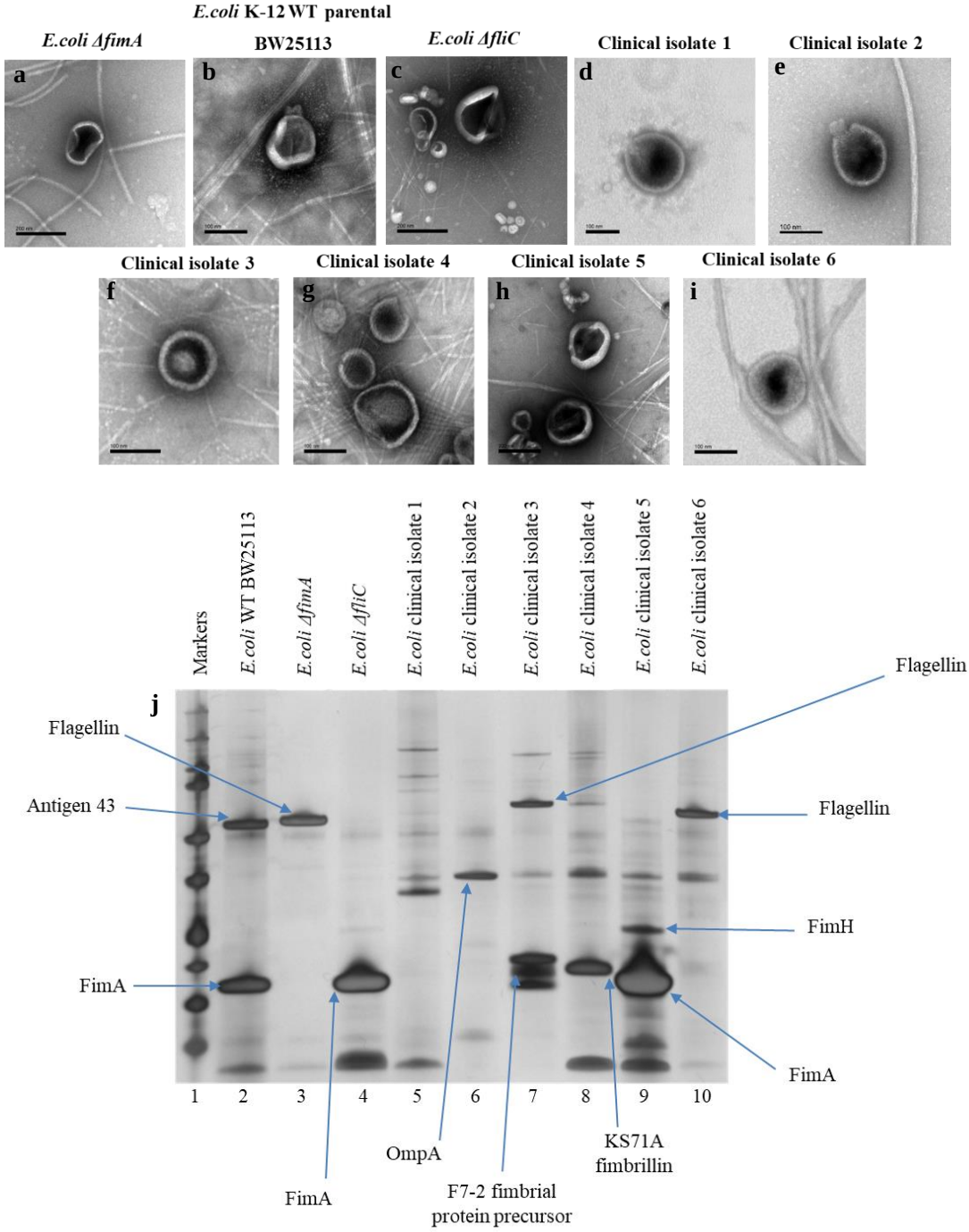


Figure 4.25 TEM images and protein profiles of OMVs purified from six *E. coli* clinical isolates, BW25113, *ΔfimA* and *ΔfliC* strains

TEM analysis of purified OMVs from the following *E. coli* strains: *ΔfimA* (**a**), Parental BW25113 (**b**), *ΔfliC* (**c**), Clinical isolate 1 (**d**), Clinical isolate 2 (**e**), Clinical isolate 3 (**f**), Clinical isolate 4 (**g**), Clinical isolate 5 (**h**), Clinical isolate 6 (**i**). A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the OMV protein profile. Bands of interest were identified by mass spectrometry and are labelled above (**j**). Full mass spectrometry results can be found in Appendix C.14.

A Western blot was performed using the samples from Figure 4.25 to detect FimA monomer, FimA polymer and Flagellin in the OMV samples (Figure 4.26). For a direct comparison of the SDS-PAGE gel and Western blot see Appendix C.15**d**.

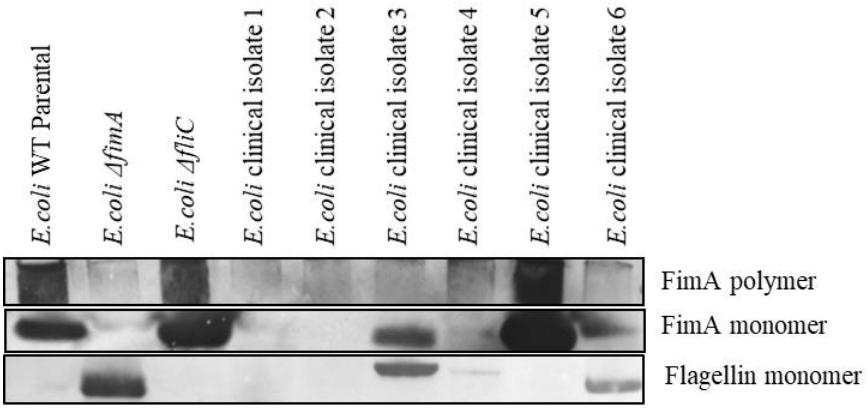


Figure 4.26 Comparison of FimA/Flagellin packaging in OMVs purified from six *E. coli* clinical isolates, BW25113, *ΔfimA* and *ΔfliC* strains

A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. Purified OMV samples were probed using the following antibodies anti-FimA monomer, anti-FimA polymer and anti-Flagellin. For original Western blot images, see Appendix C.15.

Two proteins related to fimbriae were detected in OMVs from Clinical isolates 3 and 4. A BLAST protein alignment was performed to see if these proteins were similar to FimA and could have been mis-identified. Clinical isolate 3 OMVs contain F7-2 fimbrial protein. However, this only has 34% amino acid identity to FimA. Similarly, Clinical isolate 4 OMVs were found to contain KS71A fimbrillin which has 33% amino acid similarity with FimA (see Appendix C.16 for BLAST protein alignments). Although both are fimbriae-associated proteins, it appears that they were not a mis-identified FimA monomer. Lastly, the Flagellin band in the OMVs of Clinical isolate 3 is higher than that of Clinical isolate 6 and *ΔfimA*. However, it should be noted that Clinical isolate 3 is a UPEC strain where Flagellin has a molecular weight of 61 kDa rather than 51 kDa (see Appendix C.17 for further discussion). The mutually exclusive packaging of FimA and Flagellin is summarised in Table 4.4. Clinical isolates 1, 2 and 4 have neither FimA or Flagellin present in the OMVs. Clinical isolate 3 has both FimA and Flagellin present and Clinical isolates 5 and 6 have FimA and Flagellin monomers packaged in a mutually exclusive way.

Table 4.4 Mutual exclusivity of FimA and Flagellin packaging in OMVs from various *E. coli* clinical isolate strains

Clinical isolate number	Strain name	Number of antibiotics strain is known to be resistant to	FimA monomer in OMVs?	Is Flagellin monomer in OMVs?	Is packaging mutually exclusive?
1	MS207	0	No	No	Neither present
2	MS10	4	No	No	Neither present
3	MS1	0	Yes	Yes	No
4	MS343	0	No	No	Neither present
5	MS190	5	Yes	No	Yes
6	MS234	3	No	Yes	Yes

Table 4.5 summarises the mutual exclusive packaging of FimA and Flagellin monomer in *E. coli* OMVs from all strains of interest. The results of this Table are discussed in Section 4.3.3.

Table 4.5 Final summary tables to compare mutual exclusivity of FimA and Flagellin packaging in OMVs from various *E. coli* strains (K-12 and B strains). Table **a** is a summary of the findings from all strains tested. Table **b** summarises the percentage of *E. coli* strains that give rise to OMVs with FimA and Flagellin packaged in a mutually exclusive way. Table gives **c** the percentage of *E. coli* strains that give rise to OMVs with FimA and Flagellin packaged in a mutually exclusive way **excluding** from the data set any strains do not contain FimA or Flagellin in their OMVs.

a

***E.coli* strain name**	**FimA monomer in OMVs?**	**Flagellin monomer in OMVs?**	**Is packaging mutually exclusive?**
WT parental BW25113	Yes	No	Yes
ΔfliC	Yes	No	Yes
ΔfimA	No	Yes	Yes
ΔfimC	No	Yes	Yes
ΔfimB	No	Yes	Yes
ΔfimE	Yes	Yes	No
ΔfimF	No	Yes	Yes
ΔfimG	Yes	Yes	No
ΔfimH	Yes	Yes	No
ΔfimD	No	No	Neither present
ΔfimI	Yes	Yes	No
ΔfimZ	Yes	No	Yes
WT MG1655	Yes	No	Yes
FimB-LacZ fusion	No	Yes	Yes
Fimbriae locked on	Yes	Yes	No
BL21	No	No	Neither present
BL21 (DE3)	No	No	Neither present
ΔlrhA	Yes	Yes	No
ΔfliD	Yes	No	Yes
fliS	Yes	No	Yes
flhA	Yes	No	Yes
Clinical isolate 1	No	No	Neither present
Clinical isolate 2	No	No	Neither present
Clinical isolate 3	Yes	Yes	No
Clinical isolate 4	No	No	Neither present
Clinical isolate 5	Yes	No	Yes
Clinical isolate 6	No	Yes	Yes

b

Criteria	**Number of strains that meet the criteria (27 strains studied)**	**% of total strain number studied that meet the criteria**
OMVs contain **neither** FimA or Flagellin	6	22
OMVs have **mutually exclusive** packaging of FimA and Flagellin monomer	14	52
OMVs have both FimA and Flagellin **packaged together**	7	26

c

Criteria	Number of strains that meet the criteria (/21 strains)	% of total strain number studied that meet the criteria
OMVs have **mutually exclusive** packaging of FimA and Flagellin monomer	14	67
OMVs have both FimA and Flagellin **packaged together**	7	33

4.2.6. Are Type 1 fimbriae and/or flagella co-purified with the *E. coli* OMVs?

It could be argued that the SDS-PAGE gels and Western blots are detecting FimA monomer and Flagellin monomer from co-purified fimbriae and flagella respectively and not the OMVs. For example, it could be argued that during the SDS-PAGE process, co-purified fimbriae and/or flagella may depolymerise to give rise to the FimA and Flagellin monomers. However, this section will outline the evidence that suggests that this is not true.

The following questions were addressed:

1. Is FimA monomer packaged into OMVs independently of fimbriae production? Similarly, is Flagellin monomer packaged into OMVs independently of flagella production?
2. Is the packaging of FimA and Flagellin into OMVs directly linked to fimbriae and flagella expression on the parent cells?

4.2.6.1 Identification of fimbriae and/or flagella co-purified with the OMVs

Using TEM to identify fimbriae and flagella

All the strains in Tables 4.5**a-c** were analysed to determine if fimbriae and/or flagella were co-purified with the OMVs from each strain. TEM images alone were used to detect flagella in OMV samples as they are very distinctive (discussed in Section 3.2.1.5). Figure 4.27 shows TEM images of OMVs known to be co-purified with either flagella or fimbriae. This can be used as a reference point for later decisions on how to differentiate between the two by eye.

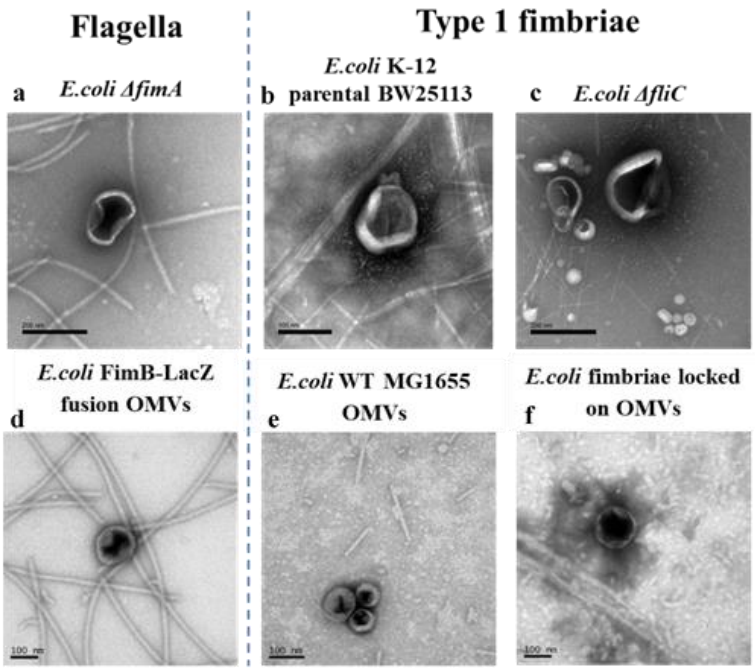


Figure 4.27 TEM images of OMVs purified from six *E. coli* strains to compare co-purification of flagella and fimbriae

TEM analysis of purified OMVs from *E. coli* strains: **a** *ΔfimA*, **b** Parental BW25113, **c** *ΔfliC*, **d** FimB-LacZ fusion, **e** MG1655 and **f** fimbriae production locked on strain.

Figure 4.27 shows that flagella are thicker and longer than fimbriae and are also curvy/wavy. Type 1 fimbriae were found to be short, thin and straight appendages compared with the flagella. This agrees with the previous findings in Section 3.2.1.5.

Western blotting with anti-polymerised FimA to detect fimbriae

Additional methods were used to confirm the presence of fimbriae along with EM images. As previously described, FimA monomers polymerise to form the main structural subunit of fimbriae. These polymers are so stable that they do not depolymerise during SDS-PAGE and are therefore too large to migrate through the gel (see Appendix C.18 for further details on the stability of fimbriae). For this reason, polymerised FimA was detected in the wells of Western blots when probing with the anti-FimA polymer antibody. As polymerised FimA is the main structural subunit of fimbriae, detection of polymerised FimA can be used to indicate the presence of fimbriae. OMVs purified from *ΔfimA* were always run as a negative control and *ΔfliC* OMVs were always used as a positive control (Figure 4.28).

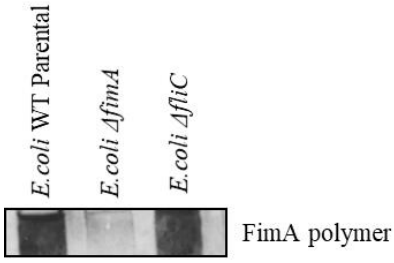


Figure 4.28 Polymerised FimA positive and negative Western blot controls

A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. Purified OMV samples were probed using anti-FimA polymer antibody. Image was cropped from Figure 4.27 to show positive and negative controls for this antibody.

Fimbriae agglutination test with *E. coli* cells to detect fimbriae

In order to determine whether *E. coli* cells (from which the OMVs are purified from) express functional and intact fimbriae, a simple yeast agglutination test can be performed. The *E. coli* cells of interest were mixed 1:1 with *Saccharomyces cerevisiae* cells on a glass microscope slide. If fimbriae are present, FimH (the adhesin on the tip of Type 1 fimbriae) adheres to the *S. cerevisiae* cells. This causes the agglutination of the yeast cells which can be visualised by light microscopy Figure 4.29 **a** and **b**.

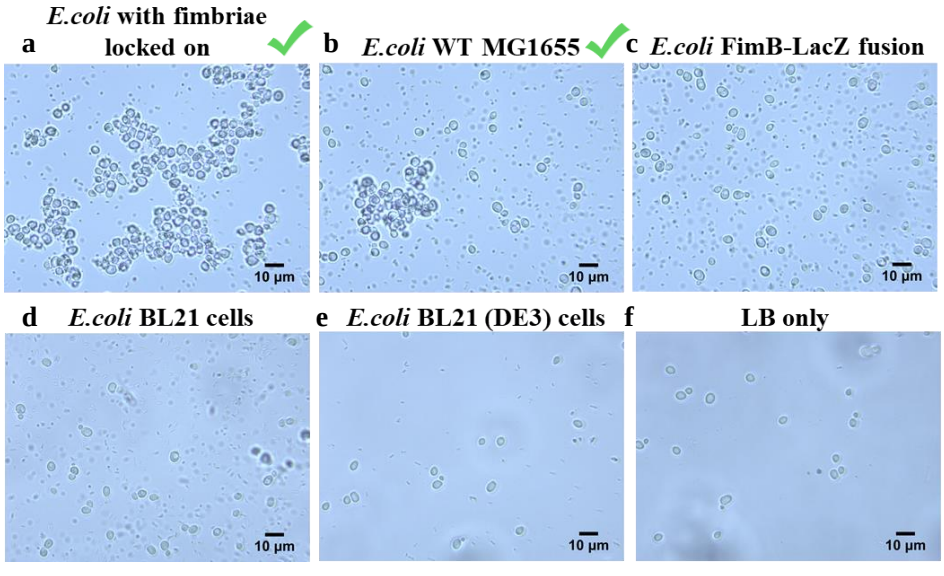


Figure 4.29 Yeast agglutination test to detect fimbriae expression on *E. coli* cells

Saccharomyces cerevisiae were mixed 1:1 with the following *E. coli* strains: fimbriae locked on (**a**), MG1655 (**b**), FimB-LacZ fusion (**c**), BL21 (**d**), BL21 (DE3) (**e**), LB only (**f**). Yeast cell agglutination was visualised using light microscopy at 400x magnification. Green ticks indicate yeast cell agglutination and therefore fimbriae.

For yeast agglutination test of all other strains, see Appendix C.19. All the evidence for whether *E. coli* strains are producing fimbriae and/or flagella are summarised in Table 4.6.

Table 4.6 Summary table to confirm which strains produce fimbriae and/or flagella using all available strain evidence. Purple indicates which strains express fimbriae only, yellow indicates which strains produce flagella only and orange represents strains that produce both fimbriae and flagella together.

***E.coli* strain name**	**Evidence for T1F expression in strain**			**Evidence for flagella expression in strain: EM images**	**Final conclusion for whether T1F and/or flagella are expressed**
	EM images	**Anti-polymerised FimA antibody (Western blot)**	**Yeast agglutination test**		
WT parental BW25113	Yes	Yes	Yes	No	Fimbriae only
ΔfliC	Yes	Yes	Yes	No	Fimbriae only
ΔfimA	No	No	No	Yes	Flagella only
ΔfimC	No	No	No	Yes	Flagella only
ΔfimB	No	No	No	Yes	Flagella only
ΔfimE	Yes	Yes	Yes	No	Fimbriae only
ΔfimF	No	No	No	Yes	Flagella only
ΔfimG	No	No	No	Yes	Flagella only
ΔfimH	No	No	No	Yes	Flagella only
ΔfimD	No	No	No	No	Neither
ΔfimI	Yes	Yes	Yes	Yes	Both
ΔfimZ	Yes	Yes	No	No	Fimbriae only
WT MG1655	Yes	Yes	Yes	No	Fimbriae only
FimB-LacZ fusion	No	No	No	Yes	Flagella only
Fimbriae locked on	Yes	Yes	Yes	No	Fimbriae only
BL21	No	No	No	No	Neither
BL21 (DE3)	No	No	No	No	Neither
ΔlrhA	No	Yes	Yes	Yes	Both
ΔfliD	Yes	Yes	Yes	No	Fimbriae only
ΔfliS	Yes	Yes	Yes	No	Fimbriae only
ΔflhA	Yes	Yes	Yes	No	Fimbriae only
Clinical isolate 1	No	Unclear	No	No	Neither
Clinical isolate 2	No	Unclear	No	Yes	Flagella only
Clinical isolate 3	Yes	Unclear	Yes	No	Fimbriae only
Clinical isolate 4	Yes	Unclear	No	No	Fimbriae only
Clinical isolate 5	Yes	Yes	Yes	No	Fimbriae only
Clinical isolate 6	No	Unclear	No	Yes	Flagella only

In order to address the two questions outlined at the start of Section 4.26, the packaging of FimA and Flagellin into OMVs should be compared to whether the parent cell produces fimbriae and/or flagella. Table 4.7 addresses the questions: if fimbriae is produced in the parent cell, is FimA monomer always packaged into the OMVs? Similarly, if flagella are produced in the parent cell, is Flagellin monomer always packaged into OMVs?

4.2.6.2 Is the packaging of FimA/Flagellin monomers into OMVs independent of the production of fimbriae and/or flagella on the parent *E. coli* strain?

Table 4.7 Summary table to compare the packaging of FimA and Flagellin monomers into OMVs with the production of fimbriae and/or flagella in the parental strain. The criteria for a 'match' is explained in Table **a** and the results are found in **b**. Type 1 fimbriae is abbreviated to 'T1F'.

a

FimA/Flagellin monomer present in OMVs	T1F/Flagella present on cell
FimA monomer only	T1F expression only
Flagellin monomer only	Flagella expression only
FimA **and** Flagellin monomer	T1F **and** flagella expression
No FimA or Flagellin monomer	**No** T1F or flagella expression

b

E.coli strain name	FimA monomer in OMVs?	Flagellin monomer in OMVs?	Fimbriae or Flagella co-purified with the OMVs	Is it a match?
WT parental BW25113	Yes	No	Fimbriae only	Yes
ΔfliC	Yes	No	Fimbriae only	Yes
ΔfimA	No	Yes	Flagella only	Yes
ΔfimC	No	Yes	Flagella only	Yes
ΔfimB	No	Yes	Flagella only	Yes
ΔfimE	Yes	Yes	Fimbriae only	No
ΔfimF	No	Yes	Flagella only	Yes
ΔfimG	Yes	Yes	Flagella only	No
ΔfimH	Yes	Yes	Flagella only	No
ΔfimD	No	No	Neither	Yes
ΔfimI	Yes	Yes	Both	Yes
ΔfimZ	Yes	No	Fimbriae only	Yes
WT MG1655	Yes	No	Fimbriae only	Yes
FimB-LacZ fusion	No	Yes	Flagella only	Yes
Fimbriae locked on	Yes	Yes	Fimbriae only	No
BL21	No	No	Neither	Yes
BL21 (DE3)	No	No	Neither	Yes
ΔlrhA	Yes	Yes	Both	Yes
ΔfliD	Yes	No	Fimbriae only	Yes
ΔfliS	Yes	No	Fimbriae only	Yes
ΔflhA	Yes	No	Fimbriae only	Yes
Clinical isolate 1	No	No	Neither	Yes
Clinical isolate 2	No	No	Flagella only	No
Clinical isolate 3	Yes	Yes	Fimbriae only	No
Clinical isolate 4	No	No	Fimbriae only	No
Clinical isolate 5	Yes	No	Fimbriae only	Yes
Clinical isolate 6	No	Yes	Flagella only	Yes

There are 7 instances out of 27 strains in total (26%) where packaging of FimA and/or Flagellin monomers into OMVs is independent of whether fimbriae or flagella are expressed on the parent cell. These examples will be discussed further in Section 4.3.3.

4.2.7. Can FimA be used to target proteins and other molecules for delivery within OMVs?

A construct of *E. coli* MG1655 strain with a FimA-GFP fusion protein was available to use from Professor Sander Tans from AMOLF in the Netherlands (Adiciptaningrum *et al.* 2009). In this strain, GFP was inserted within the chromosomal *fimA* DNA sequence, which is the first gene of the *fim* operon (Adiciptaningrum *et al.* 2009). This results in the production of GFP-FimA protein fusion instead of FimA. See Appendix C.20 for full details about the FimA-GFP fusion strain.

4.2.7.1 Visualisation of purified OMVs from the *E. coli* FimA-GFP fusion strain using TEM

The OMVs purified from the MG1655 FimA-GFP fusion strain and the WT MG1655 strain were visualised by TEM (Figure 4.30). OMVs isolated from *E. coli* WT MG1655 are co-purified with fimbriae which the parent cells express. Interestingly, the FimA-GFP fusion strain produces flagella instead of fimbriae.

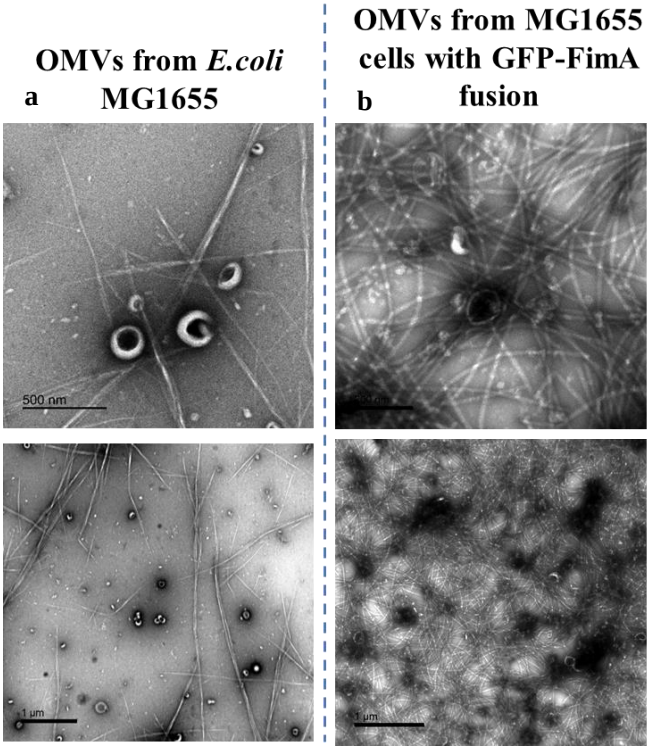


Figure 4.30 TEM images of OMVs purified from *E. coli* MG1655 strain and FimA-GFP fusion strain

TEM images of purified OMVs from *E. coli* strains: **a** MG1655 **b** MG1655 GFP-FimA fusion strain

Immunogold labelling of resin-embedded OMVs

Immunogold labelling showed co-localisation of both FimA and GFP in the OMVs, which indicates that the GFP-FimA fusion protein was successfully delivered. Flagellin also appeared to be detected within the OMVs too (Figure 4.31). Photos **g** and **h** show how the immunogold labels are localised to the sites containing the embedded OMVs and are not spread evenly over the grid. Additional TEM photos can be found in Appendix C.21.

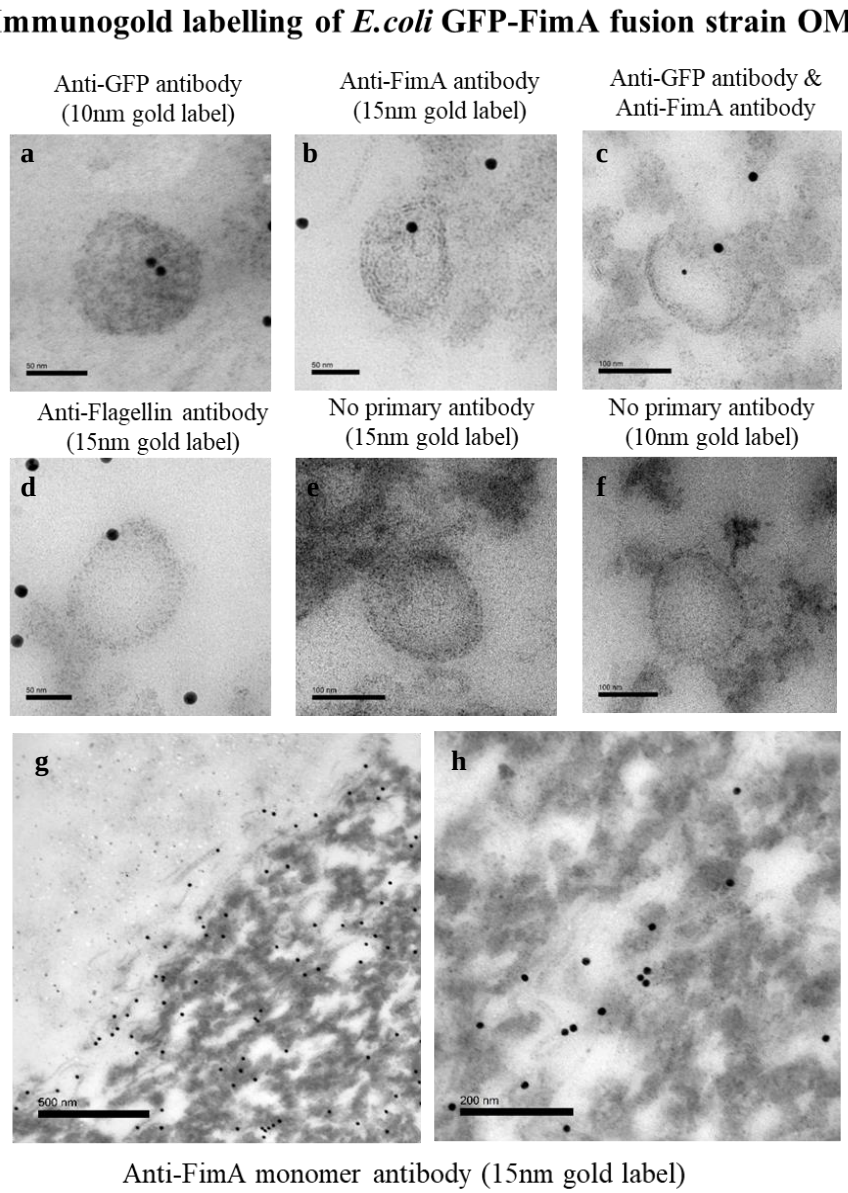


Figure 4.31 Immunogold labelling of embedded *E. coli* FimA-GFP strain OMVs

TEM analysis of thin-sectioned OMVs embedded in resin. The sections were immunogold labelled and probed with: anti-GFP antibody (**a**) anti-FimA monomer antibody (**b, g and h**), anti-FimA monomer and anti-GFP antibodies mixed (**c**), anti-Flagellin antibody (**d**). As a negative control, the embedded OMVs were incubated in TBST only (no primary antibody). The samples were then incubated with the following secondary antibodies: 15 nm gold label (**e**) or 10 nm gold label (**f**)

The *E. coli* FimA-GFP fusion strain cells were embedded in resin and the sections were immunogold labelled to show the location of the FimA-GFP protein and Flagellin (Figure 4.32).

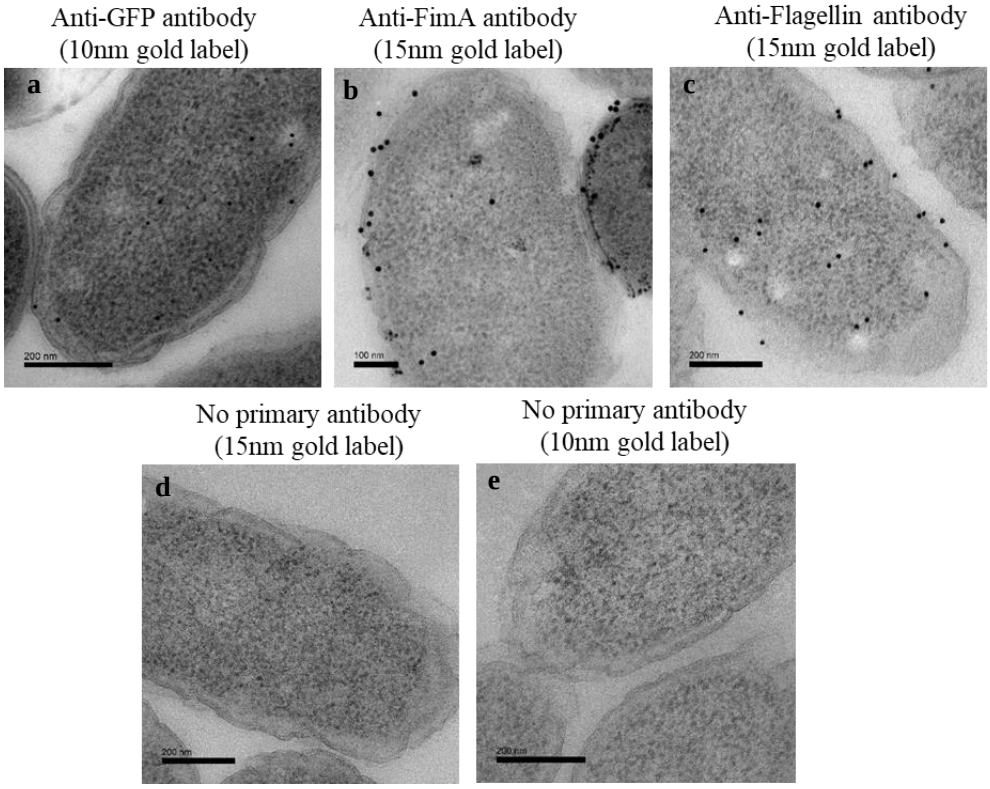


Figure 4.32 Immunogold labelling of embedded *E. coli* FimA-GFP strain cells

TEM analysis of thin-sectioned and cells embedded in resin. The sections were immunogold labelled and probed with: anti-GFP antibody (**a**) anti-FimA monomer antibody (**b**), anti-FimA monomer and anti-GFP antibodies mixed (**c**), anti-Flagellin antibody (**d**) and TBST only as a negative control (**e** and **f**).

4.2.7.2 Co-localisation of both FimA and GFP in the OMVs

Western blots also confirmed co-localisation of both FimA and GFP in the OMVs, which indicated that the GFP-FimA fusion protein was successfully delivered. Proteins were purified from the OM and periplasm of the GFP-FimA fusion strain and MG1655 strain (without the protein fusion). This was compared to the OMV protein profile and whole cell profile (Figure 4.33).

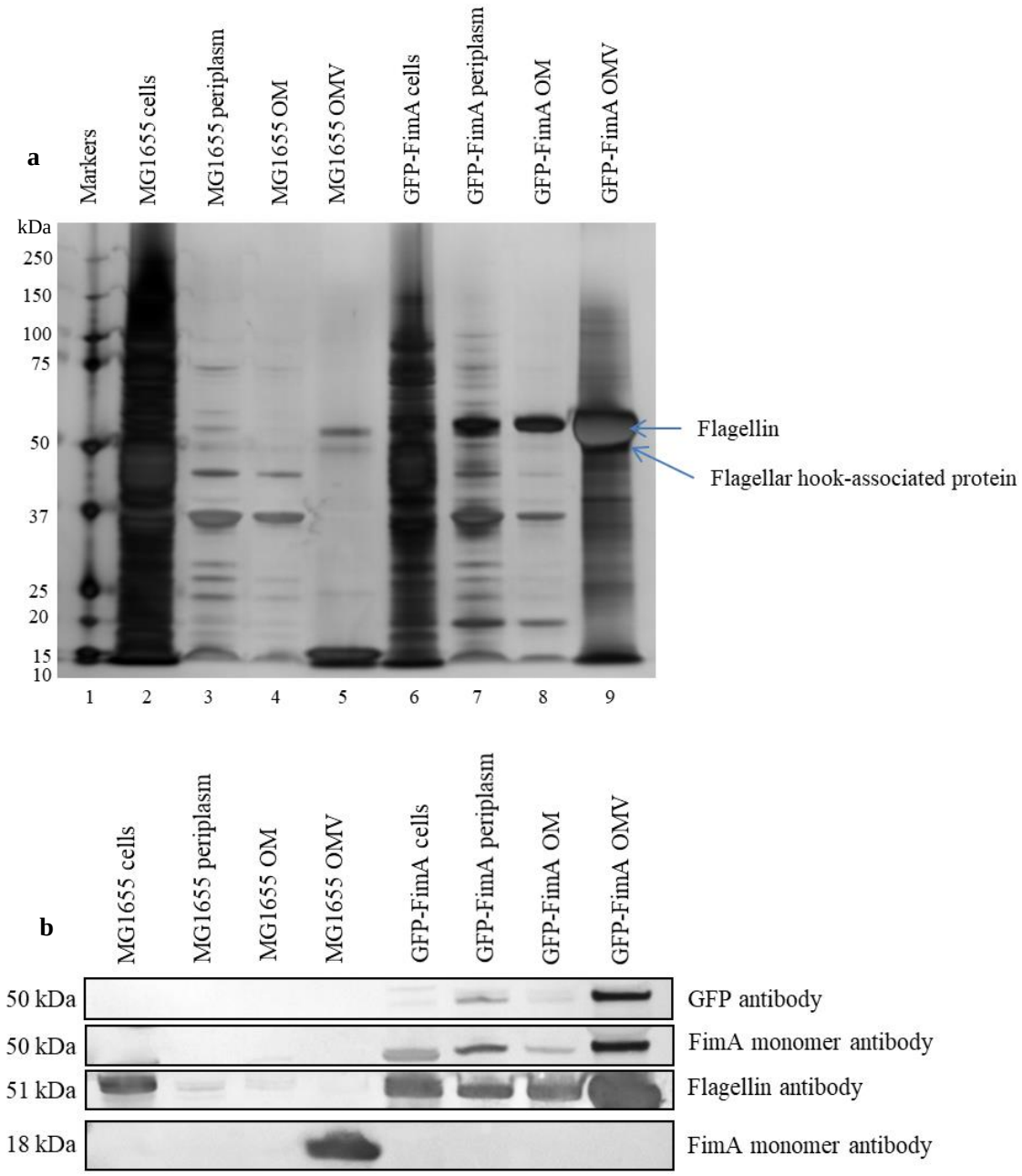


Figure 4.33 Detection of FimA, Flagellin and GFP in MG1655 and the MG1655 GFP-FimA protein fusion strain

Proteins were purified from the OM and periplasm of the cells and compared to the OMVs and whole cells. A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was silver stained and the two bands labelled were excised from the gel and identified by mass spectrometry (**a**). Western blots were performed on these samples using the following antibodies: anti-GFP, anti-FimA monomer and anti-Flagellin (**b**). Mass spectrometry data and original Western blot images can be found in Appendix C.22.

The Western blots confirmed that there was a protein present in the OMV sample at approximately 50 kDa which was detected by both the anti-GFP antibody and anti-FimA monomer antibody. This was further evidence that the FimA-GFP protein had successfully been delivered to the OMVs. This protein

was also detected in the periplasm meaning that the GFP fusion to FimA did not disrupt the export of the protein to the periplasm or the signals necessary to package the protein within the OMVs.

In the WT MG1655 strain, FimA monomer (18 kDa) was packaged into the OMVs but not Flagellin (51 kDa). OMVs from the GFP-FimA fusion strain did not contain the FimA monomer (18 kDa) due to its absence in the FimA-GFP protein fusion strain. As seen in OMVs from the *ΔfimA* strain, the GFP-FimA fusion caused packaging of Flagellin (51 kDa) into OMVs and not FimA monomer. The mutually exclusive packaging of FimA and Flagellin remained in the OMVs.

4.2.7.3 Is the FimA-GFP fusion protein protected within the OMVs?

The Proteinase K test was performed using the OMVs purified from the FimA-GFP fusion strain (Figure 4.34).

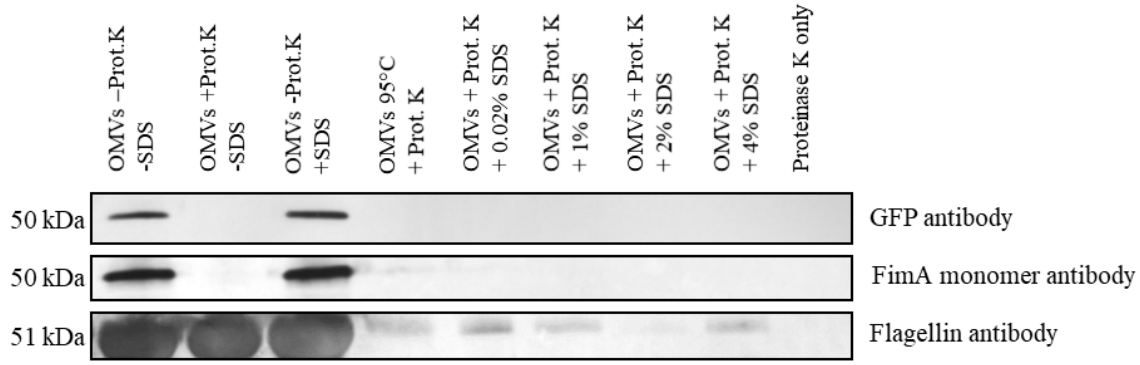


Figure 4.34 Proteinase K test on OMVs from *E. coli* FimA-GFP fusion strain

OMVs were incubated in the presence and absence of 10 µg/mL Proteinase K and various concentrations of SDS for 30 mins at 37ºC. 5 mM was added to inhibit Proteinase K and samples were incubated for another 30 mins at 37ºC. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The samples were used for Western blotting and probing with the following antibodies: anti-GFP, anti-FimA monomer and anti-Flagellin. Original Western blot images can be found in Appendix C.23.

The Proteinase K test was performed on the OMVs from the *E. coli* FimA-GFP fusion strain. As explained previously, the sample containing 'OMVs + Proteinase K – SDS' reveals which proteins are protected within the OMVs. Flagellin was protected within the OMVs from degradation by Proteinase K but the FimA-GFP fusion protein was degraded. This may be because the addition of the GFP to the FimA protein disrupted how the FimA monomer protein is usually packaged within the OMVs. For example, when GFP is fused to FimA, the protein is more than double its usual molecular weight and therefore it could be more exposed to degradation by proteases than usual. Furthermore, applying the Proteinase K test to FimA has previously been unsuccessful (Section 4.2.2). The FimA monomer may be packaged into a different part of the OMVs where it is less protected from Proteinase K (for example, it could be OM-associated). Therefore, the degradation of the FimA-GFP protein in this Proteinase K test should not be used as evidence either way for whether the FimA-GFP fusion protein is OMV-associated.

4.2.8. Can FimA be used to target proteins and other molecules for delivery within OMVs using an alternative method (ie. expression of the fusion protein on a plasmid)?

4.2.8.1 Targeting a FimA-mNeon green fusion protein to *E. coli* OMVs

After the success of the FimA-GFP fusion protein targeting to the OMVs, expression of a FimA fusion protein using a plasmid was trialled. If successful, this could be very useful therapeutically for targeting proteins of interest to *E. coli* OMVs. A pJB005 plasmid (details in Section 2.12.1) was produced which resulted in the synthesis of a FimA-mNeon green fusion protein when induced in a competent *E. coli* K-12 strain. The plasmid was selectable by using the antibiotic chloramphenicol. The plasmid was used to transform competent *E. coli ΔfimA* cells and *E. coli* WT parental BW25113 cells which were induced to produce the FimA-mNeon green fusion protein. The OMVs were purified and the protein profile was compared from both *E. coli* strains. The first aim was to see if the FimA-mNeon green fusion protein had been packaged into the OMVs successfully. The second aim was to see if the re-introduction of FimA changed the proteins packaged within the OMVs. A pJB005 plasmid containing a FimC-mNeon green fusion protein was also produced. However, this plasmid was never used due to time constraints.

Expression of a plasmid containing FimA-Neon green protein fusion in *E. coli* BW25113 parental and *ΔfimA* strains

The plasmid was transformed into competent *E. coli ΔfimA* and *E. coli* WT parental BW25113 cells. One colony from each successful transformation was used to produce an overnight strain, which was then used to inoculate 2 identical flasks containing LB with chloramphenicol. When the OD600 of the cells was 0.25-0.3 (early stationary phase), IPTG was added to one of the flasks. The other was not induced as a negative control. When the induced cells had reached an OD600 of approximately 1.0, OMVs were purified using the standard protocol (see Section 2.6.1). The OMVs were visualised using TEM (Figure 4.35). It appears that induction with IPTG causes hypervesiculation (comparing **a** and **b** to **c** and **d**).

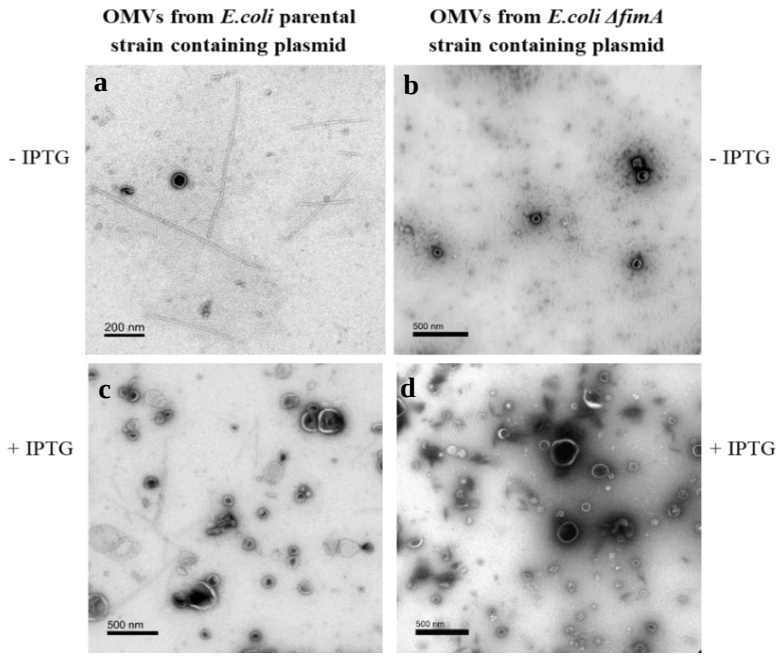


Figure 4.35 TEM images of OMVs purified from *E. coli* strains expressing pJB005 plasmid

The plasmid was transformed into competent *E. coli ΔfimA* and *E. coli* WT parental BW25113 cells. When the OD600 of the cells was 0.25-0.3 (early stationary phase), IPTG was added to one of two identical flasks containing either *E. coli* WT Parental BW25113 or *ΔfimA* competent cells (**c** and **d**). The other was not induced as a negative control (**a** and **b**). When the induced cells had reached an OD600 of approximately 1.0, OMVs were purified using the standard protocol and visualised using TEM.

A Western blot was performed on the purified OMVs and compared to the whole cell protein profile. This was to detect the FimA-mNeon green fusion protein using an anti-mNeon green antibody (Figure 4.36 and Appendix C.25).

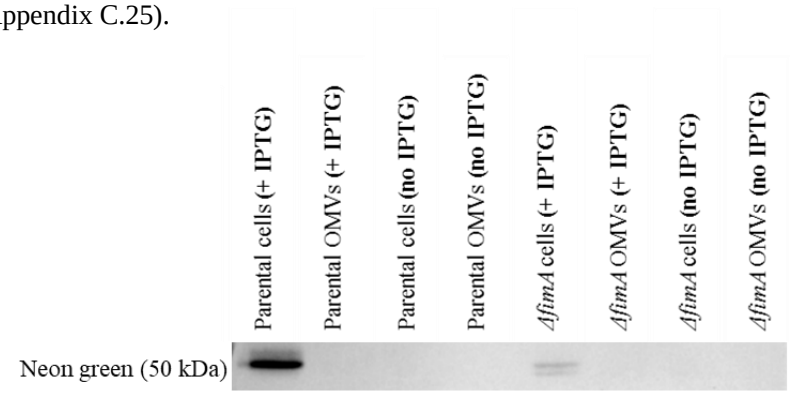


Figure 4.36 Detection of FimA-mNeon green fusion protein in OMVs purified from *E. coli* strains expressing pJB005 plasmid

The plasmid was used to transform competent *E. coli ΔfimA* and *E. coli* WT parental BW25113 cells. When the OD600 of the cells was 0.25-0.3 (early stationary phase), IPTG was added to one of two identical flasks containing either *E. coli* WT parental BW25113 or *ΔfimA* competent cells. The other was not induced as a negative control. When the induced cells had reached an OD600 of approximately 1.0, OMVs were purified using the standard protocol. A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. A Western blot was performed on these samples using anti-mNeon green antibody.

Although, the FimA-mNeon green protein was expressed when the cells were induced with IPTG, there was no indication that the fusion protein was packaged in the OMVs.

Is the FimA-mNeon green fusion protein transported to the periplasm?

To investigate further, transformed *E. coli ΔfimA* and *E. coli* WT parental BW25113 cells were grown and induced with IPTG as done previously. However, this time periplasmic proteins and OM proteins were also isolated from the cells as well for comparison, The OMVs were visualised by TEM as done previously (Figure 4.37).

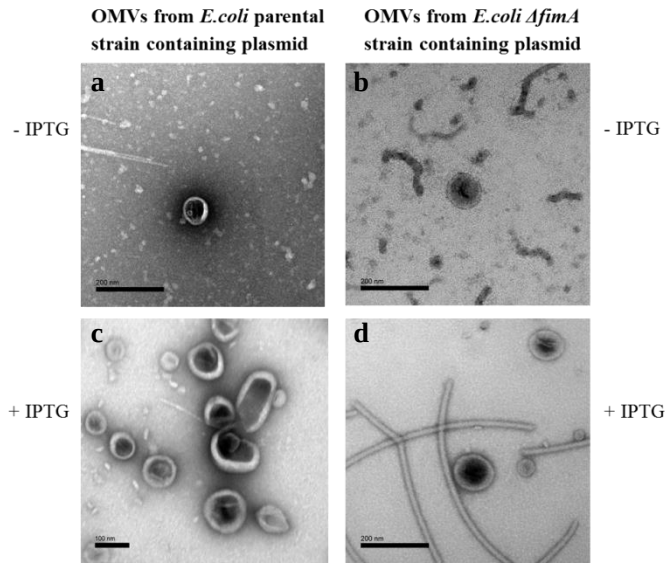


Figure 4.37 TEM images of OMVs purified from *E. coli* strains expressing pJB005 plasmid

The plasmid was transformed into competent *E. coli ΔfimA* and *E. coli* WT parental BW25113 cells. When the OD600 of the cells was 0.25-0.3 (early stationary phase), IPTG was added to one of two identical flasks containing either *E. coli* WT Parental BW25113 or *ΔfimA* competent cells (**c** and **d**). The other was not induced as a negative control (**a** and **b**). When the induced cells had reached an OD600 of approximately 1.0, OMVs were purified using the standard protocol and visualised using TEM.

A Western blot was performed to detect whether the FimA-mNeon green fusion protein had been transported to the periplasm (Figure 4.38 and Appendix C.26).

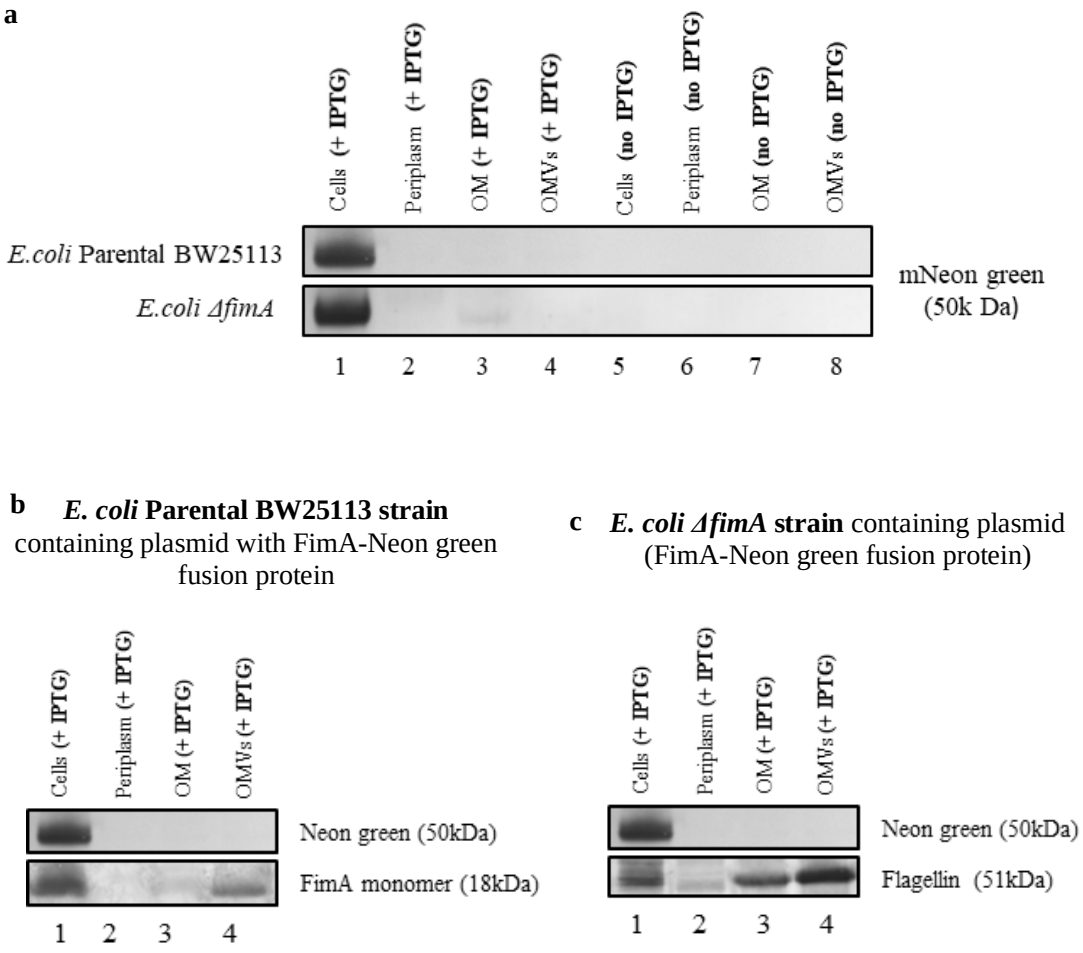


Figure 4.38 Detection of FimA-mNeon green fusion proteins from *E. coli* strains expressing pJB005 plasmid

Proteins were purified from the OM and periplasm of the cells and compared to the OMVs and whole cells, A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. Western blots were performed on these samples using the following antibodies: anti-Neon green (**a**), anti-FimA monomer (**b**) and anti-Flagellin (**c**).

Figure 4.38 (**a)** shows that the FimA-mNeon green protein was expressed as the band was present in the whole cell samples after induction with IPTG (Lane 1). However, the protein was not transported to the periplasm in either strain and was therefore not found in the OM or OMVs either (Lanes 2-4). The *E. coli* WT Parental BW25113 strain OMVs always contain FimA. An anti-FimA monomer antibody was used to check that the FimA monomer was still packaged into *E. coli* WT parental BW25112 OMVs successfully in the conditions trialled. If the conditions were not correct for FimA monomer to be packaged, this may be the reason that the FimA-mNeon green fusion protein was not packaged into the OMVs. However, Lane 4 of Figure 4.38 (**b**) does have a band representing FimA monomer in the

OMVs. The usual 18 kDa FimA was successfully delivered to the OMVs in these conditions but the FimA-mNeon green protein was not. *E. coli ΔfimA* OMVs normally contain Flagellin so an anti-Flagellin antibody was used to see if the Flagellin had successfully been packaged within the OMVs. Lane 4 of Figure 4.38 (**c**) shows that Flagellin was packaged as normal. Although FimA and Flagellin monomers were packaged as usual into the OMVs, the FimA-mNeon green protein was not transported to the periplasm and was not packaged within the OMVs. This is discussed further in Section 4.3.5.

4.2.8.2 mNeon green fused with FimA signal peptide

SDS-PAGE gels and Western blots have shown FimA at a molecular weight of 18 kDa and not 15 kDa. FimA is synthesised *in vivo* in a precursor form with an N terminal signal peptide sequence that allows it to be exported across the IM using the Sec translocase in the Sec pathway (Natale, *et al*. 2008). After this has occurred, the FimA signal peptide sequence is cleaved off to make a 15 kDa protein. Interestingly, the FimA monomer in OMVs has always been found at 18 kDa and never 15 kDa. This makes it possible that the signal peptide sequence remains on the FimA protein if it is destined to be packaged into OMVs (rather than into fimbriae). To test this hypothesis, a FimA signal peptide sequence was added to the N terminus of a mNeon green protein through cloning into a pJB005 plasmid (see Section 2.12) to see if the protein was packaged into the *E. coli* OMVs.

The plasmid was transformed into competent *E. coli ΔfimA* and *E. coli* WT parental BW25113 cells. Two identical flasks containing LB with chloramphenicol were inoculated with a transformed colony. When the OD600 of the cells was 0.25-0.3 (early stationary phase), IPTG was added to one of the flasks. The other was not induced as a negative control. When the induced cells had reached an OD600 of approximately 1.0, OMVs were purified using the standard protocol. The OMVs were visualised using TEM (Figure 4.39).

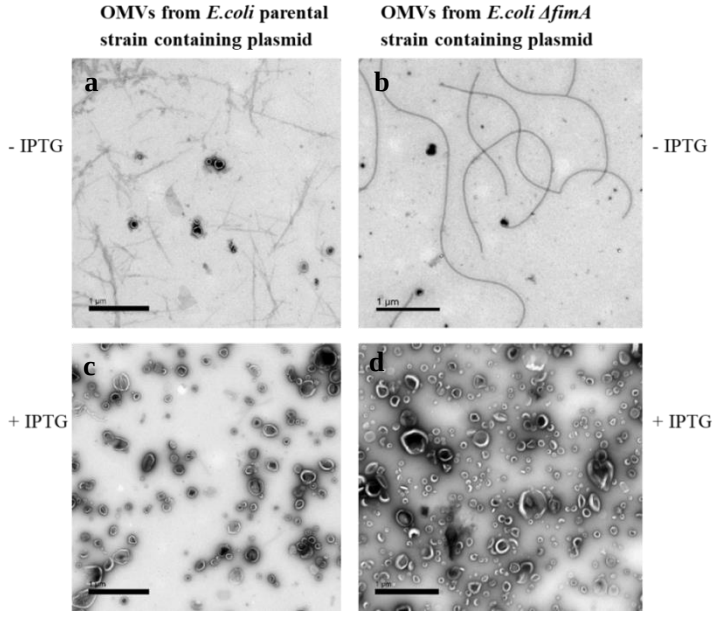


Figure 4.39 TEM images of OMVs purified from *E. coli* strains expressing pJB005 plasmid

The plasmid was transformed into competent *E. coli ΔfimA* and *E. coli* WT parental BW25113 cells. When the OD600 of the cells was 0.25-0.3 (early stationary phase), IPTG was added to one of two identical flasks containing either *E. coli* WT parental BW25113 or *ΔfimA* competent cells (**c** and **d**). The other was not induced as a negative control (**a** and **b**). When the induced cells had reached an OD600 of approximately 1.0, OMVs were purified using the standard protocol and visualised using TEM.

Interestingly, induction by IPTG appeared to cause hypervesiculation of the strain and a reduction in production of fimbriae and flagella. This was seen previously with expression of the FimA-mNeon green fusion protein (Figure 4.35). The OMV protein profiles were visualised using SDS-PAGE (Figure 4.40).

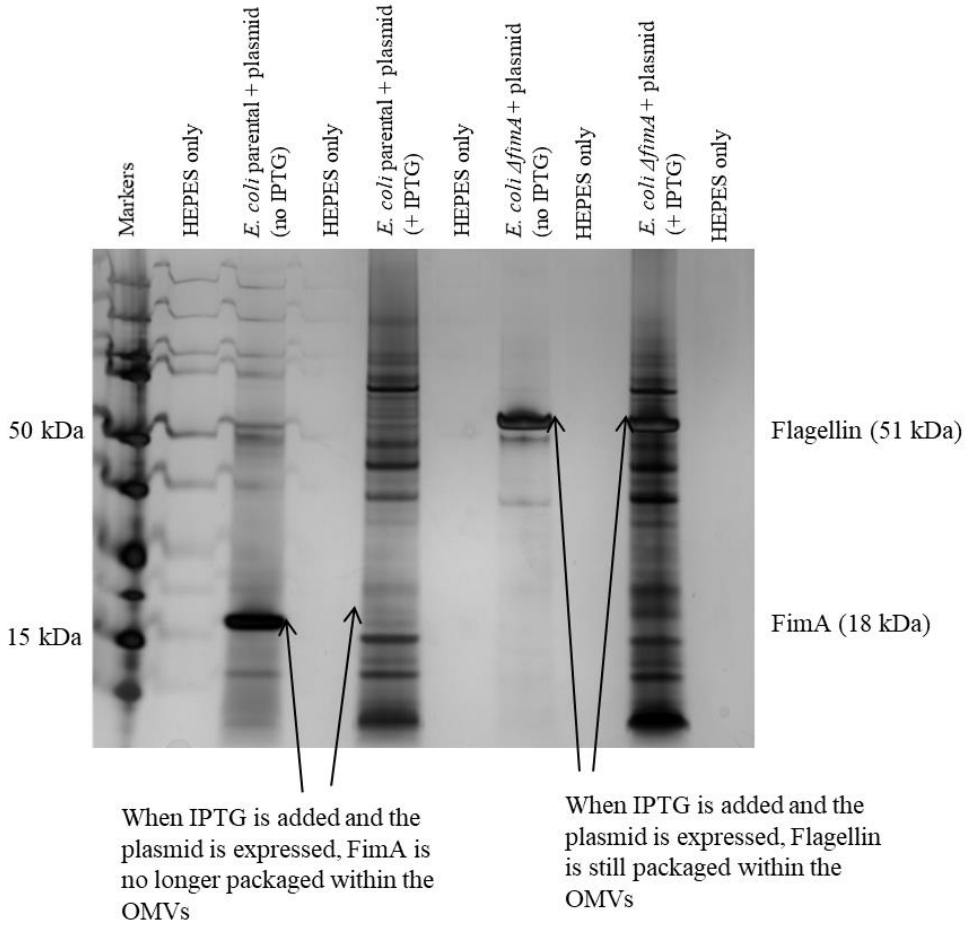


Figure 4.40 Detection of FimA-mNeon green fusion proteins in OMVs from *E. coli* strains expressing pJB005 plasmid

The plasmid was transformed into competent *E. coli ΔfimA* and *E. coli* WT parental BW25113 cells. When the OD600 of the cells was 0.25-0.3 (early stationary phase), IPTG was added to one of two identical flasks containing either *E. coli* WT parental BW25113 or *ΔfimA* competent cells. The other was not induced as a negative control. When the induced cells had reached an OD600 of approximately 1.0, OMVs were purified using the standard protocol A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was silver stained.

Induction using IPTG appeared to disrupt the regulation of which proteins were packaged to the OMVs. After IPTG was added in the *E. coli* parental WT BW25113 strain, FimA monomer was no longer packaged into the OMVs. There were also many more additional proteins packaged with no particular enrichment of any protein. Similarly, in the *ΔfimA* strain, there were many more proteins packaged into the OMVs after induction. Interestingly, Flagellin was still enriched in the OMVs from the *ΔfimA* strain. This is further evidence that FimA and Flagellin are packaged into the OMVs using different mechanisms as disruption of FimA does not also mean disruption of Flagellin. Due to time contraints, this experiment was not optimised further.

4.3 Discussion

4.3.1 Comparison of OMVs purified from *E. coli* K-12 vs B strains

4.3.1.1 *E. coli* B strains hypervesiculate compared to K-12 strains (Section 4.2.1)

The number of OMVs released from the engineered and proprietary BL21 strains was higher than *E. coli* WT strains. The possible reasons for this are discussed below.

Gene knockouts that may lead to hypervesiculation of B strains

Kulp and Kuehn (2015) determined the vesiculation production for the whole CGSC Keio collection library of *E. coli* mutant strains. The study found approximately 150 new genes, which are thought to be involved in OMV production (Kulp, Kuehn. 2015). The study indicated that a gene mutation that disrupts outer membrane structures such as LPS leads to hypervesiculation. These genes are outlined in Supplementary tables S1-S5 (Kulp, Kuehn. 2015).

OmpC and OmpF

It has previously been found that OMV production increased when there were disruptions in genes involved in outer membrane protein expressions, the synthesis of peptidoglycan and the σ^E envelope stress response. In this study, they found that disruption of the outer membrane porin proteins OmpC and OmpF genes gave increased vesiculation levels (McBroom *et al.* 2006). In *E. coli* BL21 strains, the *ompC* gene contains an insertion element making it non-functional. OmpC is not produced, which could contribute to the increased hypervesiculation of this strain (Marisch *et al.* 2013).

cAMP and ppGpp

Cyclic adenosine monophosphate (cAMP) is a derivative of ATP and is an indicator of metabolic stress in the cell. In an environment with low concentrations of glucose, levels of cAMP increase. cAMP can form a complex with a regulatory protein called cAMP receptor protein (CRP). The binding of CRP to cAMP causes a conformational change to form an active transcriptional regulator. CRP controls the transcription initiation of over 100 promoters (Zhan *et al.* 2008). These genes which are regulated by CRP are mainly involved in the catabolism of non-glucose carbon sources. As *E. coli* preferentially uses glucose as a carbon source, the CRP regulon is activated when glucose is absent in the environment. In this way, elevated cAMP levels are an indicator of glucose starvation. A study in 2013 found that levels of cAMP in *E. coli* B strains (BL21) were consistently higher than in K-12 strains despite the presence of glucose in the media (Marisch *et al.* 2013). It is possible that CRP-cAMP play a role in causing hypervesiculation in B strains either directly (by activation of specific genes that induce hypervesiculation) or indirectly (due to the cell being in a state of stress).

Similarly, the nucleotide (guanosine pentaphosphate) ppGpp is a signalling molecule which causes up-regulation of many of the genes involved in the stringent stress response. Some of these genes encode proteins needed for amino acid biosynthesis and uptake from the environment in order for the cell to

survive harsh conditions. *E. coli* B strains were also found to contain high levels of ppGpp compared with the K-12 strains, indicating that the cells are in a state of stress (Marisch *et al.* 2013). The higher levels of ppGpp are another indicator that *E. coli* B strain cells are in states of stress, which could be the reason for their hypervesiculation.

4.3.1.2 *E. coli* K-12 OMVs are enriched with specific proteins unlike OMVs from B strains (Section 4.2.1)

OMVs from *E. coli* BL21 and BL21 (DE3) strains contain many more proteins than *E. coli* K-12 WT OMVs and there are no dominant bands on the SDS-PAGE gels. In *E. coli* K-12 WT OMVs, specific proteins (identified as FimA and Flagellin) appear to be selectively included and excluded. The appearance of the OMVs from *E. coli* BL21 (DE3) in the TEM photos match those in the literature (Figure 2A of Bartolini *et al.* 2013). The protein profile of BL21 and BL21 (DE3) OMVs is not clear from the literature. For example, OMVs have been isolated from *E. coli* BL21 *ΔompA* and an SDS-PAGE gel has been run to show the OMV protein profile. However, the strain contains a deletion of OmpA and expression of recombinant proteins of interest, which means that they are not directly comparable to the results in this study (Fantappie *et al.* 2014). After reviewing the available evidence in the literature, it appears that *E. coli* B strain cells are producing and secreting OMVs as part of a stress response. The cargo packaged is non-specific whereas *E. coli* K-12 strain OMVs is a more regulated process with careful enrichment and exclusion of certain proteins such as FimA and Flagellin monomers (as explained in Section 4.3.1.1 above).

4.3.2 Evidence that FimA and Flagellin are packaged into *E. coli* K-12 OMVs (Sections 4.2.1 - 4.2.6)

4.3.2.1 Methods used as evidence that FimA and Flagellin are packaged within *E. coli* K-12 OMVs

Firstly, the immunogold labelling of *E. coli* OMVs embedded in resin confirmed the presence of FimA and Flagellin (Figures 4.6 and 4.31). Secondly, SDS-PAGE gels, Western blots and mass spectrometry identified FimA and Flagellin in the periplasmic fraction of *E. coli* cells and their respective purified OMV samples (Figures 4.7-4.8). Lastly, the Proteinase K showed that Flagellin monomer is OMV-associated and protected within the OMVs (Figures 4.9 and 4.11).

4.3.2.2 The Proteinase K test cannot be used to prove that FimA monomer is packaged into K-12 OMVs (Section 4.2.1)

According to ExPASy peptide cutter tool, Proteinase K should cut at 108 cleavage sites within the FimA monomer protein (see Appendix C.6). However, it was not possible to optimise the Proteinase K test in a way that would allow the degradation of FimA. Only two conditions were found that made FimA susceptible to Proteinase K degradation which are summarised below.

Firstly, TCA precipitation of the OMV sample prior to the Proteinase K test made the FimA monomer susceptible to degradation. It may be that the FimA monomers within OMVs are in a conformation where all cleavage sites are sequestered from proteases. As discussed previously, TCA causes a protein to denature in a way that it loses its secondary structure (Koontz *et al.* 2014). This change in protein conformation may make the protease cleavage sites now accessible to Proteinase K and allow degradation.

Secondly, the creation of the fusion protein FimA-GFP resulted in a protein that was now susceptible to degradation by Proteinase K under the usual assay conditions. ExPASy peptide cutter tool indicates that GFP (GFP_AEQVI) contains 112 cleavage sites that can be cut by Proteinase K (Appendix C.24). GFP has a molecular weight of 27 kDa so adding this within the FimA protein (18 kDa) will drastically disrupt the protein conformation as well as adding 112 new cleavage sites for Proteinase K.

4.3.3.3 FimA monomer in OMVs may be in a different conformer to FimA in fimbriae

It is possible that the FimA monomer found in OMVs is a conformer of the usual FimA monomer found in fimbriae. Puorger *et al.* (2011) produced an incredibly stable variant form of FimA, which was generated to mimic the state of the usual FimA monomer in the context of the quaternary structure of the pilus rod. It was made by 'elongation of FimA at the C-terminus by its own donor strand generated a self-complemented variant (FimAa) with alternative folding possibilities that spontaneously adopts the more stable conformation' (Puorger *et al.* 2011). Perhaps the form of FimA monomer found in OMVs is in a different conformation to the usual FimA monomer (found in fimbriae), which is more resistant to proteolytic degradation (see further discussion in Section 4.3.4).

4.3.3 Discussion of the mutual exclusive packaging of FimA and Flagellin into OMVs from a variety of *E. coli* strains (Sections 4.2.3-4.2.6).

In the literature, Flagellin has already been reported as present in the lumen of *E. coli* W3110 OMVs which is a K-12 strain (Manabe *et al.* 2013). Similarly, FimA has also been found in OMVs in the literature (Bai, *et al.* 2014). However, the mutually exclusive packaging of FimA and Flagellin has not yet been addressed in the literature. One particular study indicates that pathogenic *E. coli* cells reciprocally regulate the expression of flagella and fimbriae (Cooper *et al.* 2012). It is possible that the regulation of packaging FimA and Flagellin monomers into OMVs could also be reciprocally regulated or linked to the expression of flagella and fimbriae. Table 4.8 a-c below summarises whether FimA and Flagellin monomers are packaged into the OMVs of each strain and whether the packaging is mutually exclusive.

Table 4.8 Is the packaging of FimA and Flagellin monomers into OMVs mutually exclusive?

Table **a** compares OMVs from five commonly used *E. coli* strains from Chapters 3 and 4. Table **b** compares OMVs purified from a range of *E. coli* strains with deletions of various fimbriae or flagella associated proteins. Table **c** compares OMVs purified from 6 clinical isolates. In the following tables, Type 1 fimbriae is abbreviated to 'T1F'.

a

***E.coli* strain name**	**FimA monomer in OMVs?**	**Flagellin monomer in OMVs?**	**Is packaging mutually exclusive?**	**Discussion of mutual exclusivity of FimA and Flagellin packaging within OMVs**
WT MG1655	Yes	No	Yes	MG1655 is K-12 WT strain. The OMVs contain FimA monomer but not Flagellin (same as the Keio collection WT parental strain BW25113).
FimB-LacZ fusion	No	Yes	Yes	The FimB-LacZ fusion strain contains a deletion of *fimB* which inhibits the production of T1F and so flagella is expressed instead. This leads to the packaging of Flagellin into the OMVs and not FimA.
Fimbriae locked on	Yes	Yes	No	When T1F expression is locked on, both FimA and Flagellin are packaged into the OMVs. The exclusion of Flagellin from the OMVs has been disrupted.
BL21	No	No	Neither present	All *E.coli* B strains examined do not express T1F or flagella and do not package FimA or Flagellin into the OMVs. There appears to be a loss of regulation into which proteins are packaged into the OMVs.
BL21 (DE3)	No	No	Neither present	

b

E.coli strain name	FimA monomer in OMVs?	Flagellin monomer in OMVs?	Is packaging mutually exclusive?	Discussion of mutual exclusivity of FimA and Flagellin packaging within OMVs
WT parental BW25113	Yes	No	Yes	The Parental strain of the Keio collection (from which the other knockout strains are derived) has FimA packaged in the OMVs but not Flagellin.
ΔfliC	Yes	No	Yes	Deletion of Flagellin from the *E.coli* BW25113 Parental strain does not affect the packaging of FimA.
ΔfimA	No	Yes	Yes	Deletion of *fimA* causes a switch where Flagellin is packaged instead of FimA (mutual exclusivity).
ΔfimC	No	Yes	Yes	Deletion of *fimC* causes a switch where Flagellin is packaged instead of FimA. FimC is a chaperone protein which could play a role in directing FimA monomers to be packaged within OMVs.
ΔfimB	No	Yes	Yes	FimB is a site-specific recombinase that can bind to *fimS* and switch T1F expression either on-to-off or off-to-on. When FimB is absent, all fimbriae production is locked off which means that there is no expression of FimA. In this case, Flagellin is packaged instead of FimA.
ΔfimE	Yes	Yes	No	FimE is a site-specific recombinase that can bind to *fimS* and switch T1F expression on-to-off. When FimE is absent, T1F production is locked on. As with the other strain where fimbriae production was locked on, both FimA and Flagellin are packaged within the OMVs.
ΔfimF	No	Yes	Yes	FimF binds to FimA in the formation of T1F. Perhaps FimF plays a role in the packaging of FimA to the OMVs as FimA is no longer packaged when it is absent.
ΔfimG	Yes	Yes	No	FimG and FimH are fimbrial tip proteins that have no known interaction with FimA monomer. When FimG and FimH are absent, FimA is still packaged into OMVs in the usual way. However, there are no functional T1F without the tip proteins and this leads to the expression of flagella on the cells. This also led to the packaging of Flagellin into the OMVs from these strains.
ΔfimH	Yes	Yes	No	
ΔfimD	No	No	Neither present	FimD is an OM protein that is an usher to aid the formation of T1F. When FimD is absent, neither flagella or T1F appear to be expressed on the cells and no FimA or Flagellin is packaged into the OMVs.
ΔfimI	Yes	Yes	No	The role of FimI in T1F formation is unclear from the literature. However, deletion of *fimI* led to the packaging of Flagellin as well as FimA into the OMVs.
ΔfimZ	Yes	No	Yes	FimY and FimZ independently activate the promoter which controls the expression of *fim* structural genes. Therefore, even though FimZ was absent, FimY could promote *fimA* expression and there was no effect on the OMV protein profile.
ΔlrhA	Yes	Yes	No	LrhA is a key regulator of the transcription of genes relating to flagella. Without the regulation of LrhA, flagella are expressed and Flagellin is packaged along with FimA.
ΔfliD	Yes	No	Yes	Absence of these proteins that interact with Flagellin did not affect the packaging of FimA into the OMVs.
ΔfliS	Yes	No	Yes	
ΔflhA	Yes	No	Yes	

c

E.coli strain name	FimA monomer in OMVs?	Flagellin monomer in OMVs?	Is packaging mutually exclusive?	Discussion of mutual exclusivity of FimA and Flagellin packaging within OMVs
Clinical isolate 1	No	No	Neither present	Clinical isolate 1 did not appear to express flagella or T1F. Previous analysis of the virulence genes determined that the strain was deficient in *fimA*. Neither FimA or Flagellin was packaged within the OMVs.
Clinical isolate 2	No	No	Neither present	Genetic analysis of this strain determined that FimB was non-functional and that there was no expression of T1F. Although, flagella was expressed on the cell, neither FimA or Flagellin were found in the OMVs.
Clinical isolate 3	Yes	Yes	No	This strain appeared to express fimbriae only but both FimA and Flagellin were packaged within the OMVs. F7-2 fimbrial precursor protein was also identified in the OMVs by mass spectrometry.
Clinical isolate 4	No	No	Neither present	Neither FimA or Flagellin were found in OMVs from Clinical isolate 4. However, KS71A fimbrillin protein was also found which is related to fimbriae and may have a similar function to FimA.
Clinical isolate 5	Yes	No	Yes	Clinical isolate 5 expressed T1F and is evidence of the mutually exclusive packaging of FimA (and not Flagellin) in a real-life clinical isolate *E.coli* strain.
Clinical isolate 6	No	Yes	Yes	Clinical isolate 6 expressed flagella and is evidence of the mutually exclusive packaging of Flagellin (and not FimA) in a real-life clinical isolate *E.coli* strain.

4.3.4 What is the function of packaging FimA and Flagellin into *E. coli* OMVs?

It appears that in the majority of strains, FimA and Flagellin are reciprocally regulated and are packaged into OMVs independently (see Section 4.2.6). In 2012, a study proposed that there is a reciprocal regulation of adherence (by expressing Type 1 fimbriae) and motility (by expressing flagella) in UPEC *E. coli* (Cooper *et al.* 2012). The cells are either motile or adhering, which are opposing virulence objectives but both essential for colonisation of the urinary tract (Cooper *et al.* 2012). From this evidence, it seems possible that FimA and Flagellin could also be packaged into OMVs in a way that is also reciprocally regulated.

4.3.4.1 Discussions of FimA monomer in the literature

FimA can form a stable monomer conformation

During the formation of Type 1 fimbriae, FimA monomers polymerise by donor-strand complementation (see Section 1.6.3). The FimA monomer has a donor strand which inserts into an immunoglobulin-like fold of the preceding FimA monomer to complete it. In this way, chains of FimA monomers form stable polymers. More recently, however, FimA has been found to adopt a monomeric self-complemented form. To remain stable, the self-donor strand is inserted in the opposite orientation to that during polymerisation to prevent any other FimA monomers from forming oligomers (Walczak *et al.* 2014).

FimA monomers can inhibit cell apoptosis (innate immune response)

In 2010, a study found a soluble, monomeric form of FimA that could suppress host cell apoptosis by targeting a Mitochondrial complex (Sukumaran *et al.* 2010). Many bacterial pathogens improve the chances of survival by inhibiting the apoptosis of host cells, which is part of the innate immune response. A pro-apoptotic protein called Bax is known to play a major role in the mitochondrial signalling pathway. When apoptosis is induced, Bax inserts into mitochondrial membranes and causes the release of cytochrome c into the cytosol (Heimlich *et al.* 2004). Cytochrome c then induces formation of an apoptosome which triggers a cascade of events resulting in cell apoptosis. In this study, FimA (from *E. coli* K1 supernatants) was shown to be a potent inhibitor of Bax-mediated release of cytochrome c from mitochondria (Sukumaran *et al.* 2010).

This soluble form of FimA was also found in *Shigella flexneri* and *Salmonella enterica* supernatants. It was also shown to selectively block Bax from integrating into the mitochondrial membrane (Sukumaran *et al.* 2010). Interestingly, FimA was found to later dissociate from the mitochondria, which allowed apoptosis to resume. The authors hypothesised that the bacteria may benefit from stalling apoptosis temporarily for bacterial survival then allow apoptosis to occur to facilitate dissemination (Sukumaran *et al.* 2010). They also contemplate how a soluble FimA monomer could be found in the supernatant. Based on the results of this study, one could argue that these FimA monomers are the same as those found in *E. coli* K-12 OMVs here. Their FimA monomer was also found at approximately 18 kDa in their Western blots rather than the usual 15 kDa FimA in fimbriae.

FimA monomer has been detected in OMVs from other bacterial strains

FimA has also been found in OMVs from other bacterial strains including *Porphyromonas gingivalis* (Mantri *et al.* 2015). *P. gingivalis* is a Gram-negative OMV-producing pathogen that plays a role in causing chronic periodontitis (gum disease). OMVs were found to contain virulence factors such as hemagglutinin and a protease called gingipain, which degrades cytokines and reduces inflammation.

4.3.4.2 Discussions of Flagellin monomer in the literature

Flagellin monomer has been detected in the lumen of *E. coli* K-12 OMVs as well as other bacterial strains

In 2013, a study found that an *E. coli* K-12 strain (W3110) produced OMVs that were enriched with Flagellin monomer (Manabe *et al.* 2013). In this study, the authors detected Flagellin within the lumen of the OMVs which agrees with the findings in the current study. Flagellin type B was detected in *P. aeruginosa* OMVs in this study. This agrees with another study in the literature which found Flagellin type B in OMVs from *PA01*s and S470 strain. S470 is a *P. aeruginosa* clinical isolate from the sputum of a cystic fibrosis patient, which makes this finding clinically relevant (Bauman *et al.* 2006). Similarly, Flagellin has also been found in the supernatant and OMVs from pathogenic enterotoxigenic *E. coli* (ETEC) strains (Roy *et al.* 2010).

Flagellin monomer is pro-inflammatory

Flagellin is a bacterial virulence factor that is recognised by the innate immune system. Flagellin is detected by receptors on innate immune cells which leads to the production of cytokines. In 2012, a study showed that *E. coli* K-12 Flagellin induced a pro-inflammatory immune response in mice (Zgair, 2012). Flagellin is known to bind to Toll-like receptor 5 (TLR-5) which activates NF-κB signalling in the cell (Yoon *et al.* 2017). NF-κB is a transcription factor that induces the expression of cytokines, chemokines and expression of pro-inflammatory genes. These pro-inflammatory cytokines activate the adaptive immune response which is specific for the bacterial antigen. TLR-5 receptors are found on a range of cell types including macrophages, neutrophils, lymphocytes, dendritic cells and epithelial cells (Hajam *et al.* 2017).

In the 2012 study, Flagellin was purified from depolymerising *E. coli* K-12 flagella into Flagellin monomers. To observe the effect on the immune system in the lungs of mice, Flagellin was instilled intranasally. The presence of Flagellin induced the expression and production of the pro-inflammatory cytokine's interleukin 1 beta, interleukin 6 and tumour necrosis factor alpha. High numbers of neutrophils were also recruited to the lungs after 24 hours (Zgair, 2012) as well as high levels of cytokines and chemokines. Flagellin from *E. coli* has also been reported to be recognised by TLR11 in mice (Hatai *et al.* 2016). Lastly, it appears that Flagellin is also recognised by the NAIP5/NLRC4 inflammasome, which promotes the secretion of pro-inflammatory cytokines (Hajam *et al.* 2017).

4.3.4.3 Current hypothesis

Table 4.9 below outlines how packaging FimA and Flagellin into OMVs may be beneficial to the OMV-producing cell. If an *E. coli* cell is adhering to a surface to colonise and cause infection, releasing OMVs that contain an anti-inflammatory molecule to the host system would be beneficial. Similarly, if a cell is motile, releasing a trail of OMVs containing a pro-inflammatory molecule such as Flagellin may be beneficial to cause the immune response to react to the OMVs rather than the parent cell.

Table 4.9 Hypotheses of how packaging FimA and Flagellin monomers inti OMVs may be beneficial to the parent cell.

Cell Adhering	Cell Motile
Fimbriae expressed	Flagella expressed
OMVs enriched with monomeric FimA	OMVs enriched with monomeric Flagellin
FimA monomer is anti-inflammatory	Flagellin monomer is pro-inflammatory

Current hypothesis: FimA and Flagellin monomers have opposite effects on the hosts' immune response so it makes sense to package them in a mutually exclusive way into OMVs. This is discussed further in Chapter 6.1.5.

4.3.5 How can this finding be used for therapeutic purposes?

GFP was successfully targeted to *E. coli* K-12 OMVs by protein fusion to FimA. Table 4.10 below summarises the differences between the two methods of targeting a protein of interest to the OMVs using FimA.

Table 4.10 Comparison of targeting GFP/mNeon green to OMVs by fusion with FimA using two different methods

Characteristic	OMVs from *E.coli* MG1655 GFP-FimA fusion strain	OMVs from *E.coli* MG1655 with plasmid containing FimA-Neon green protein fusion
Was the FimA fusion protein successfully targeted to the OMVs?	Yes	No
Was the FimA fusion protein transported to the periplasm?	Yes	No
Was there hypervesiculation compared to OMVs produced in the WT strain?	No	Yes
Were plasmids used to express the FimA fusion protein of interest?	No	Yes
Was IPTG added in the media?	No	Yes
Was antibiotic added to the media?	No	Yes

The experiments showed that to target a protein of interest to *E. coli* K-12 OMVs, a chromosomal insert of the protein within the *fimA* gene is required. Expression of a FimA fusion protein by plasmid caused an unexpected hypervesiculation of the strain. Furthermore, the OMVs produced appeared to contain many proteins with no particular protein dominant. Both the appearance of the OMV protein profile and hypervesiculation resembled an *E. coli* B strain rather than a K-12 strain. This could be because plasmid expression puts the cell under stress and causes hypervesiculation as in the B strains and loss of specific protein inclusion/exclusion from the OMVs. This method would need to be optimised further if plasmid expression was to be used to target cargo to OMVs for therapeutic applications.

Chapter 5

Streptomyces S4 cells secrete Membrane Vesicles containing the antifungal compound Candicidin

5.1 Introduction

5.1.1 Comparison of OMVs (from Gram-negative bacteria) to MVs (Gram-positive bacteria)

Chapter 3 and 4 focussed on OMVs that are secreted by Gram-negative bacteria. As discussed previously, OMVs are formed from the bacterial outer membrane and contain a range of cargo including some periplasmic proteins. Gram-positive bacteria do not have an outer membrane (Figure 5.1**a**) and so do not produce *outer* membrane vesicles. Instead, the vesicles produced are thought to be composed of the cytoplasmic membrane and contain cytoplasmic proteins (Figure 5.1**b**). These will be referred to as Membrane Vesicles (MVs) instead of OMVs. For details on the hypotheses for the formation of Gram-positive MVs, see Section 1.8.3.

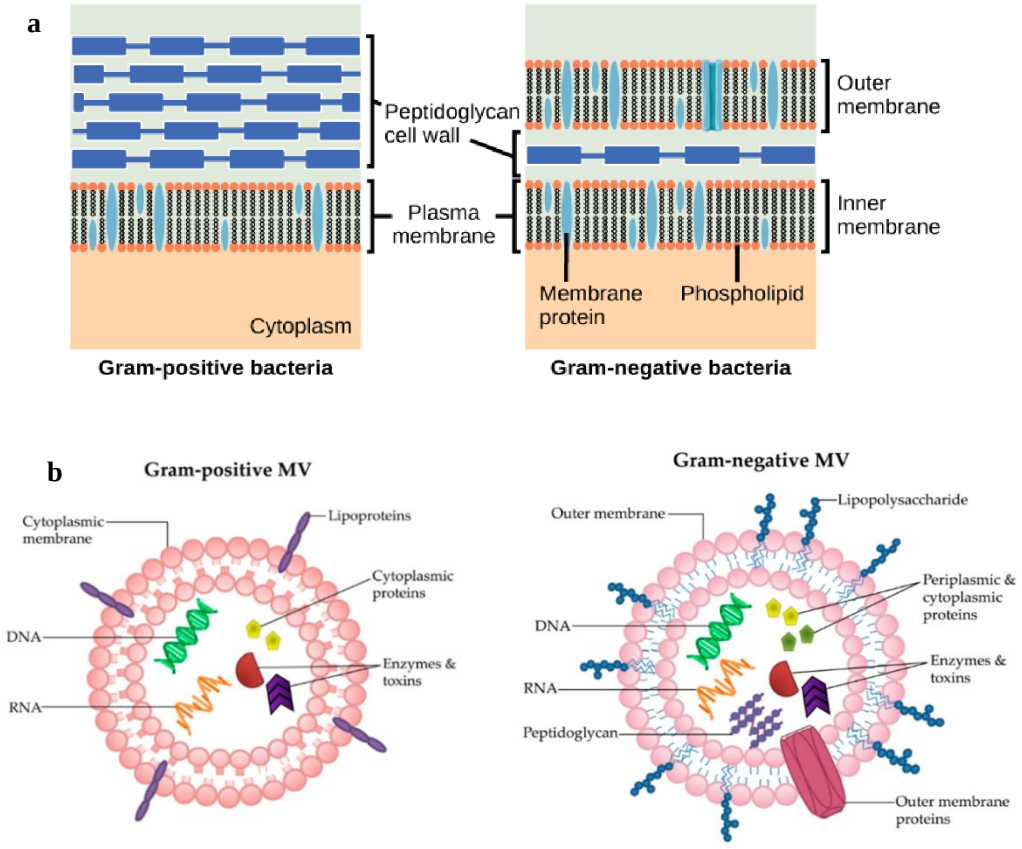


Figure 5.1 Comparison of membrane vesicles from Gram-positive and Gram-negative bacteria

Gram-positive and Gram-negative bacteria have different membrane compositions (**a**). Gram-negative bacteria produce OMVs which are formed from the Outer Membrane and contain some periplasmic proteins. MVs from Gram-positive bacteria are thought to be composed of the cytoplasmic membrane and contain cytoplasmic proteins (**b**). Image (**a**) was sourced from: http://simbac.gatech.edu/outer-membrane-proteins/. Image (**b**) sourced from: Bitto, *et al.* 2017.

5.1.2 *Streptomyces* S4 strains

As summarised in Chapter 1, membrane vesicles (MVs) have previously been purified from the Gram-positive strain *Streptomyces lividans* (Schrempf, Merling. 2015). The *Streptomyces S4* strain produces the antifungal compounds candicidin and eight antimycins (Seipke *et al.* 2011). Due to the known success of the delivery of amphotericin B within a liposome to fungal cells (Section 1.9.3), it led us to hypothesise if a similar process occurs in nature. This was tested by purifying membrane vesicles from *Streptomyces* S4 and we hypothesised that candicidin and/or antimycin is packaged and released in *Streptomyces* S4 MVs for enhanced uptake in the target cells. Professor Hutchings (UEA) kindly donated the following strains for study into MV production in *Streptomyces* S4 (Table 5.1 and Appendix D.1).

Table 5.1 Brief introduction to the *Streptomyces* S4 strains used in Chapter 5.

Strain name	Strain information	References
Streptomyces S4 wild type	The *Streptomyces* S4 strain was isolated from the attine ant species *A. octospinosus* in Panama. Attine ants cultivate fungus for food and have developed a symbiotic relationship with Actinobacteria to protect their cultured fungus from other microorganisms. *Streptomyces* S4 produces the antifungal compounds candicidin and eight antimycins which likely offer their food source protection against microfungal weeds.	Barke *et al.* 2010 Haeder *et al.* 2009 Seipke *et al.* 2011 Hopwood *et al.* 2012 McLean *et al.* 2016
Streptomyces S4 *ΔantC*	AntC encodes a nonribosomal peptide synthetase which is thought to play a role in the biosynthesis of antimycins. Deletion of the gene *antC* inhibits the synthesis of all eight antimycin compounds usually produced by *Streptomyces* S4. The antifungal compound candicidin, however, is still produced by this strain.	
Streptomyces S4 *ΔfscC*	In the *ΔfscC* strain, the polyketide synthase gene (*fscC*) is deleted. This encodes the candicidin biosynthetic module and disruption of this gene causes inhibition of candicidin production. In this strain, antimycin is still produced.	
Streptomyces S4 *ΔantCΔ fscC*	This double mutant strain does not produce candicidin or antimycins and has no antifungal activity against *C. albicans.*	

Membrane vesicles produced by Gram-positive bacteria (such as *Streptomyces*) is a new and emerging field with many unanswered questions. The standard OMV purification protocol was applied to *Streptomyces* S4 and the three mutant strains to see if MVs could be isolated.

5.1.3 Main chapter aims:

1. To compare and contrast MV composition, cargo and function from both Gram-negative and Gram-positive bacterial strains
2. To gain a deeper understanding into cross-species signalling using MVs
3. To gain insight into the function of any cargo discovered (i.e. why would this be beneficial *in vivo*?)
4. To develop and optimise new protocols for MV purification, characterisation and manipulation
5. To gain a fundamental understanding of vesiculation in Gram-positive bacteria.

6. To identify drug targets to prevent infection by MV-producing pathogens or use MVs in therapeutic applications such as drug delivery or vaccines.

5.2 Results

5.2.1 Visualisation of Membrane Vesicles (MVs) from *Streptomyces* S4 by TEM

The OMV purification protocol was applied to *Streptomyces* S4 WT and the three mutant strains to see if any MVs could be isolated (see Section 2.6.3 for further details). The resulting samples were concentrated then visualised using TEM and photos were taken at various magnifications (Figure 5.2). The TEM images indicated that MVs had been purified from all four *Streptomyces* S4 strains. The purified MVs were very similar in appearance to OMVs purified from *E. coli* and *P. aeruginosa* (Chapters 3-4) and MVs from *Streptomyces lividans* in the literature (Appendix D.2).

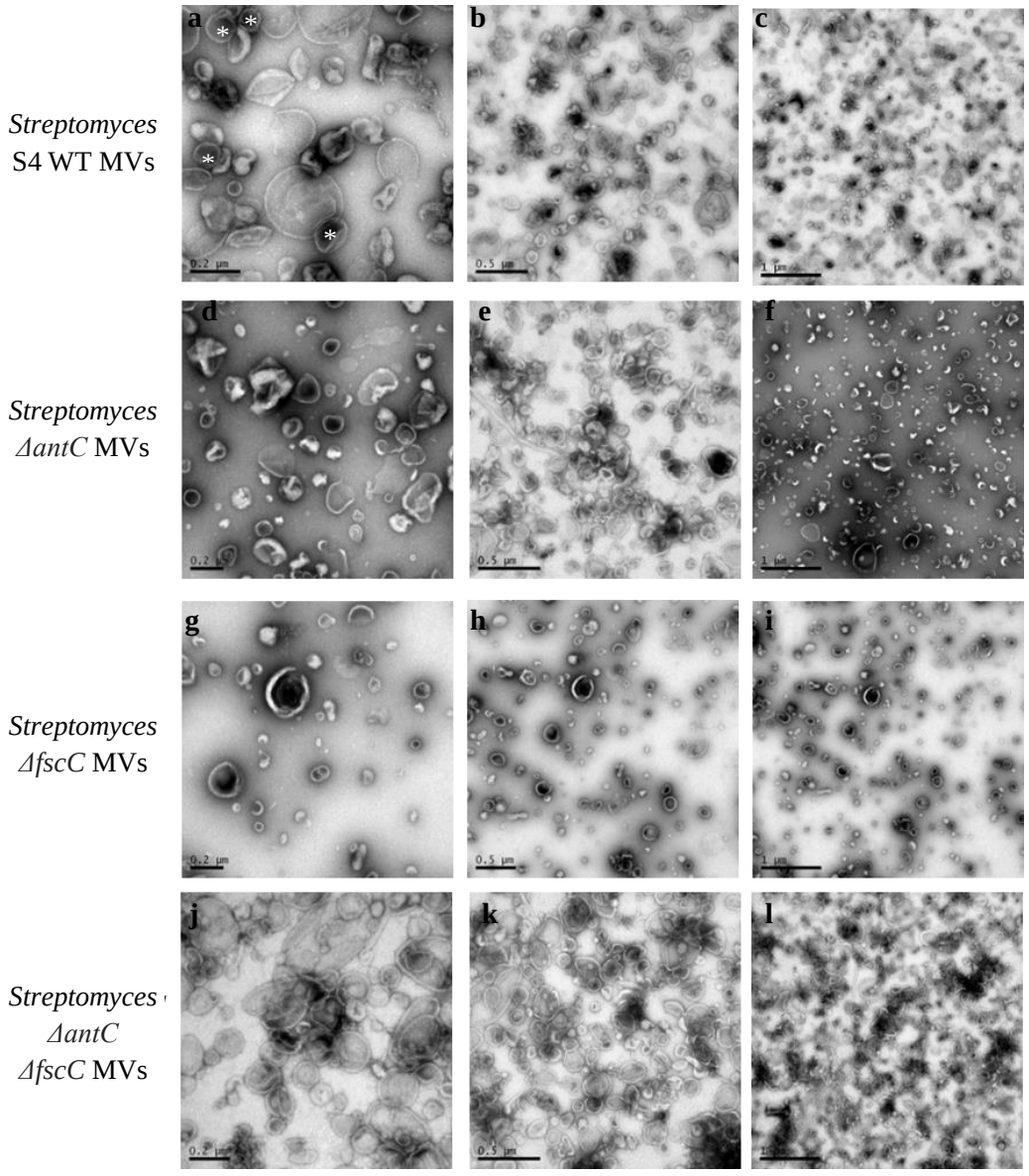
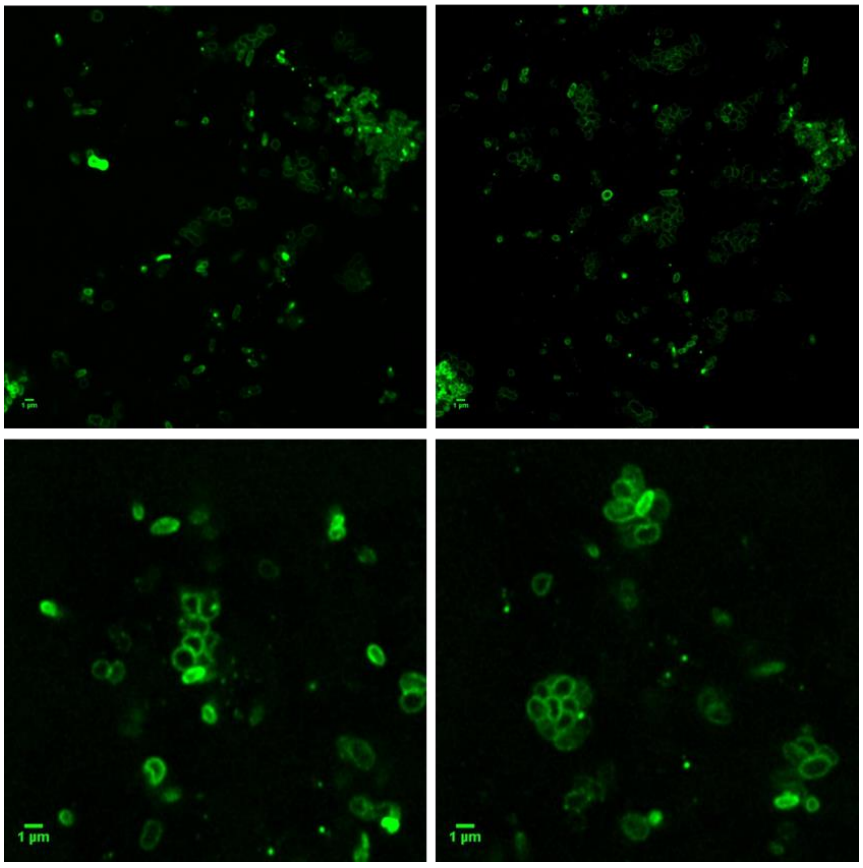


Figure 5.2. Visualisation of purified MVs from *Streptomyces* S4 strains using TEM TEM analysis of purified MVs from the following *Streptomyces* S4 strains: *Streptomyces* S4 WT **(a-c)**, *Streptomyces ΔantC* **(d-f)**, *Streptomyces ΔfscC* **(g-i)**, *Streptomyces ΔantC ΔfscC* **(j-l)**. White asterisks in image **a** indicate MVs.

5.2.2 Visualisation of *Streptomyces* S4 cells and MVs using WGA-FITC

Streptomyces S4 cells and MVs were visualised using WGA-FITC based on a protocol found in the literature (Celler *et al.* 2016). WGA refers to wheat germ agglutinin, a lectin from *Triticum vulgaris* (wheat) conjugated to the fluorescent conjugate fluorescein isothiocyanate (FITC). This product was developed for the fluorescent detection of glycoproteins containing β(1→4)-N-acetyl-D-glucosamine (Sigma-Aldrich L4895). This is found in the peptidoglycan layer of Gram-positive cell walls. In the literature, evidence suggests that Gram–positive MVs are formed from the cytoplasmic membrane without the peptidoglycan layer (see Figure 5.1). If this theory is correct, the WGA-FITC will bind successfully to the peptidoglycan on whole *Streptomyces* S4 cells but will not bind and fluoresce when applied to purified MVs. Figure 5.3 shows whole *Streptomyces* S4 cells treated with WGA-FITC. Before incubation with WGA-FITC, half of the cells were washed 3x in PBS to remove any MVs from around the cells (**a**). The other half of the cells remained unwashed in PBS and may still have MVs in the sample (**b**).

a *Streptomyces* S4 WT cells including PBS washes



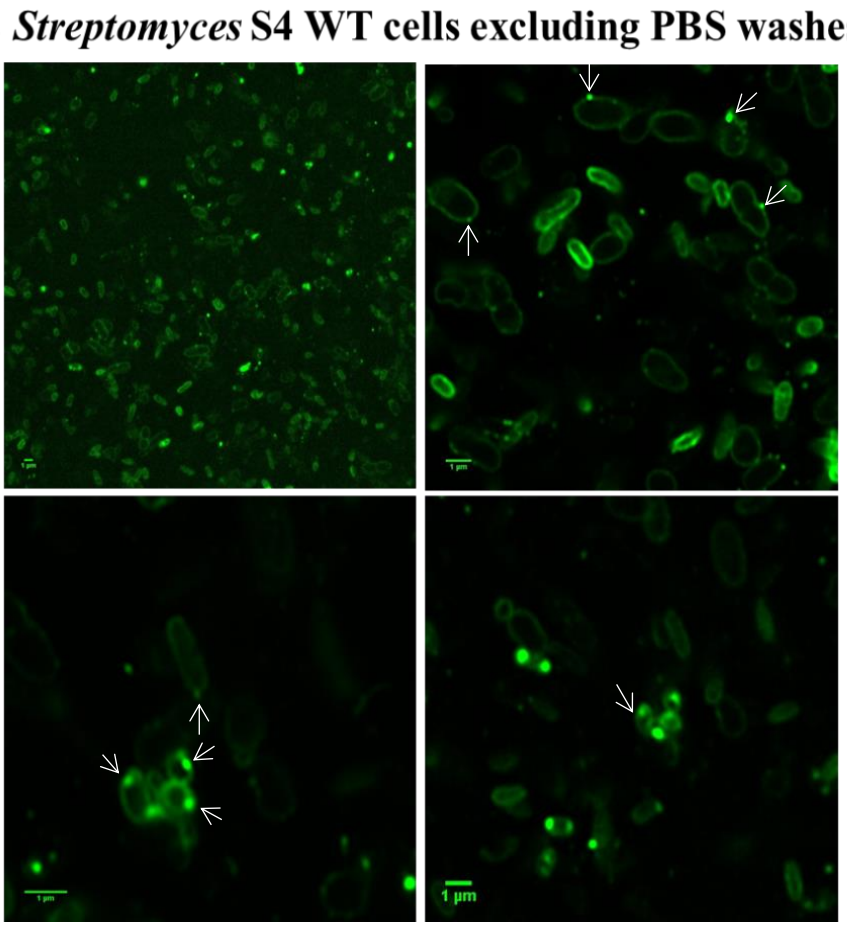


Figure 5.3. Visualisation of *Streptomyces* S4 cells and MVs using WGA-FITC.
Streptomyces S4 cells were grown for 48 hrs then concentrated by centrifugation at 13200 RPM. Half of the cells were washed 3x in PBS in an attempt to remove all MVs around the cells (**a**). The other half remained unwashed with PBS and may contain MVs (**b**). Cells were fixed in 2% (w/v) paraformaldehyde then incubated in 100 µg/mL WGA-FITC (Sigma L4895**)** in the dark for 1 hour. 15 µL cells were added onto a coverslip before being inverted onto a drop of ProLong Gold antifade mountant on a glass slide. Samples were visualised the next day by confocal microscopy (Zeiss lsm 880 with airscan) under the supervision of Matt Lee. Scale bar was added to images using Fiji (Image J). White arrows indicate potential MVs budding from the *Streptomyces* cells.

WGA-FITC successfully bound to the Gram-positive *Streptomyces* S4 cells so that they could be visualised. Furthermore, areas of intense fluorescence were found on the membranes of the cells which may be the sites of vesiculation (white arrows on Figure 5.3**b**). This appeared to be more present in the cells which had not been washed 3x with PBS (discussed further in Section 5.3.1).

Next, purified MVs from all four *Streptomyces* S4 stains were incubated with WGA-FITC (Figure 5.4). Any areas of fluorescence were very dispersed and difficult to find (Figure 5.4 **a-d**). This suggests that the MVs are composed of the cytoplasmic membrane only and do not contain peptidoglycan on their surface (Figure 5.4 **e**).

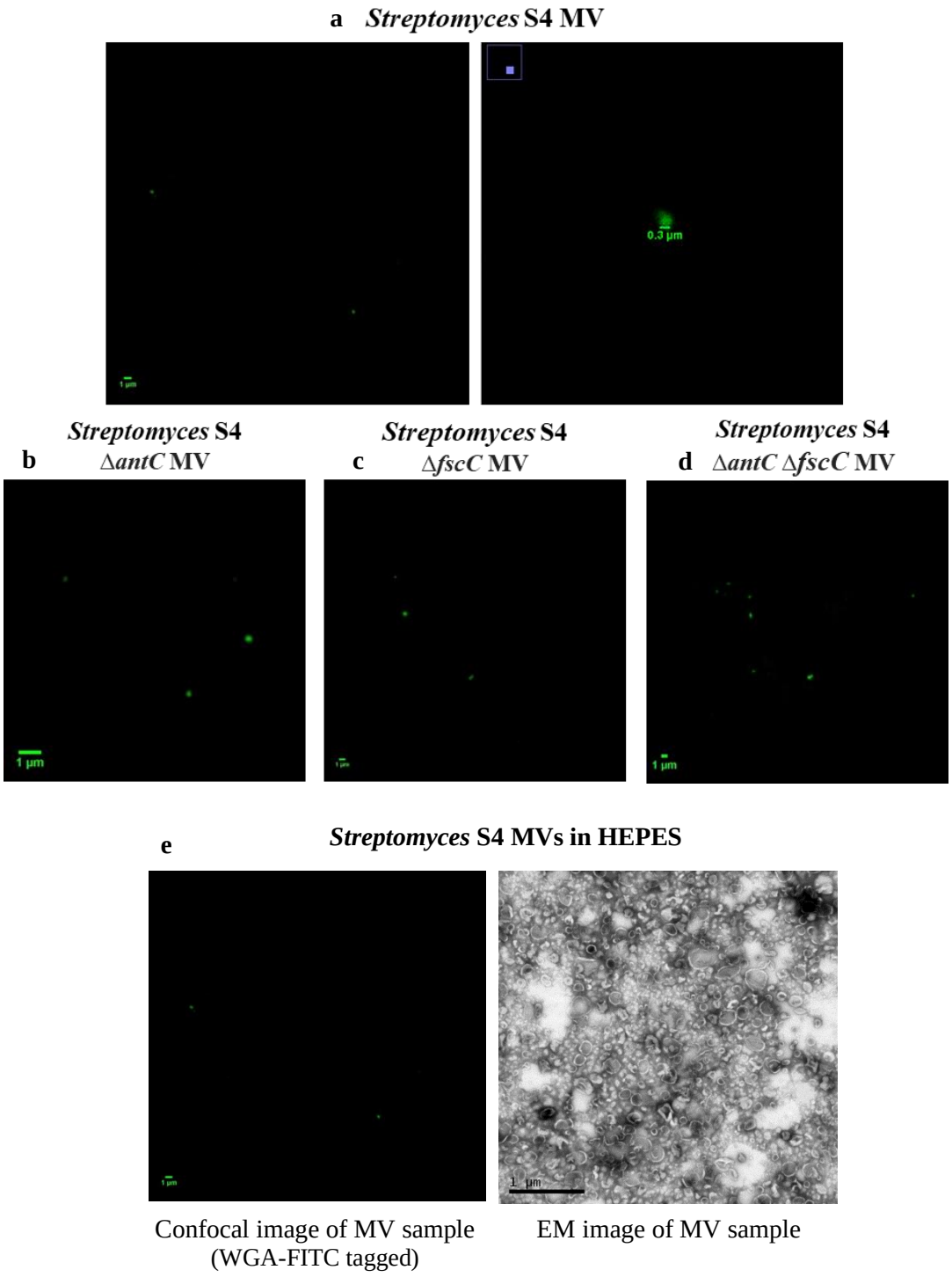


Figure 5.4. Visualisation of *Streptomyces* S4 MVs using WGA-FITC.

MVs were purified from the following strains: *Streptomyces* S4 WT (**a**), *ΔantC* (**b**), *ΔfscC* (**c**) and *ΔantC ΔfscC* (**d**). MVs were concentrated by centrifugation at 13200 RPM. MVs were fixed in 2% (w/v) paraformaldehyde then incubated in 100 µg/mL WGA-FITC (Sigma L4895**)** in the dark for 1 hour. 15 µL cells were added onto a coverslip before being inverted into a drop of ProLong Gold antifade mountant on a glass slide. Samples were visualised the next day by confocal microscopy (Zeiss lsm 880 with airscan) under the supervision of Matt Lee. Scale bar was added to images using Fiji (Image J). Lastly, the same *Streptomyces* S4 MV WT sample was concentrated by centrifugation 50x and the images of the sample taken by EM and confocal were compared (**e)**.

5.2.3 Characterisation of *Streptomyces* S4 MV proteome

5.2.3.1 Comparison of the *Streptomyces* S4 MV protein profiles

The protein profile of MVs purified from the four *Streptomyces* S4 strains was compared (Figure 5.5). The protein profile of MVs from *Streptomyces* S4 WT (lane 3), Δ*antC* (lane 5) and Δ*fscC* (lane 7) appear the same. However, the protein profile of Δ*antC* Δ*fscC* appears to contain extra proteins (lane 9).

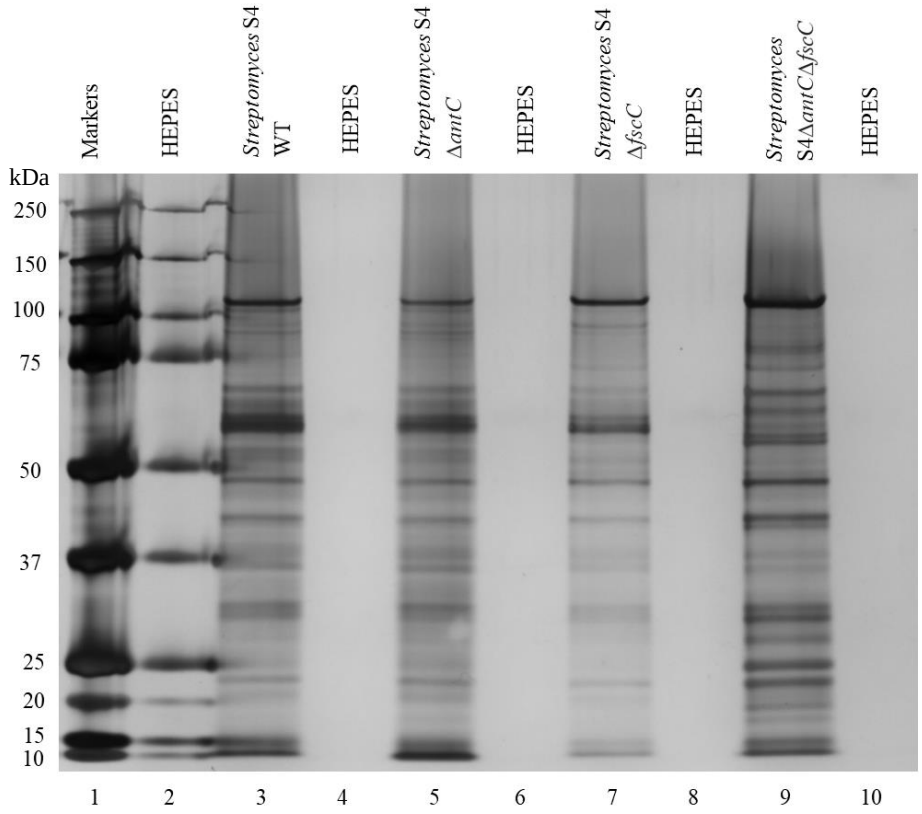


Figure 5.5 Comparison of MV protein profile from *Streptomyces* S4 and the 3 mutant strains
MVs were purified from four different *Streptomyces* S4 strains: WT, Δ*antC,* Δ*fscC* and Δ*antC* Δ*fscC*. A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the MV protein profile.

5.2.3.2 Densitometry comparison

Protein densitometry plots were generated using Fiji (Image J) to compare the protein profile of the MVs from each strain (Figure 5.6). The densitometry plots indicated that the MVs purified from the double mutant did have extra proteins compared with MVs from the other 3 strains. Perhaps the regulation of which proteins enter the MVs was disrupted when the cell no longer produces the antifungals antimycin or candicidin. However, the exact reason for this change is unclear.

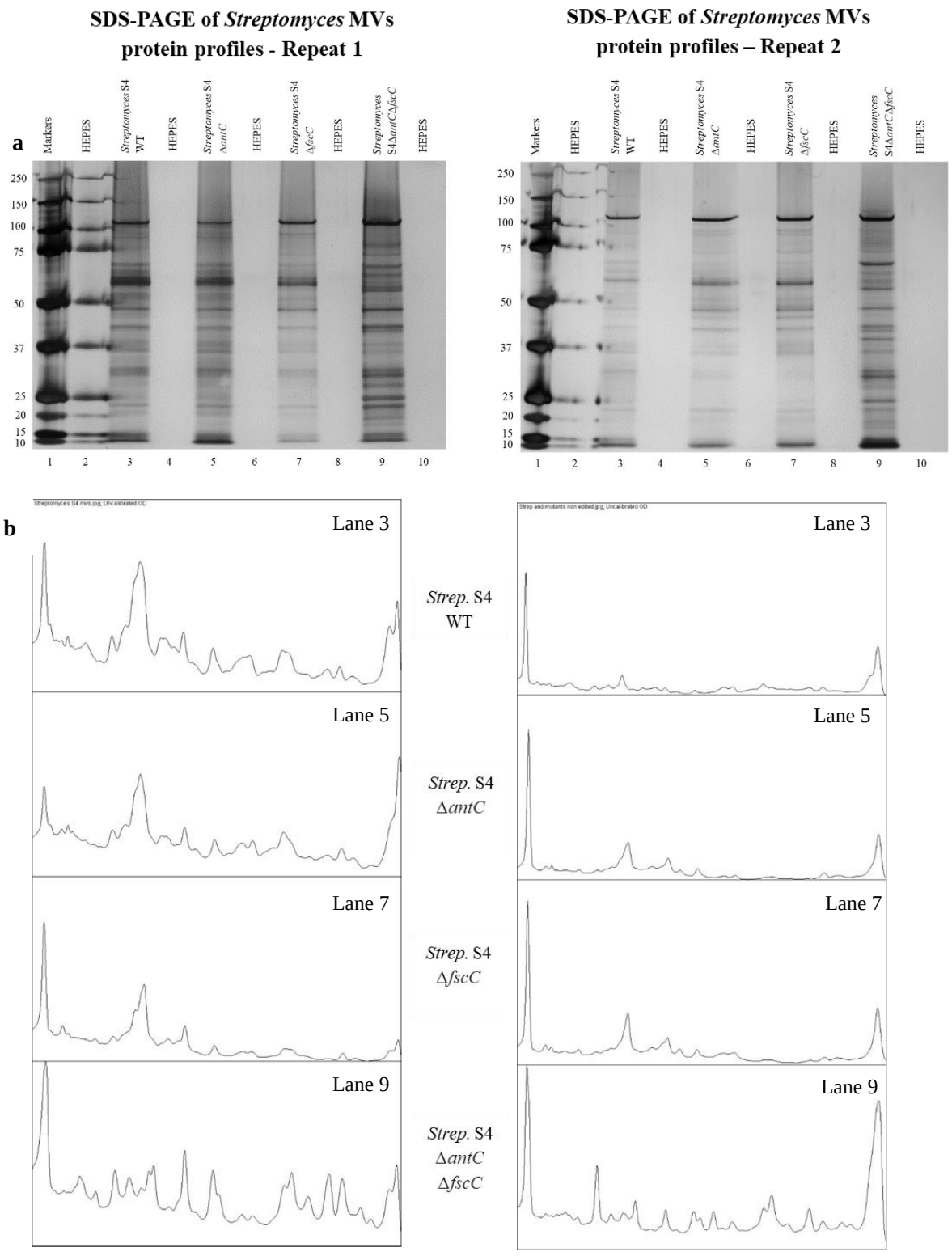


Figure 5.6 Densitometry comparison of MV protein profile from *Streptomyces* S4 and the 3 mutant strains

MVs were purified from four different *Streptomyces* S4 strains: WT, Δ*antC,* Δ*fscC* and Δ*antC* Δ*fscC.* A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the MV protein profile (**a**). Protein densitometry plots were generated from the SDS-PAGE gel photos using Fiji (Image J) (**b**).

5.2.3.3 Protein profile of *Streptomyces S4* MVs compared with the whole cell

The *Streptomyces* S4 MV protein profile was compared to the whole cell to see which proteins are enriched and excluded from *Streptomyces* S4 MVs (Figure 5.7). One band in particular appears to be enriched in *Streptomyces* S4 MVs compared to the levels in the cell (labelled with a blue arrow). This protein was later extracted and identified by mass spectrometry to be a serine protease (see Section 5.2.4).

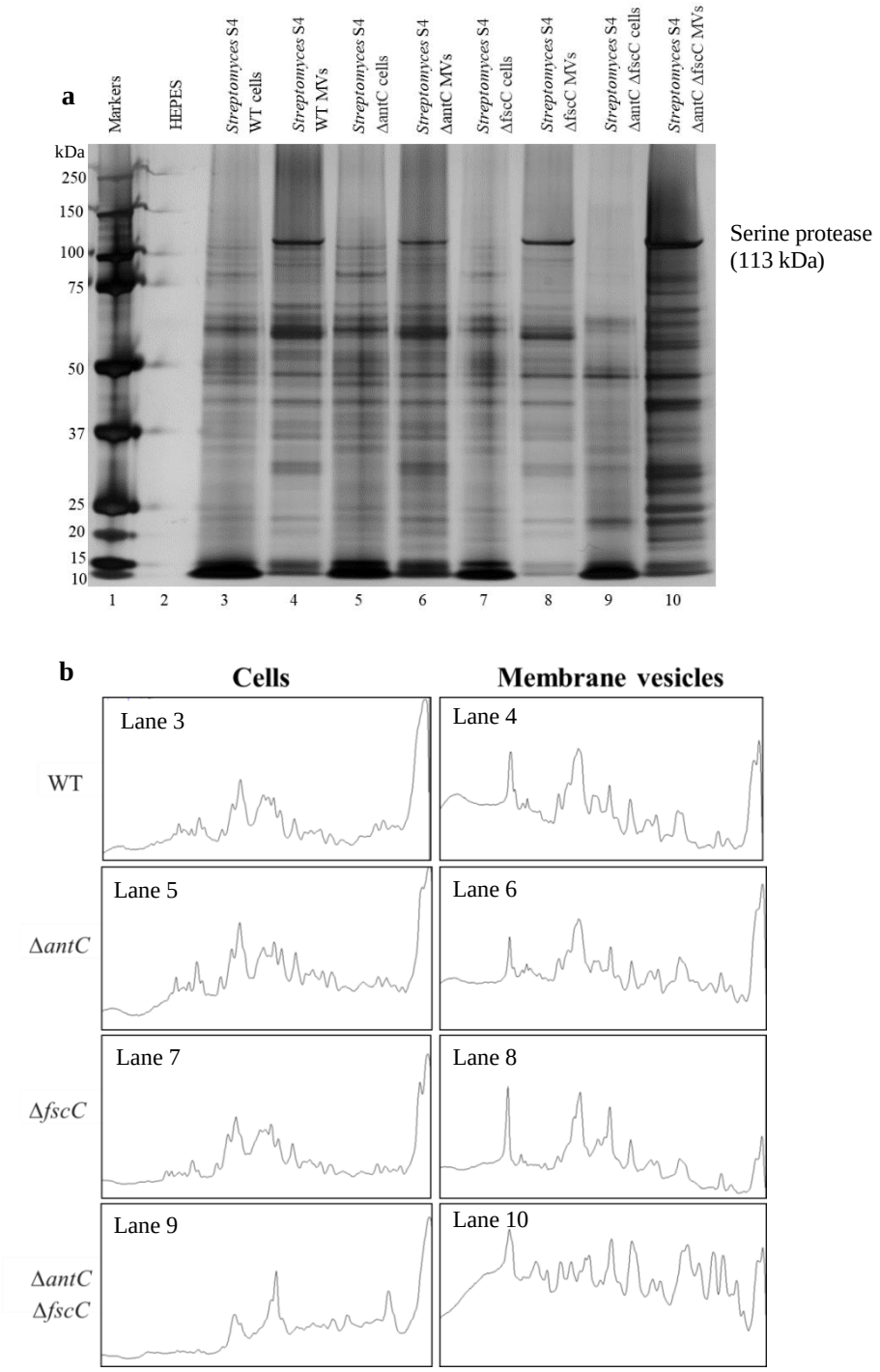
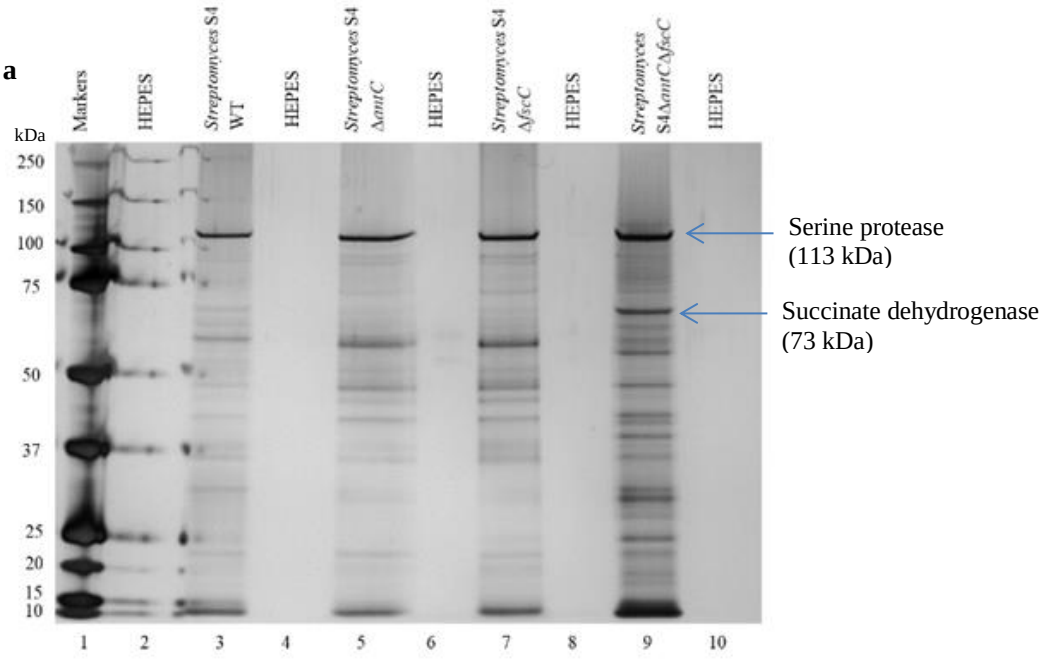


Figure 5.7 Comparison of MV protein profile from *Streptomyces* S4 and the 3 mutant strains
MVs were purified from four different *Streptomyces* S4 strains: WT, Δ*antC*, Δ*fscC* and Δ*antC* Δ*fscC* and were compared to the whole cell samples. A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the MV protein profile (**a**). Protein densitometry plots were generated from the SDS-PAGE gel photos using Fiji (Image J) (**b**).

5.2.4 Do *Streptomyces* S4 MVs contain proteases?

Two bands of interest in *Streptomyces* S4 MVs were extracted and identified by mass spectrometry (Figure 5.8). The mass spectrometry results indicate that all *Streptomyces* S4 MV samples contain a serine protease and that the Δ*antC* Δ*fscC* strain MVs contain a fumarate reductase/succinate dehydrogenase flavoprotein subunit.

a



b

Approx. MW on gel (kDa)	Protein detected (using NCBIprot database)	Score (score needed to be significant)	Protein MW (kDa)	Protein reference code for NCBI Protein database
65-72	Fumarate reductase/succinate dehydrogenase flavoprotein subunit [*Streptomyces* sp. S4].	126 (92)	72.819	WP_010639587.1
110-120	Serine protease [*Streptomyces* sp. FR-008]	183 (92)	113.459	ALM41672.1

Figure 5.8 Comparison of MV protein profile from *Streptomyces* S4 and the 3 mutant strains
MVs were purified from four different *Streptomyces* S4 strains: WT, Δ*antC*, Δ*fscC* and Δ*antC* Δ*fscC*. A Bradford assay was performed and all samples were standardised to be the same protein concentration. TCA precipitation was used to concentrate samples prior to loading on an SDS-PAGE gel. The SDS-PAGE gel was run then silver stained to visualise the MV protein profile (**a**). The two proteins labelled were extracted and identified by mass spectrometry (**b**).

5.2.4.1 Release of proteases from *Streptomyces* S4 MVs when SDS is added

In Section 3.2.5, *E. coli* OMVs were incubated with SDS, which appeared to cause disruption to the membrane of the OMVs and release of active proteases from within. These proteases degraded some of the OMV-associated proteins (and flagella) and changed the OMV protein profile. This method was applied to purified *Streptomyces* S4 WT MVs to see if they contain active proteases (Figure 5.9). *Streptomyces* S4 WT MVs were purified and resuspended in 10 mM HEPES buffer. Half of the purified MVs were resuspended in 10 mM HEPES buffer only (Lanes 3-6). The other half were resuspended in 10 mM HEPES buffer then filtered through a 0.22 µm filter (Lanes 7-10). Many of the bands disappeared when various concentrations of SDS were added (Lanes 4-6 compared to Lane 3 and Lanes 8-10 compared to Lane 7). This indicates that proteases are present which are released when MVs are disrupted by SDS. One of these proteases could be the serine protease detected by mass spectrometry.

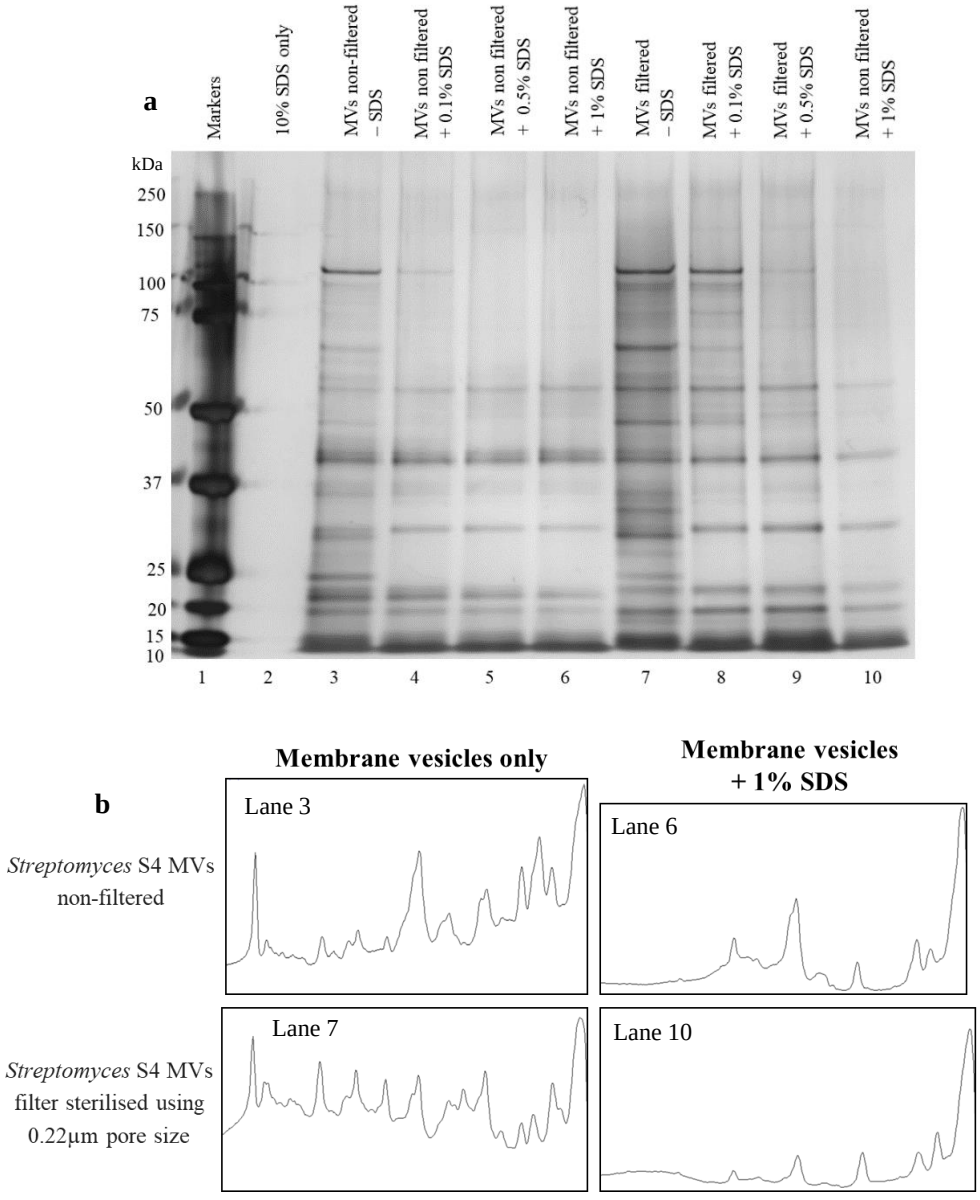


Figure 5.9 Do *Streptomyces* S4 MVs contain proteases?
Streptomyces S4 WT MVs were incubated with various concentrations of SDS at 37 ºC for 60 mins. The samples were run on an SDS-PAGE gel then silver stained to visualise the MV protein profile (**a**). Protein densitometry plots were generated from the SDS-PAGE gel photos using Fiji (Image J) (**b**).

5.2.5 Are any antifungal agents present within the purified *Streptomyces* S4 MVs?

5.2.5.1 Positive control: Candicidin

In order to determine if *Streptomyces* S4 MVs contained any antifungal compounds, a method to observe the effect on the growth of *Candida albicans* was developed. This was initially trialled with various concentrations of candicidin (purchased from Bio-Australis) and antimycin (purchased from Sigma-Aldrich). Candicidin was dissolved in absolute ethanol and diluted to give various concentrations of candicidin ranging from 200 µg/mL to 1 µg/mL. 10 µL was spotted on to a LB agar plate and left to soak in. This was then overlayed with *C. albicans* mixed in with LB 0.5% (w/v) agar and left to set and the diameter of the zones of inhibition (ZOI) were measured the next day (Figure 5.10). The ZOI are defined as the areas where *C. albicans* growth is inhibited and the agar is transparent and clear.

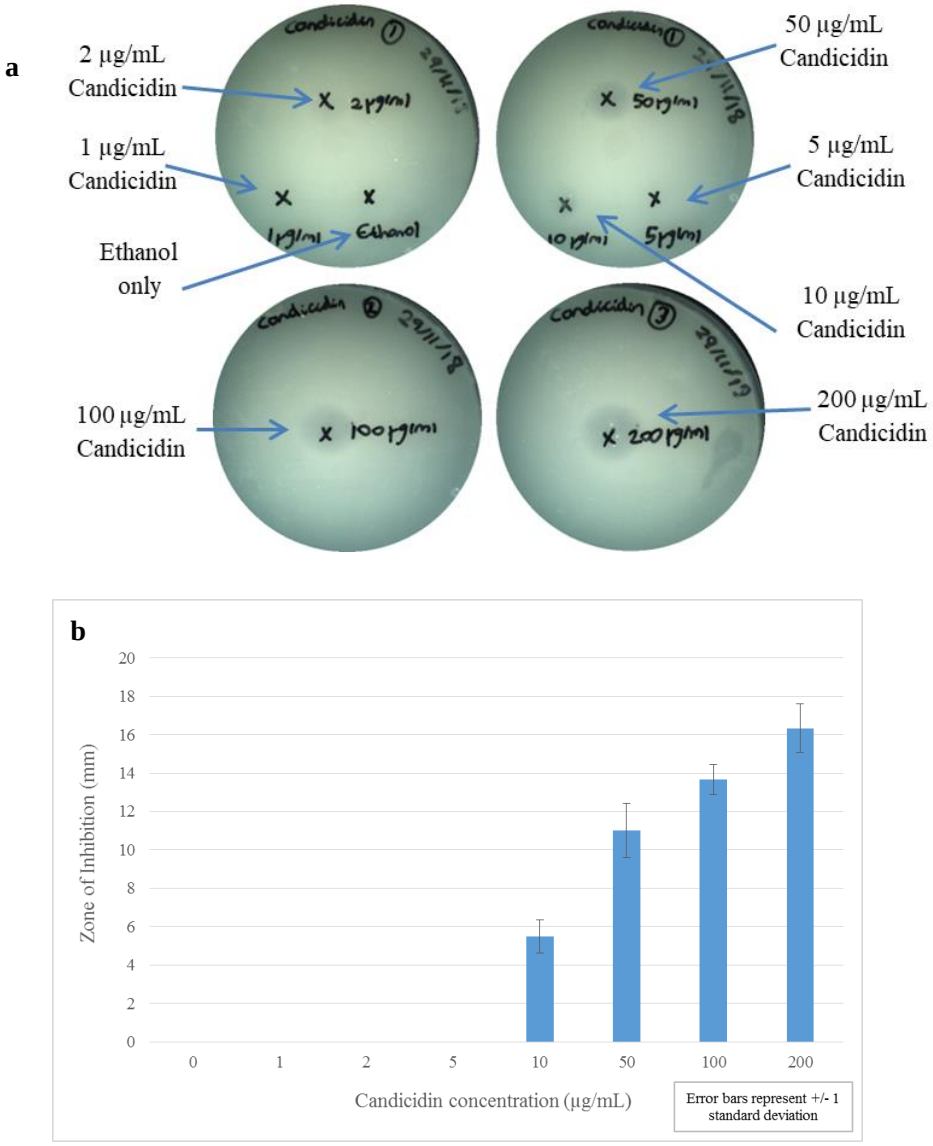


Figure 5.10 Effect of varying concentrations of candicidin on *C. albicans*
Candicidin was resuspended in ethanol then diluted to give various concentrations ranging from 200 µg/mL-1 µg/mL. 10 µL candicidin was added to LB plates and left to soak/dry for a minimum of 2 hrs at room temperature. 10 µL ethanol only was used as a negative control. This was then overlayed with *C. albicans* mixed in with LB 0.5% (w/v) agar and left to set. Plates were incubated at 37 ºC for 18 hrs and the diameter of the ZOI was measured (**a**). All plates were prepared in triplicate and the average ZOI was calculated and presented as a graph (**b**).

5.2.5.2 Positive control: Antimycin

The experiment above was repeated with antimycin purchased from Sigma-Aldrich (Figure 5.11).

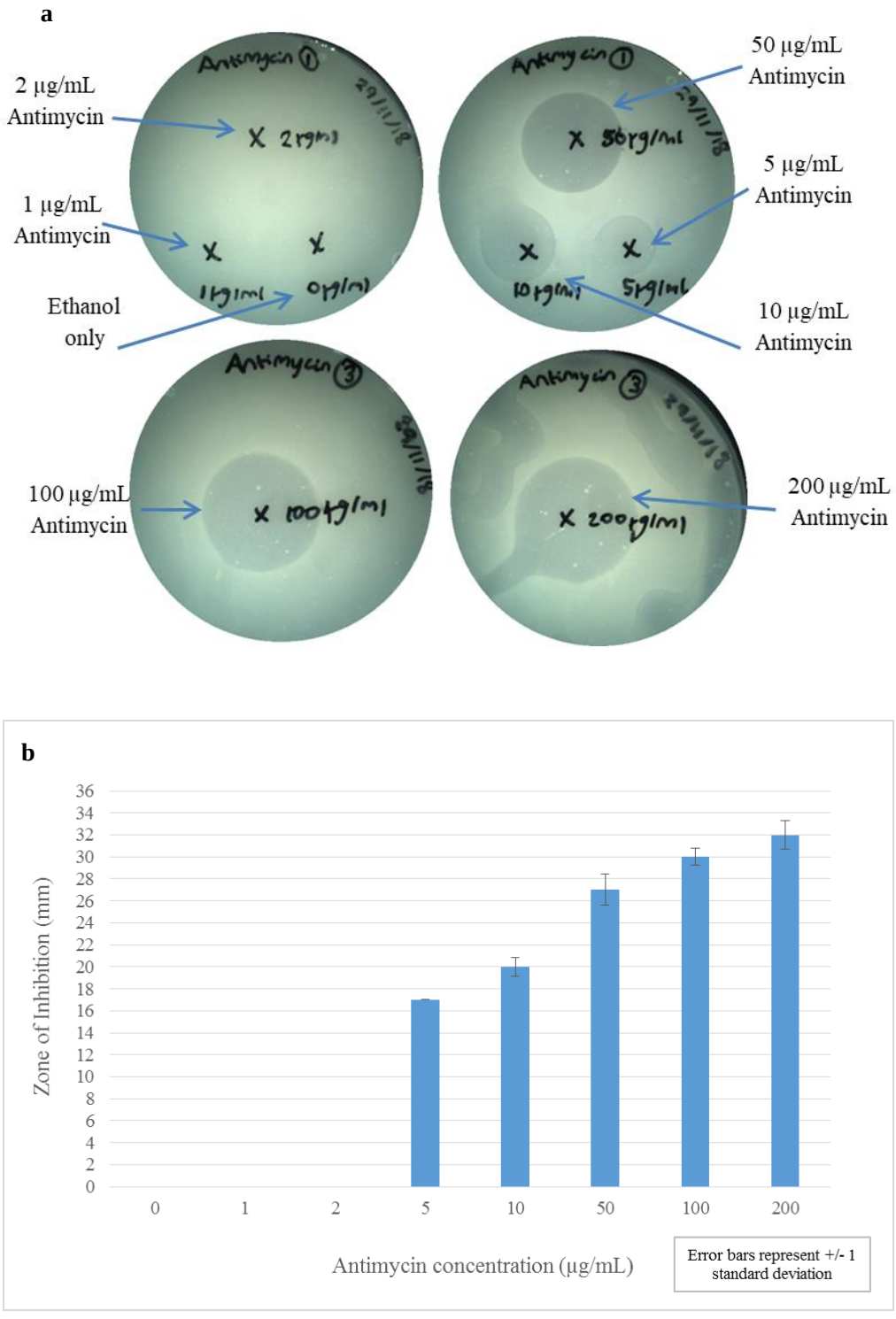


Figure 5.11 Effect of varying concentrations of antimycin on *C. albicans*
Antimycin was resuspended in ethanol then diluted to give various concentrations ranging from 200 µg/mL-1 µg/mL. 10 µL antimycin was added to LB plates and left to soak/dry for a minimum of 2 hrs at room temperature. 10 µL ethanol only was used as a negative control. This was then overlayed with *C. albicans* mixed in with LB 0.5% (w/v) agar and left to set. Plates were incubated at 37 ºC for 18 hrs and the diameter of the ZOI was measured (**a**). All plates were prepared in triplicate and the average ZOI was calculated and presented as a graph (**b**).

5.2.5.3 Effect of *Streptomyces* S4 cells on *C. albicans* growth

This method was then trialled by observing the ZOI caused by the four *Streptomyces* S4 strains overlayed with *C. albicans*. As expected, ZOI were produced from *Streptomyces* S4 WT strain, Δ*antC* and Δ*fscC* but not from the Δ*antC* Δ*fscC* strain (Figure 5.12).

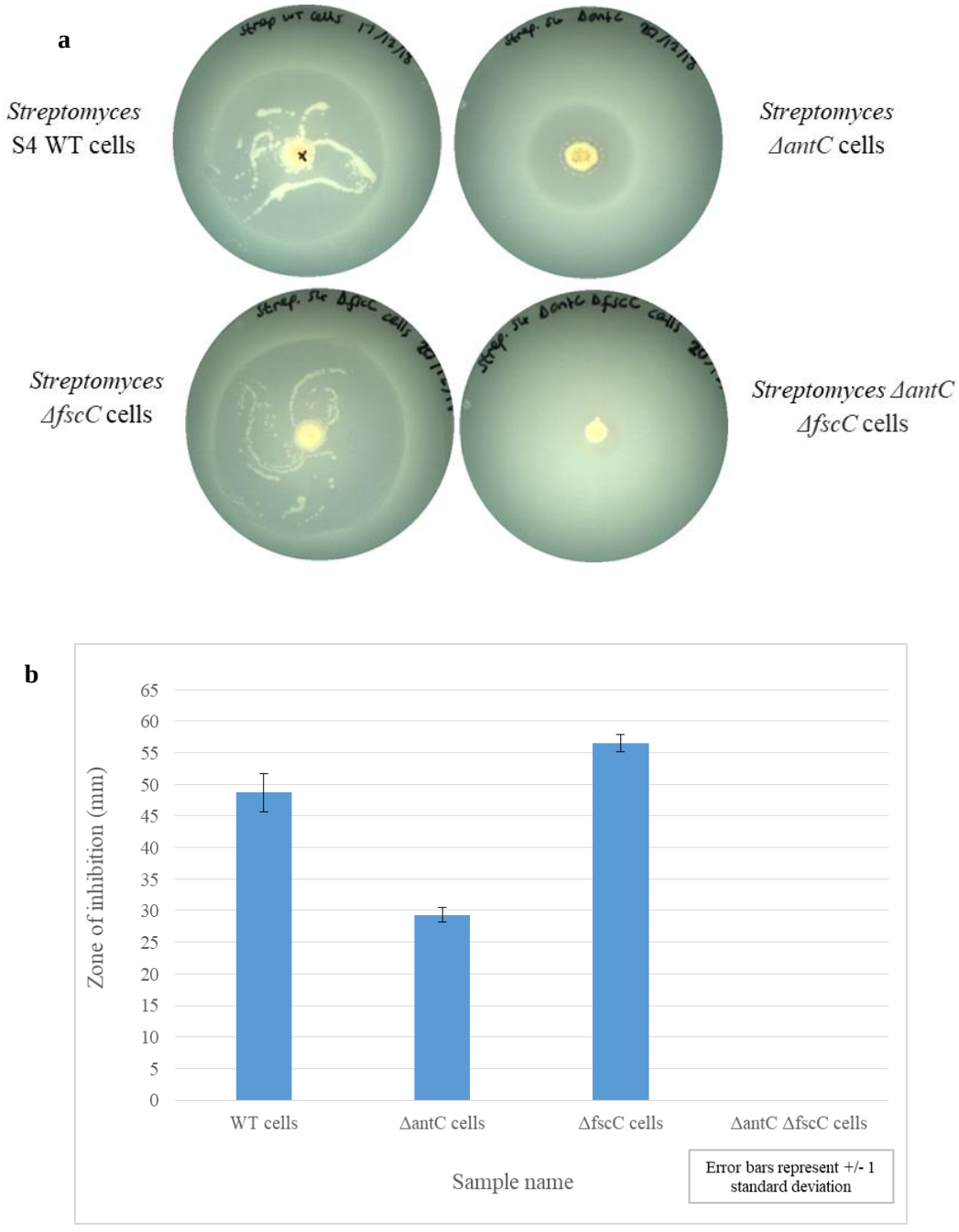


Figure 5.12. Effect of *Streptomyces* S4 cells on *C. albicans* growth
Streptomyces S4 colonies (from MS plates) were inoculated into TSB:YEME (two media mixed 1:1) grown up for 72 hrs. 5 µL of this culture was spotted at the centre of a LB agar plate and left to soak/dry for 2 hrs. Plates were incubated at 30ºC for 72 hrs to allow growth of *Streptomyces*. *C. albicans* mixed with LB 0.5% agar was overlayed on to the plates and left to set. Plates were incubated at 37ºC for 18 hrs and the diameter of the ZOI was measured (**a**). All plates were prepared in triplicate and the average ZOI was calculated and presented as a graph (**b)**.

5.2.5.4 Effect of *Streptomyces* S4 MVs on *C. albicans* growth

Purified MVs from the four *Streptomyces* S4 strains were concentrated and added to a LB agar plate and left at room temperature to dry/soak in. This was then overlayed with *C. albicans* mixed in with LB 0.5% (w/v) agar and left to set (see Section 2.13.1 for full protocol). Plates were incubated at 37ºC for 18 hours and the diameter of the ZOI measured using a ruler. The aim of this was to determine if the antifungal compounds candicidin or antimycin were present within any of the purified MVs by measuring the ZOI produced (Figure 5.13**a**). Each experiment was done in triplicate and the zones of inhibition were measured in mm. The findings were summarised as a bar chart for comparison (Figure 5.13**b**).

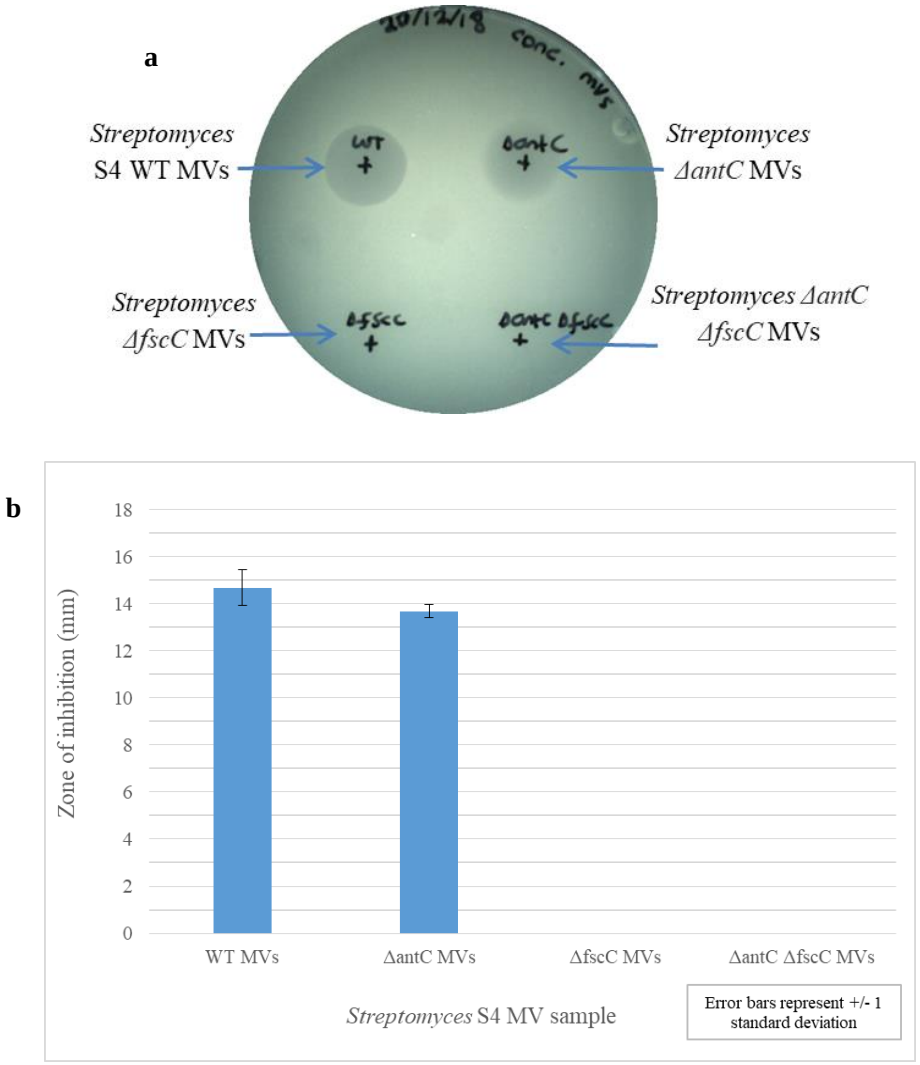


Figure 5.13 Do *Streptomyces* S4 MVs contain antifungals?
5 µL concentrated MVs (in 10 mM HEPES buffer) were added to LB plates and left to soak/dry for at room temperature. *C. albicans* mixed with LB 0.5% (w/v) agar was overlayed on to the plates and left to set. Plates were incubated at 37ºC for 18 hrs and the diameter of the ZOI was measured (**a**). All plates were prepared in triplicate and the average ZOI was calculated and presented as a graph (**b**).

The results of Figure 5.13 suggest that *Streptomyces* S4 MVs contain the antifungal compound candicidin and not antimycin (see Table 5.2) which is a novel finding.

Table 5.2 Interpretation of results from Figure 5.13 indicating that *Streptomyces* S4 MVs contain candicidin

Strain purified MVs originated from	Is a zone of inhibition produced with *C. albicans*?	Explanation for the result
Streptomyces S4 wild-type	✓	The *Streptomyces* S4 strain produces both of the antifungal compounds candicidin and antimycin. The MVs must contain at least one of these compounds as there's a zone of inhibition against *C. albicans.*
Streptomyces S4 *ΔantC*	✓	The *Streptomyces ΔantC* strain produces candicidin but not antimycin as its production is inhibited. There is a zone of inhibition produced so the MVs must contain candicidin.
Streptomyces S4 *ΔfscC*	✗	The *Streptomyces ΔfscC* strain produces antimycin but not candicidin. The MVs must not contain antimycin as otherwise there would be a zone of inhibition against *C. albicans.*
Streptomyces S4 *ΔantCΔ fscC*	✗	The *Streptomyces ΔantCΔ fscC* strain does not produce candicidin or antimycin so as expected, the MVs have no zone of inhibition against *C. albicans.*

5.2.5.5 Further comparison of MVs from all *Streptomyces* S4 strains

To explore this further, *Streptomyces* S4 MVs were treated in 3 different ways to observe the effect on the ZOI. *Streptomyces* MV pellets were resuspended in 5 mL 10 mM HEPES buffer and filter sterilised as part of the OMV purification procedure. 5 µL of this sample was added to LB agar plates labelled as the 'MV 1x concentration' sample (Figure 5.14 **a**). The MVs were then concentrated 25x by pelleting the MVs and resuspending in a smaller volume of 10 mM HEPES buffer to give an 'MV 25x concentrated' sample (**b**). Lastly, the MVs were pelleted and washed 3x in 10 mM HEPES buffer to remove anything that is not MV-associated (**c**).

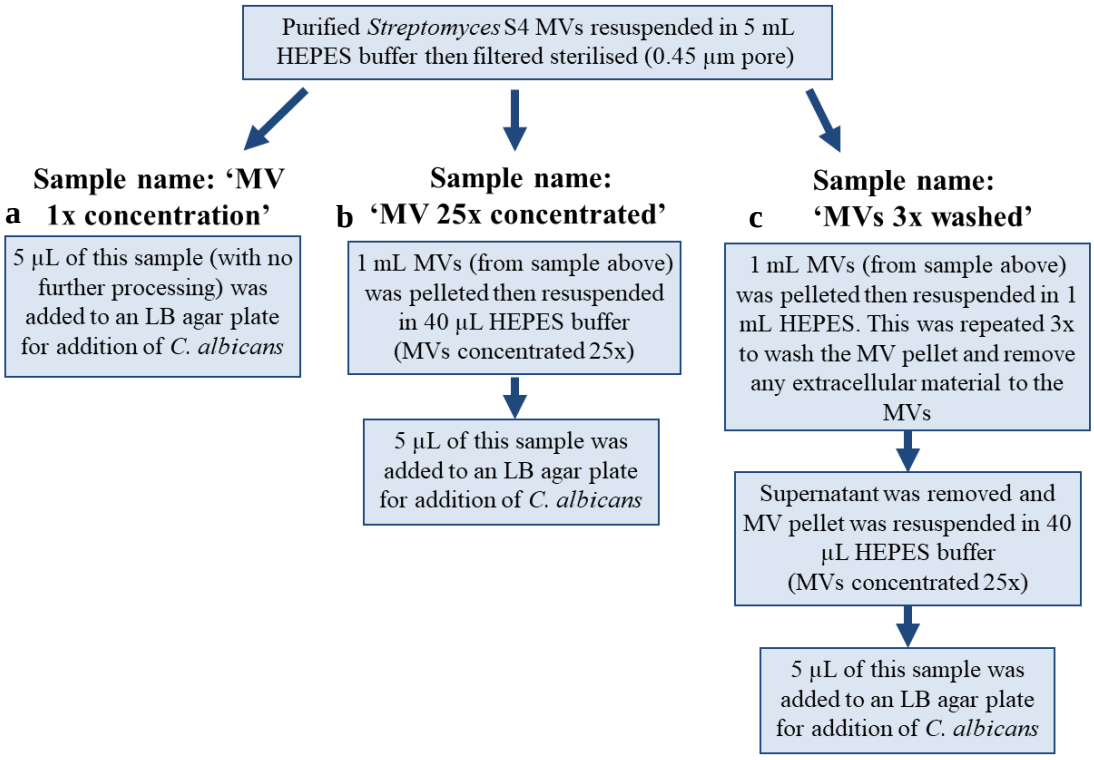


Figure 5.14 Preparation of MVs for zone of inhibition plates with *C. albicans* *Streptomyces* S4 MVs were treated in 3 different ways to observe the effect on the ZOI. The preparation of the following samples was summarised: 'MV 1x concentration' (**a**), 'MV 25x concentrated' (**b**) and 'MVs 3x washed' (**c**).

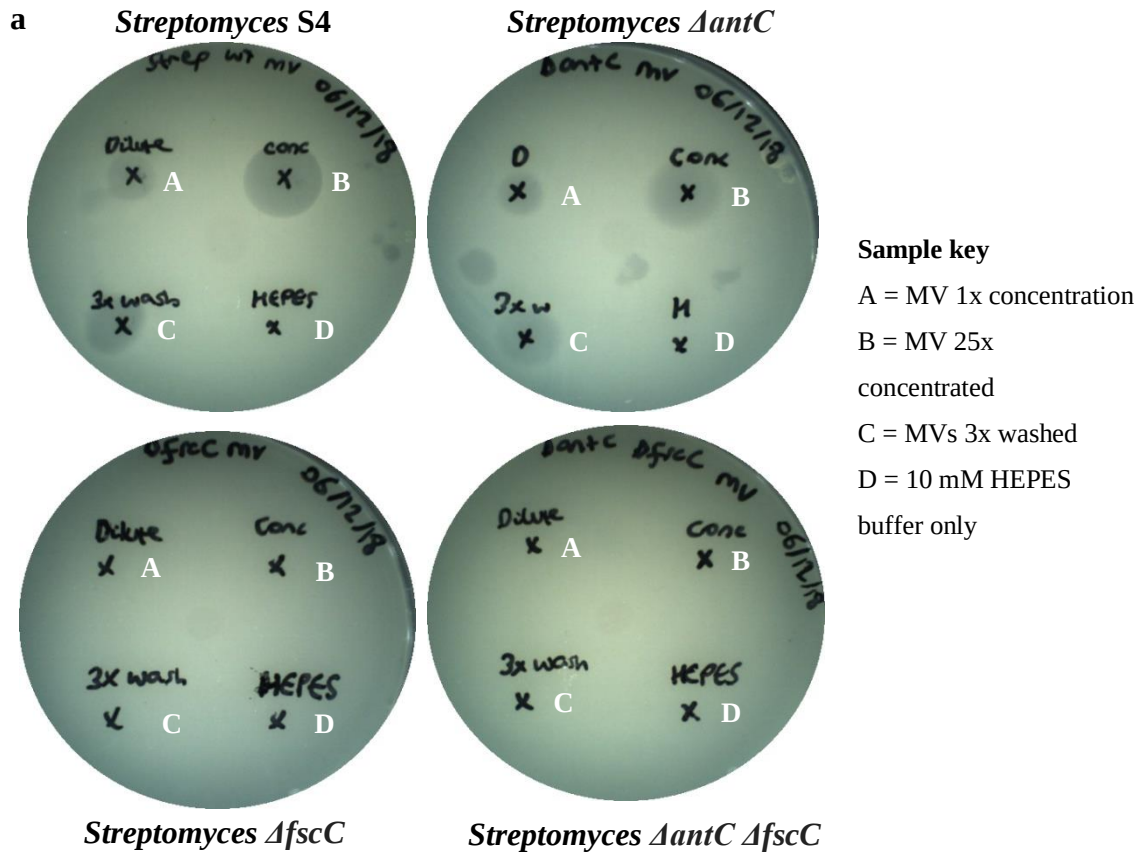


Figure 5.15 Do *Streptomyces* S4 MVs contain antifungals?
Streptomyces S4 MVs were treated in 3 different ways to observe the effect on the ZOI. 5 µL of the following MV samples were added to the LB plates: 'MV 1x concentration', 'MV 25x concentrated' and 'MVs 3x washed'. *C. albicans* mixed with LB 0.5% (w/v) agar was overlayed on to the plates and left to set. Plates were incubated at 37ºC for 18 hrs and the diameter of the ZOI was measured (**a**). All plates were prepared in triplicate and the average ZOI was calculated and presented as a graph (**b**).

The results from Figure 5.15 still indicate that the *Streptomyces* S4 WT MVs contain candicidin. Although the MVs are concentrated 25x, the ZOI produced is only slightly larger for the 'MV 25x concentrated' sample compared to the 'MV 1x concentration' sample and is not proportional (discussed in Section 5.3.3). Lastly, the MV pellets were also washed 3x with 10 mM HEPES buffer to remove any non-OMV associated contaminants and the ZOI remained. This is evidence that the candicidin is MV-associated and this was explored further.

5.2.5.6 Is Candicidin MV-associated?

In order to find further evidence that candicidin is MV-associated, *Streptomyces* S4 MVs were washed 1x, 2x and 3x with 10 mM HEPES buffer. This was to remove any extracellular material from around the MVs so that any antifungal activity observed was from MVs only. Figure 5.16 summarises how the wash steps were performed and the procedure used to generate each sample and Figure 5.17 shows the results.

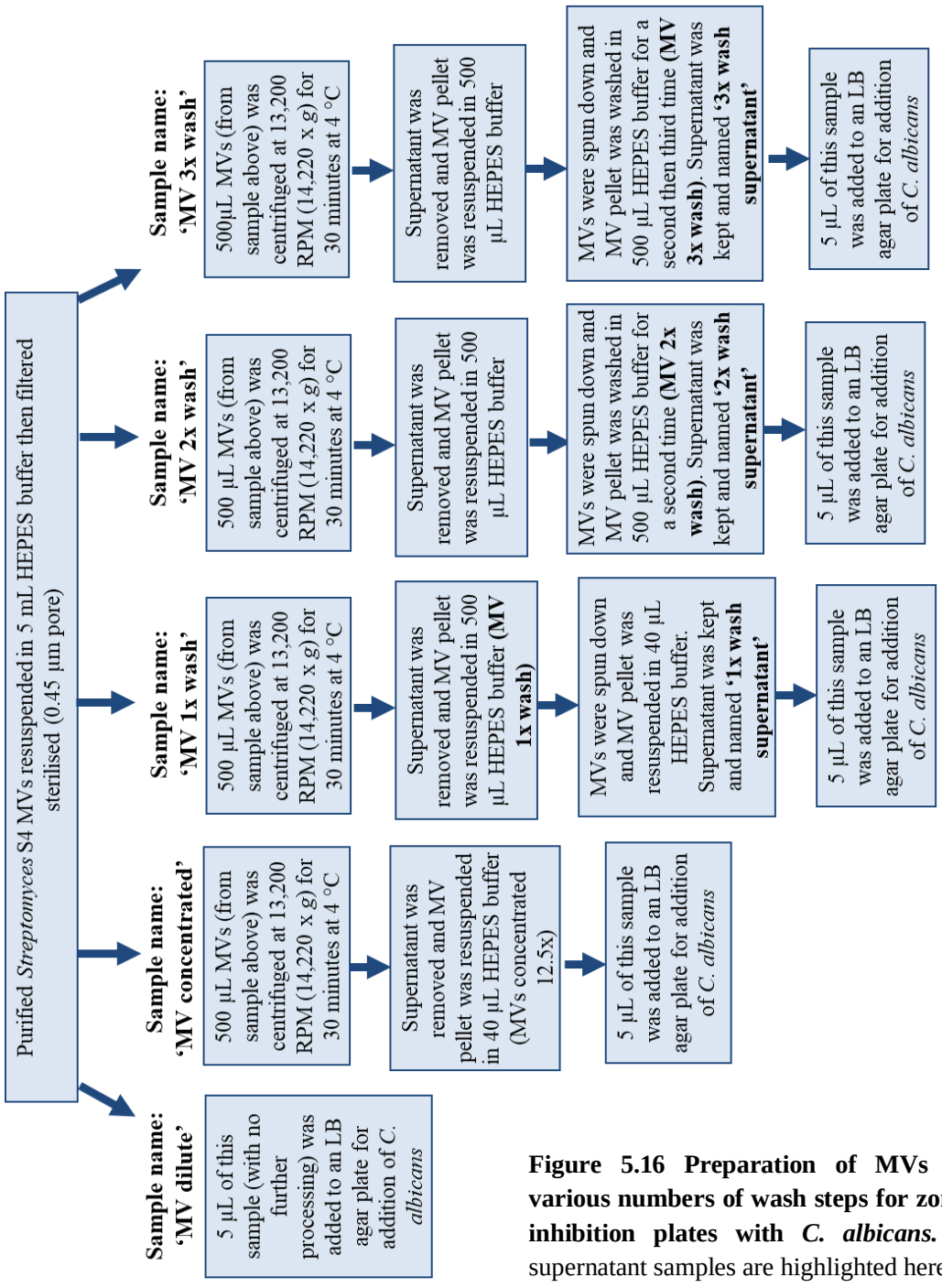
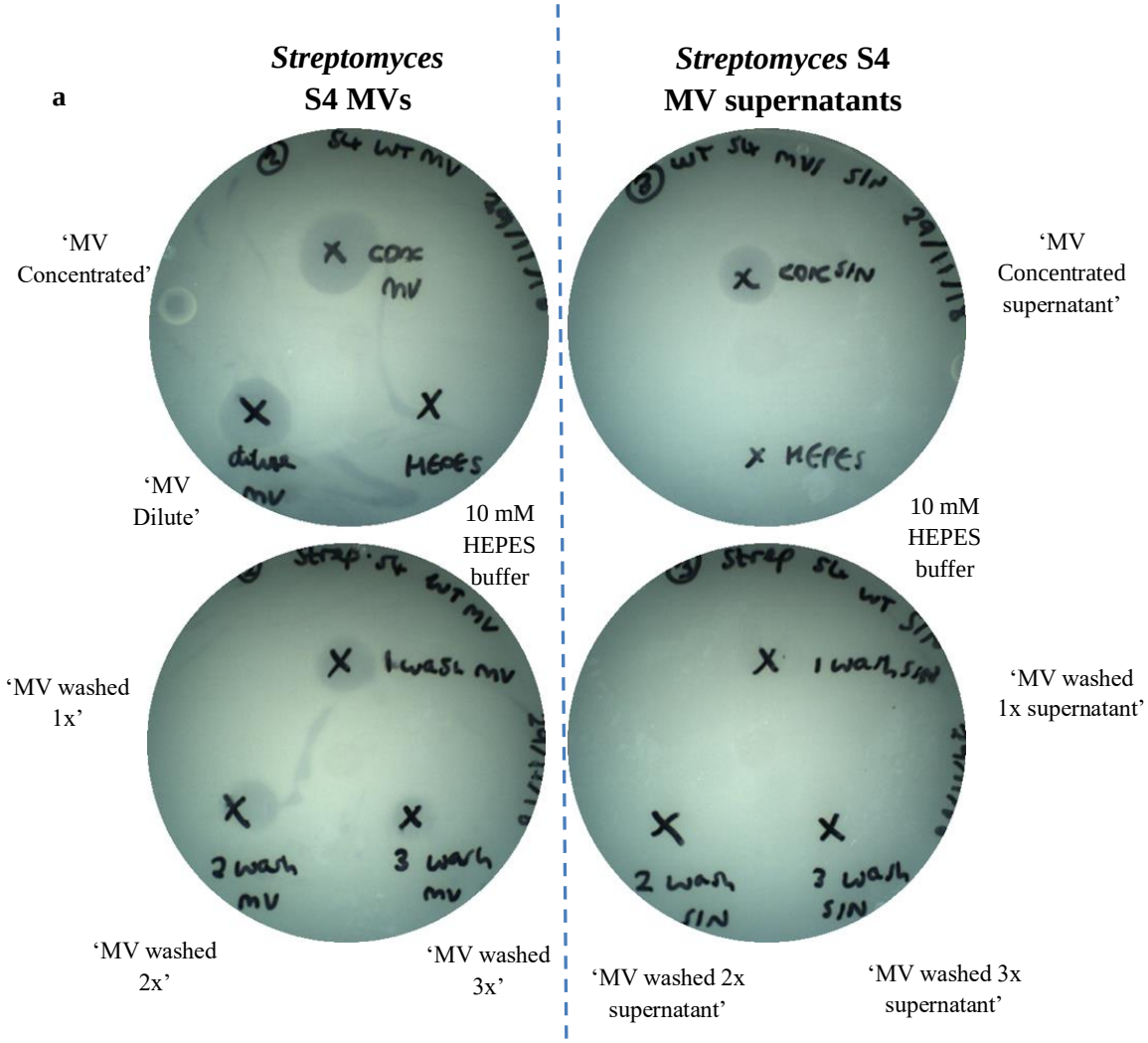
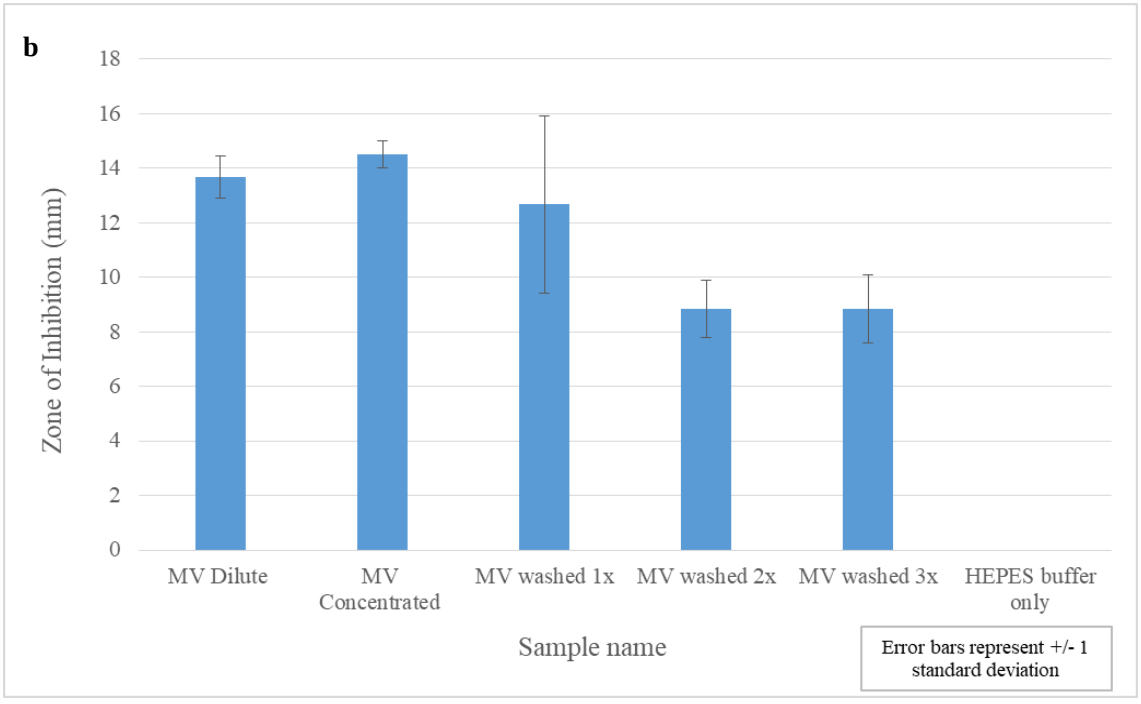


Figure 5.16 Preparation of MVs with various numbers of wash steps for zone of inhibition plates with *C. albicans*. The supernatant samples are highlighted here too.


a
Streptomyces S4 MVs
Streptomyces S4 MV supernatants
'MV Concentrated'
'MV Concentrated supernatant'
'MV Dilute'
10 mM HEPES buffer
10 mM HEPES buffer
'MV washed 1x'
'MV washed 1x supernatant'
'MV washed 2x'
'MV washed 3x'
'MV washed 2x supernatant'
'MV washed 3x supernatant'


b
Zone of Inhibition (mm)
18
16
14
12
10
8
6
4
2
0
MV Dilute
MV Concentrated
MV washed 1x
MV washed 2x
MV washed 3x
HEPES buffer only
Sample name
Error bars represent +/- 1 standard deviation

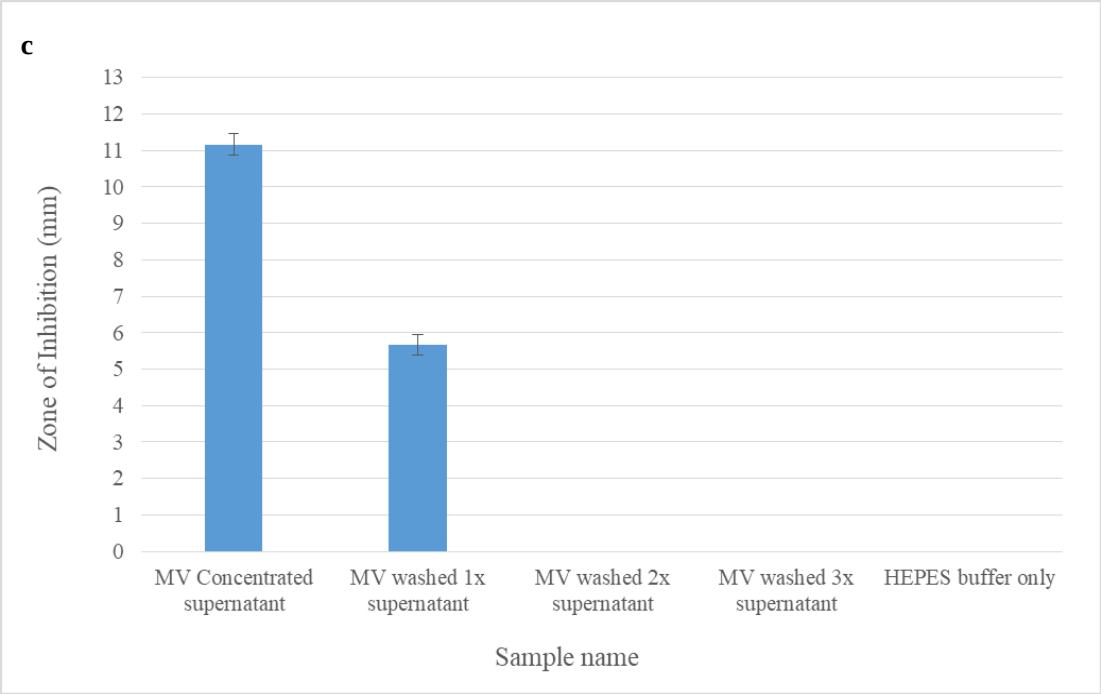


Figure 5.17 Do *Streptomyces* S4 MVs contain antifungals? Various numbers of washes of the MV pellet with 10 mM HEPES buffer

Streptomyces S4 MVs were washed up to 3x in 10 mM HEPES buffer to observe the effect on the ZOI. 5 µL of the following MV samples were added to the LB plates: 'MV Dilute', 'MV Concentrated', 'MV washed 1x', 'MV washed 2x' and 'MV washed 3x' as well as 5 µL of their corresponding supernatants (as explained in Figure 5.15). *C. albicans* mixed with LB 0.5% (w/v) agar was overlayed on to the plates and left to set. Plates were incubated at 37ºC for 18 hrs and the diameter of the ZOI was measured (**a**). All plates were prepared in triplicate and the average ZOI was calculated and presented as a graph (**b-c)**.

Streptomyces S4 WT MVs still appear to contain candicidin. As in Figure 5.17, ZOI produced for the 'MV concentrated' sample compared to the 'MV dilute' sample is not proportional. The *Streptomyces* S4 MVs were washed in 10 mM HEPES buffer either 1x, 2x or 3x. The supernatant was also taken each time and added to the LB plates to see if there was a ZOI with *C. albicans*. The supernatants from the concentrated MV sample and the supernatant after 1 wash in 10 mM HEPES buffer contained candicidin and/or antimycin as there was a ZOI. However, after two or three washes in 10 mM HEPES buffer, the MV supernatants do not contain candicidin or antimycin as there is no ZOI produced. This indicates that anything extracellular to the MVs (e.g. antifungals or lysed cells in the supernatant) has now been washed away with the 10 mM HEPES buffer. Therefore, this provides further evidence that the ZOI found for the MVs after two or three washes is genuinely MV-associated.

5.2.6 Detection of candicidin in *Streptomyces* S4 MVs by Ultraviolet–visible spectroscopy (UV-Vis)

Candicidin and antimycin can both be identified by their distinct UV-Vis spectra (see Appendix D.3 for examples in the literature). Candicidin and antimycin were purchased and dissolved in absolute ethanol which was then diluted to give various concentrations. These samples were run on the spectrophotometer (Agilent Technologies Cary 60 UV-Vis) to give the UV-Vis spectra characteristic to candicidin and antimycin (Figure 5.18). These spectra were used as positive controls to detect candicidin (and/or antimycin) in *Streptomyces* S4 MVs.

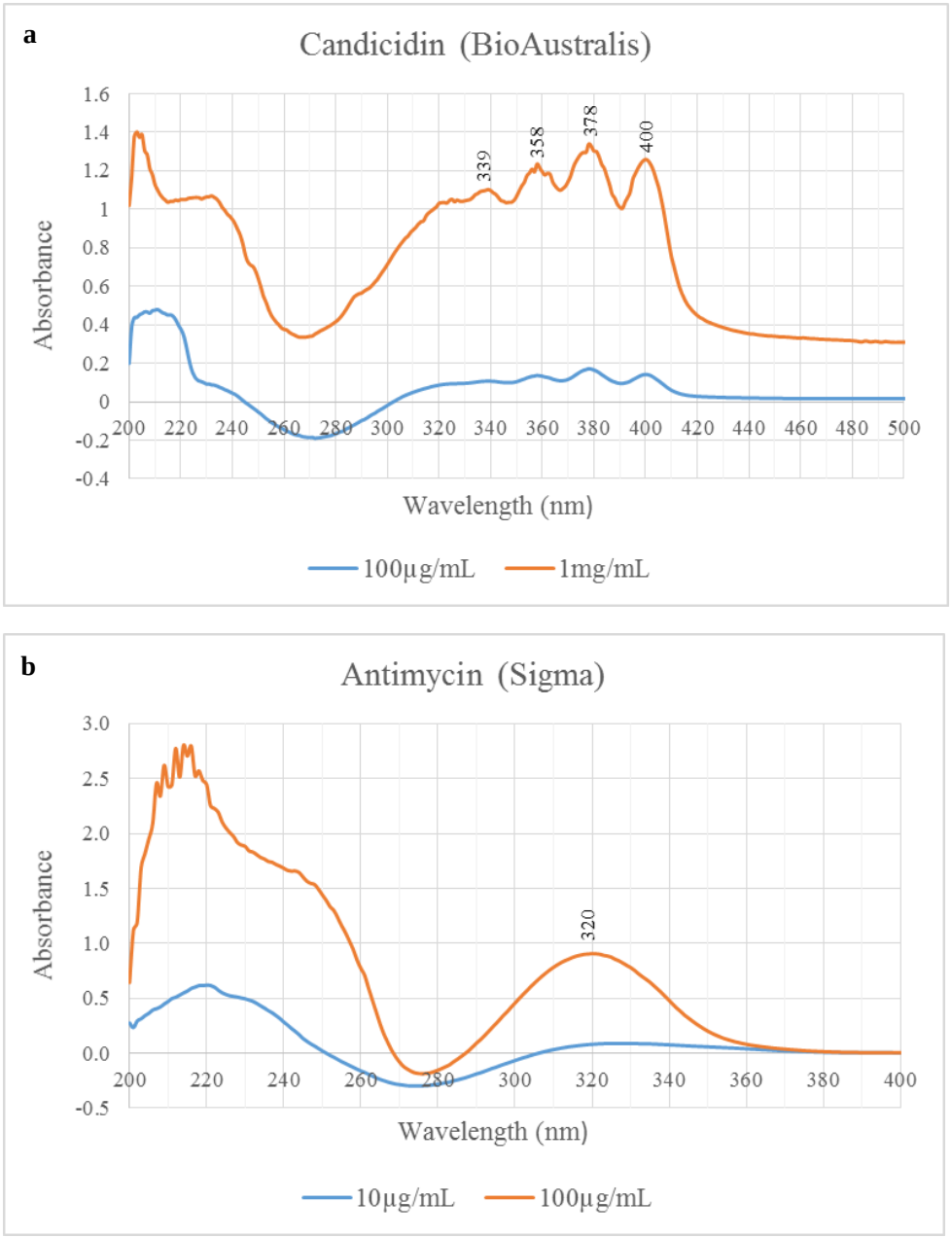


Figure 5.18 UV-Vis spectra of candicidin and antimycin
500 µL samples were loaded on to the spectrophotometer (Agilent Technologies Cary 60 UV-Vis) in quartz cuvettes to give the UV-Vis spectra. Candicidin dissolved at 1 mg/mL which gave the clearest spectra and there was a proportional decrease in Absorbance when 100 µg/mL was run (**a**). Antimycin dissolved at 1 mg/mL gave the clearest spectra and there was a proportional decrease in Absorbance when 100 µg/mL was run (**b**). The peaks that are 'characteristic' of each antifungal compound is labelled above.

5.2.6.1 Extraction of candicidin from *Streptomyces* S4 MVs was trialled using 3 different methods

Detection of candicidin and/or antimycin was then investigated in *Streptomyces* S4 MV samples. Initially, *Streptomyces* S4 MVs resuspended in 10 mM HEPES buffer were trialled on the UV/Vis spectrophotometer with no success (see Appendix D.4). Three methods were trialled to extract the antifungal compounds from the other contaminating MV components using butanol (Figure 5.19). Methods to extract candicidin using butanol were based on a method in the literature which was used to extract candicidin from the supernatant of *Streptomyces* S4 strains for LC-MS (Seipke *et al.* 2011).

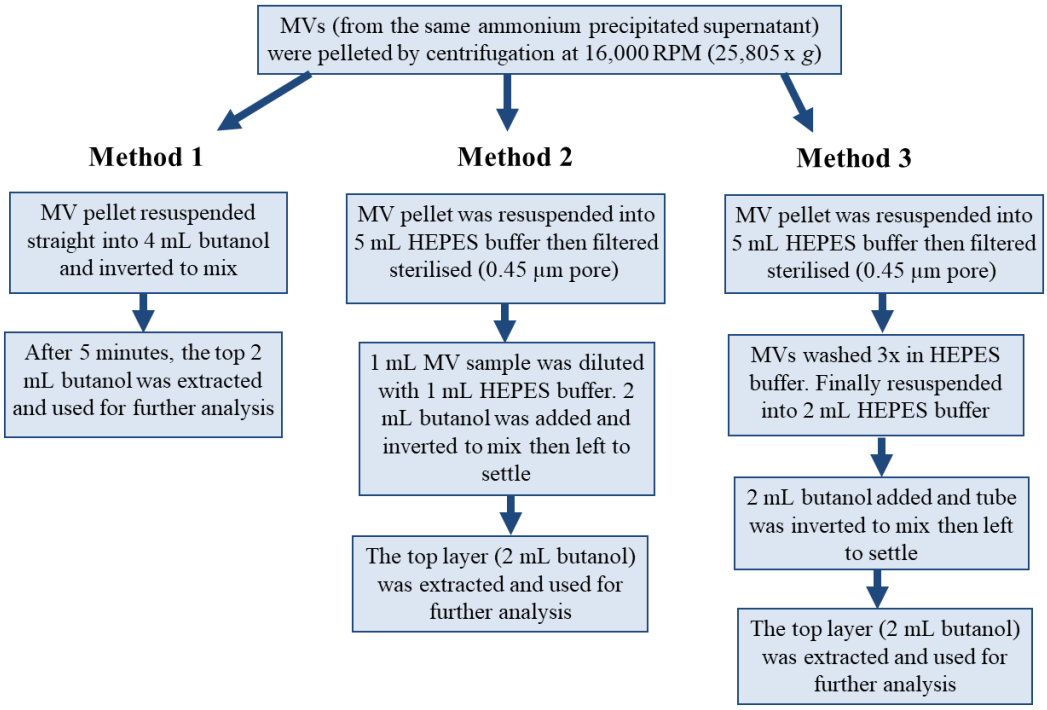


Figure 5.19 Different methods of butanol extraction trialled on *Streptomyces* S4 MVs to identify candicidin

In Method 1, MVs were resuspended directly into butanol then the butanol was extracted for analysis. In Method 2, MVs were resuspended into 10 mM HEPES buffer first then extracted using butanol. In Method 3, MVs were washed 3x in 10 mM HEPES buffer then extracted using butanol

Butanol and 10 mM HEPES buffer only were run on the spectrophotometer to visualise the UV-Vis spectra as negative controls (Figure 5.20).

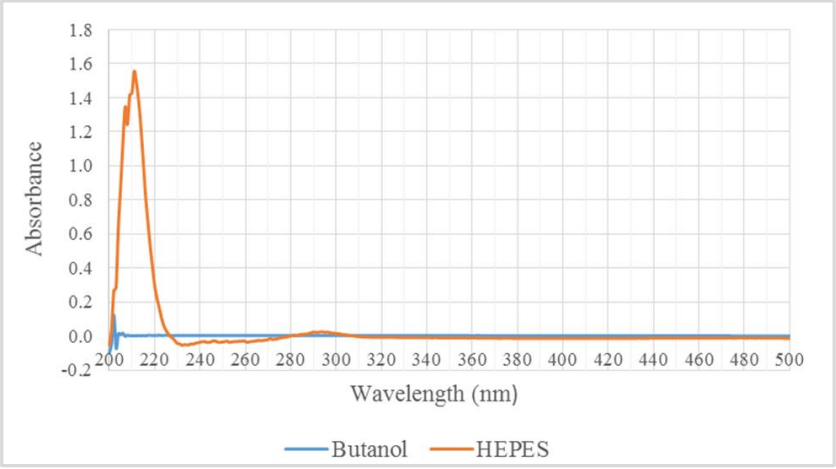


Figure 5.20 UV-Vis spectra of butanol and 10 mM HEPES buffer
500 µL butanol or 10 mM HEPES buffer were loaded on to the spectrophotometer (Agilent Technologies Cary 60 UV-Vis) in quartz cuvettes to give the UV-Vis spectra.

5.2.6.2 Candicidin extraction Method 1 (MV pellet resuspended directly into butanol)

At the last step of the MV purification protocol, MV pellets were resuspended directly into butanol (rather than into 10 mM HEPES buffer) and ran on the spectrophotometer to generate the UV-Vis spectra (Figure 5.21).

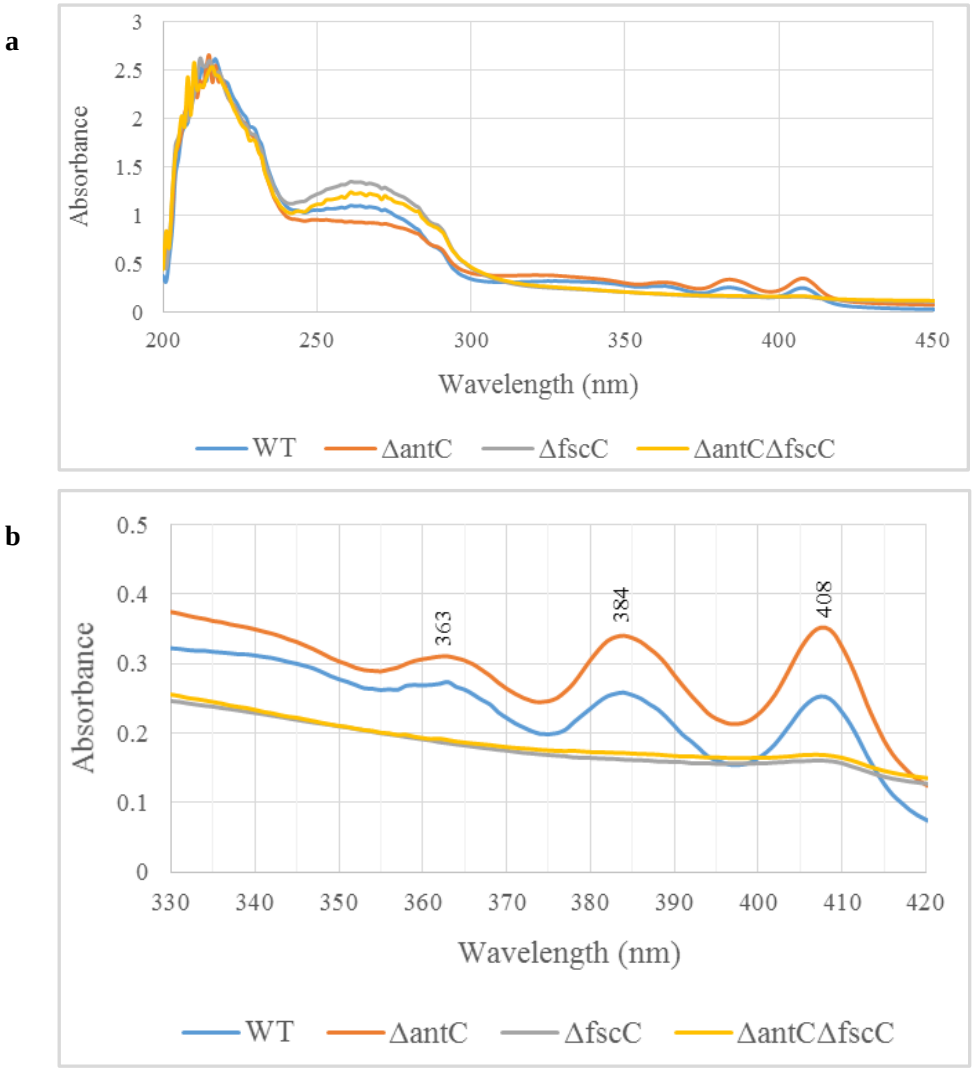


Figure 5.21 UV-Vis spectra of *Streptomyces* S4 MVs resuspended directly into butanol
Streptomyces S4 MVs were purified using the standard protocol but the MV pellets were resuspended directly into butanol. 500 µL MVs (extracted in butanol) were loaded on to the spectrophotometer (Agilent Technologies Cary 60 UV-Vis) in quartz cuvettes to give the UV-Vis spectra. The Absorbance range used was 200-450 nm (**a**) and the area of interest was enlarged to see the characteristic Candicidin peaks (**b**).

MVs from *Streptomyces* S4 and *ΔantC* gave peaks characteristic of candicidin. There was no indication of candicidin in MVs from the *ΔfscC* or from the *ΔantC ΔfscC* strain and there was no indication of antimycin in MVs from any of the strains. The ZOI plates in Section 5.2.5 showed that MVs from *Streptomyces* S4 and *ΔantC* gave a ZOI against *C. albicans*. This indicated that candicidin was present in the MVs which agree with the findings here. Another method to detect candicidin in the MVs involved resuspending the MVs in 10 mM HEPES buffer first then extracting using butanol. The results of using Methods 2 and 3 to identify candicidin can be found in Appendix D.5-D.6.

5.3 Discussion

5.3.1 *Streptomyces* S4 secretes MVs which were purified and characterised (Sections 5.2.1-5.2.6)

5.3.1.1 Presence of *Streptomyces* S4 MVs were confirmed by TEM

The OMV purification protocol was applied to the *Streptomyces* S4 WT strain and the three mutant strains (*ΔantC*, *ΔfscC* and *ΔantC ΔfscC*). The resulting samples were visualised by TEM. There appeared to be vesicles which were the same shape, size and appearance as OMVs purified from *E. coli* and *P. aeruginosa* in Chapters 3-4. The purified MVs were also very similar to those purified from *Streptomyces lividans* in the literature (Schrempf, Merling. 2015).

5.3.1.2 *Streptomyces* S4 MVs are composed of the cytoplasmic membrane

WGA-FITC bound successfully to the peptidoglycan on *Streptomyces* S4 WT cells and the cells could clearly be seen. WGA-FITC did not bind and fluoresce when added to purified MVs. The most likely explanation for this is that the MVs are composed of the cytoplasmic membrane of the cells with no peptidoglycan layer as suggested in the literature (Figure 5.1). This could be resolved further by using a specialised EM grid with coordinates so that the same area can be used for confocal microscopy. MVs can be tagged with WGA-FITC then the same grid can be negatively stained and used for TEM. The images can be overlayed and this would determine the percentage of the MV population that is stained with WGA-FITC.

Figure 5.3 shows whole *Streptomyces* S4 cells treated with WGA-FITC. Before incubation with WGA-FITC, half of the cells were washed 3x in PBS to remove any MVs from around the cells (**a**). The other half of the cells remained unwashed in PBS and may still have MVs in the sample (**b**). Areas of intense fluorescence were found on the membranes of the cells, which may be points of MV biogenesis. These were seen on the cells that were unwashed in PBS but not on the washed cells. It is currently unclear why these areas fluoresce more intensely than the membranes alone. Perhaps the peptidoglycan layer in these areas is being broken down (in order for the MVs to bulge from the cytoplasmic membrane) and the WGA-FITC binds more giving greater fluorescence.

5.3.1.3 MV protein profile

SDS-PAGE gels were run to show the protein profile of the purified *Streptomyces* S4 MVs. The densitometry plots indicated that the MVs purified from the double mutant (*ΔantC ΔfscC*) did have extra proteins compared with MVs from the other 3 strains. It appears that there is less regulation of which proteins enter the MVs when the cell no longer produces antimycin or candicidin. However, the exact reason for this change is unclear.

5.3.2 *Streptomyces* S4 MVs contain proteases (Section 5.2.4)

The protein profile of the MVs from *Streptomyces* S4 and the three mutants were compared to that of the MV-producing cells. One protein in particular appeared to be enriched in the *Streptomyces* S4 MVs compared with the cells. This was identified by mass spectrometry to be a serine protease. Serine proteases have been found within OMVs in the literature although not yet in *Streptomyces* MVs. The serine protease VesC has been found within OMVs from *Vibrio cholerae* (Mondal *et al.* 2016). VesC was found in an active form within the OMVs and was suspected of play a role in the intestinal colonisation of *V. cholerae* in adult mice. VesC also induced a 'proinflammatory response in human cultured intestinal epithelial cells' (Mondal *et al.* 2016). The serine protease detected in the MVs should be characterised further to determine its function *in vivo*.

Lastly, Figure 5.9 provided evidence of active proteases within the *Streptomyces* S4 MVs. When SDS was added to the MVs, the protein profile changed. SDS is a detergent and is known to disrupt the membrane of OMVs. It is likely that the MVs are disrupted after SDS addition and any active proteases (including the serine protease) were released. These proteases may have caused degradation of other proteins and altered the protein profile.

5.3.3 *Streptomyces* S4 MVs contain Candicidin (Section 5.2.5-5.2.6)

5.3.3.1 Positive controls

Candicidin and antimycin were used as positive controls to optimise the development of the *C. albicans* ZOI plates. The Minimum Inhibitory Concentration (MIC) of candicidin was determined to be 10 µg/mL and the MIC of antimycin was found to be 5 µg/mL using this method. In the literature, the MICs were reported as antimycin (0.125 µg/mL) and candicidin (2 µg/mL) against *C. albicans* (McLean *et al.* 2016). MICs in this paper were determined by growing *C. albicans* in 96 well plates with various concentrations of candicidin and antimycin. In Chapter 5, zones of inhibition were only measured when the growth of *C. albicans* was fully inhibited and the agar was clear and transparent. At some of the lower concentrations of candicidin and antimycin, zones of inhibition were starting to develop but were not measured due to the criteria chosen. This made this method of determining the MIC less accurate than the 96 well plate method in the literature.

5.3.3.2 Effect of *Streptomyces* S4 MVs on *C. albicans* growth

MVs were added to a LB agar plate which was then overlayed with *C. albicans* (in LB 0.5% agar). This was to determine if the antifungals candicidin or antimycin were present within the MVs purified from each *Streptomyces* strain. Each strain of *Streptomyces* was also grown on a LB agar plate and tested in the same way as the MVs. Zones of inhibition were produced when an antifungal was present that was active against *C. albicans*. Table 5.3 summarises the main findings from the plates which indicate that *Streptomyces* S4 MVs contain candicidin.

Table 5.3 Summary of findings from the effect of *Streptomyces* S4 cells and purified MVs on *C. albicans*

Sample	Is a zone of inhibition produced with *C. albicans*?			
	***Streptomyces* S4 wild-type**	***Streptomyces* S4 *ΔantC***	***Streptomyces* S4 *ΔfscC***	***Streptomyces* S4 *ΔantCΔ fscC***
Bacterial cells	✓	✓	✓	✗
Purified membrane vesicles	✓	✓	✗	✗
Conclusions drawn	WT cells are known to produce candicidin and antimycin. MVs contain at least 1 antifungal as there was a zone of inhibition	*ΔantC* cells produce candicidin only (no antimycin). MVs must contain candicidin as there was a zone of inhibition	*ΔfscC* cells produce antimycin only (no candicidin). MVs must **not** contain antimycin as there was no zone of inhibition	*Streptomyces* S4 *ΔantCΔ fscC* cells do not produce antimycin or candicidin so there was no zone of inhibition with the cells or the MVs

In order to find further evidence that candicidin is MV-associated, *Streptomyces* S4 MVs were washed up to 3x with 10 mM HEPES buffer. This was to remove any extracellular material (for example lysed cells) from around the MVs so that any antifungal activity observed was from MVs only. Even after three washes with 10 mM HEPES buffer, the ZOI was still present which could be evidence that the candicidin is genuinely MV-associated.

5.3.3.3 Detection of Candicidin in *Streptomyces* S4 MVs by UV-Vis

Candicidin and antimycin were purchased and run on the spectrophotometer (Agilent Technologies Cary 60 UV-Vis) to give their characteristic UV-Vis spectra. These spectra were used as positive controls to detect candicidin (and/or antimycin) in *Streptomyces* S4 MVs as they were almost identical to those found in the literature (Seipke *et al.* 2011).

Streptomyces S4 MVs resuspended in 10 mM HEPES buffer were added to the spectrophotometer but no peaks were found in the UV-Vis spectra. Three different methods to extract candicidin using butanol were developed. These were based on a method used to extract candicidin from the supernatant of *Streptomyces* S4 strains for LC-MS (Seipke *et al.* 2011). The optimum method to identify candicidin was to resuspend the MVs into butanol instead of 10 mM HEPES buffer. This gave a clear UV-Vis spectra characteristic of candicidin found in MVs from *Streptomyces S*4 WT and *ΔantC.* These results are in agreement with the results of the ZOI plates with *C. albicans.*

5.3.3.4 Identification of candicidin by mass spectrometry

MALDI-TOF and LC-MS have both been trialled to detect candicidin in the *Streptomyces* S4 MV samples. Unfortunately, this has been unsuccessful so far including with the candicidin purchased from Bio-Australis. The reason for this is unclear as the purchased candicidin is active against *C. albicans*

and produces a UV-Vis spectrum characteristic of candicidin so appears to be correct. Optimisation of LC-MS to detect candicidin is currently ongoing.

5.3.3.5 Limitations of the ZOI with *C. albicans*

Measuring the zones of inhibition using *C. albicans* is a good method to confirm if an antifungal is present within the MV samples or not. The diameter of the ZOI, however, is not necessarily representative of the concentration of candicidin in the MVs as it is too variable. The ZOI can vary if the 5 μL sample has not dried fully before addition of *C. albicans* as the sample can smudge and distort the ZOI shape. Also, ZOI are not always an exact circle and the diameter measured can vary depending on which part is measured. Lastly, the ZOI was only measured when the LB agar was fully clear and transparent. Sometimes the start of a ZOI was visible but was not measured due to the criteria as it was not fully transparent.

5.3.4 Wider Implications

Streptomyces S4 cells appear to secrete MVs containing the antifungal candicidin which is a novel discovery. Within the MVs, candicidin is in a concentrated and protected form for release into the environment. *Streptomyces* S4 has a symbiotic relationship with attine ants which use the antifungals produced by the bacteria to keep their cultivated food free from fungal contamination. Packaging candicidin within a vesicle may increase the uptake of candicidin by the target cells and enhance the killing. This is a similar method to the AmBisome where ampthotericin B is packaged in a liposome for enhanced uptake (see Section 1.9.3). Although the proteome analysis of the MVs was more limited than for the OMVs in Chapters 3 and 4, a serine protease was detected in *Streptomyces* S4 MVs. This could potentially be used as a fusion protein to target cargo to MVs. Lastly, study of MV biogenesis in *Streptomyces* S4 may lead to enhancing the delivery of anti-fungal drugs within vesicles. This will be discussed further in Chapter 6.

Chapter 6

Final Discussion

6.1 Main conclusions

6.1.1 What are the best methods to purify and characterise OMVs?

6.1.1.1 A protocol was developed that successfully purifies OMVs from bacterial cultures

The OMV protocol was used successfully to purify OMVs from a wide range of *E. coli* and *P. aeruginosa* strains. The protocol was also applied to the Gram-positive organism *Streptomyces* S4 and allowed purification of MVs with minimal modifications. It was confirmed that live bacterial cells were not co-purified with OMVs using this protocol and that the choice of PES or SFCA membrane type for filtration of the supernatant did not affect the OMV purity, yield or proteome. Purified *P. aeruginosa* OMVs were separated from flagella by buoyant density ultracentrifugation but this was not successful for *E. coli* K-12 OMVs. However, separation of OMVs from co-purified fimbriae and/or flagella has also been an unresolved issue for others in the literature (Figure 3.34).

6.1.1.2 *E. coli* and *P. aeruginosa* OMV characterisation (Gram-negative bacteria)

Purified OMVs were visualised by TEM and were very similar in size and appearance to those in the literature (Figure 3.17). The proteins found within the purified OMVs were identified by SDS-PAGE gels and mass spectrometry and Western blotting was used to confirm the presence of FimA monomer, Flagellin monomer and OmpA in *E. coli* OMV samples. Purified OMVs were confirmed as being whole and intact by using a Proteinase K test. The Proteinase K test was optimised during this study and also identified which proteins were protected within the OMVs from extracellular proteases. Purified *E. coli* K-12 OMVs were found to contain active proteases capable of degrading flagella. Methods of OMV quantification trialled were using a Bradford assay, a NanoPhotometer 50 and DLS. It became apparent that the presence of fimbriae or flagella co-purified with OMVs causes errors in DLS readings (Figure 3.32) and that the protein concentration was below the detection limit for NanoPhotometer 50 (Section 3.2.6.1). For this reason, a Bradford assay was used for OMV quantification in this study.

6.1.1.3 *Streptomyces* S4 MV characterisation (Gram-positive bacteria)

MVs were purified from *Streptomyces* S4 and the three mutant strains: *ΔantC, ΔfscC* and *ΔantC ΔfscC*. The purified MVs were visualised using TEM and were found to be similar in diameter and appearance to purified *Streptomyces lividans* MVs in the literature (Schrempf, Merling. 2015). Confocal microscopy using WGA-FITC indicated that the MVs are composed of the cytoplasmic membrane with no peptidoglycan layer present (Section 5.2.2). Furthermore, a serine protease was found within all of the purified S4 MVs which was identified by SDS-PAGE gels and mass spectrometry. Lastly, a reproducible bioassay was developed to measure the antifungal effect of *Streptomyces* S4 MVs against *C. albicans.*

6.1.2 What are the differences in (O)MV composition, cargo and function from both Gram-negative and Gram-positive bacterial strains?

6.1.2.1 *E. coli* OMV cargo

OMVs from *E. coli* wild type and recombinant strains have different protein profiles

The number of OMVs released from the engineered *E. coli* B strains was higher than the *E. coli* K-12 WT strains. Moreover, *E. coli* K-12 OMVs are enriched with specific proteins unlike OMVs from B strains which appear to contain many proteins with no particular proteins enriched.

FimA and Flagellin monomers are packaged into *E. coli* K-12 OMVs

One of the most convincing pieces of evidence to support the packaging of FimA and Flagellin monomers into OMVs is the immunogold labelling of OMVs embedded in resin (probed with anti-FimA monomer and anti-Flagellin antibody). Secondly, SDS-PAGE gels and Western blots repeatedly showed FimA and/or Flagellin monomers in the periplasmic fraction of *E. coli* cells and their respective purified OMV samples. Lastly, the Proteinase K tests repeatedly gave evidence that the Flagellin monomer is protected (most likely structurally) within the OMVs. Unfortunately, the Proteinase K test could not be used as evidence that FimA monomer is packaged into K-12 OMVs. This is because FimA monomer was not degraded by Proteinase K under the usual assay conditions.

FimA and Flagellin monomers are packaged into *E. coli* K-12 OMVs in a mutually exclusive way unless there are mutations in the OMV-producing cell

E. coli K-12 strains MG1655 and the Keio collection parental strain BW25113 produce Type 1 fimbriae and package FimA monomer into their OMVs. Deletions in certain fimbriae or flagella-associated genes caused alterations to the FimA/Flagellin packaging into OMVs as follows:

- If Type 1 fimbriae production is disrupted in an *E. coli* MG1655 or BW25113 strain, the cell switches to production of flagella. This results in the packaging of Flagellin into OMVs instead of FimA.
  Examples: FimB-LacZ fusion strain, *ΔfimA, ΔfimB, ΔfimF* strains.
- Certain mutations in the *E. coli* cell lead to a dysregulation in the packaging of Flagellin and FimA monomers in a way that both are packaged together.
  Examples: Fimbriae locked on strain, *ΔfimE, ΔfimG, ΔfimH, ΔfimI, ΔlrhA* strains
- Mutations in proteins relating to flagella biosynthesis did not affect the cells production of Type 1 fimbriae or the packaging of FimA into the OMVs.
  Examples: *ΔfliD, ΔfliS, ΔflhA* strains
- If neither flagella or fimbriae are expressed on a cell, neither FimA or Flagellin were packaged into the OMVs.
  Examples: *E. coli* Parental B strain, BL21 and BL21 (DE3), *ΔfimD* strains

It was noted that deletion of either FimC or FimF caused the cell to produce flagella rather than Type 1 fimbriae. This resulted in the packaging of Flagellin into OMVs and not FimA. Both FimC and FimF interact with FimA monomers during the formation of Type 1 fimbriae. This could suggest that FimC and FimF play a role in preparing FimA monomers for incorporation into OMVs. It is also possible that the chaperone protein FimC delivers the FimA monomers to the site needed for incorporation into OMVs.

Current theory on the reason for the mutually exclusive packaging of FimA and Flagellin

It appears that in the majority of *E. coli* K-12 strains, FimA and Flagellin are reciprocally regulated and are packaged in OMVs independently of each other. FimA and Flagellin monomers have opposite effects on the hosts' immune response so it makes sense to package them in a mutually exclusive way into OMVs. Table 6.1 outlines other studies in the literature that have found FimA and Flagellin monomers in OMVs.

Table 6.1 Summary of findings and hypotheses for the mutually exclusive packaging of FimA and Flagellin

Characteristics	Cell Adhering	Cell Motile
Extracellular appendages	Type 1 fimbriae	Flagella
Protein OMVs are most enriched with	Monomeric FimA	Monomeric Flagellin
Effect of protein on the immune system	FimA monomer is anti-inflammatory	Flagellin monomer is pro-inflammatory
Evidence of effect of FimA/Flagellin monomer on host immune response from the literature	Sukumaran *et al* . 2010 Heimlich *et al*. 2004	Zgair, 2012 Yoon *et al*. 2017 Hatai *et al*. 2016 Hajam *et al*. 2017

Clinical *E. coli* K-12 isolate study

Finally, six *E. coli* clinical isolates were examined to see if the purified OMVs contained FimA and/or Flagellin monomers. Three of the six clinical isolate strain OMVs contained neither FimA or Flagellin. One of the clinical isolates contained both FimA and Flagellin packaged together. One clinical isolate expressed Type 1 fimbriae and packaged FimA monomer into the OMVs (but not Flagellin). Another clinical isolate expressed flagella and produced OMVs containing Flagellin (but not FimA). This makes these findings clinically relevant and will be discussed further in Section 6.3.1. However, it does highlight that the mutually exclusive packaging of FimA and Flagellin into OMVs is not a clear-cut story and that there is further research needed to understand the cargo selection and exclusion process.

6.1.2.2 *Streptomyces* S4 MV cargo

All evidence found suggests that *Streptomyces* S4 MVs contain candicidin. There were zones of inhibition formed against *C. albicans* when purified MVs from both *Streptomyces* S4 wild type and *ΔantC* strains were added to the plates. However, there were no zones of inhibition formed from MVs isolated from *ΔfscC* and *ΔantC ΔfscC* strains. As the *ΔantC* strain cannot produce antimycin, candicidin must be the antifungal compound present within the MVs (see Section 5.3.3 for full explanation). Three different methods were developed to extract candicidin from the *Streptomyces* S4 MVs using butanol. This gave a clear UV-Vis spectra characteristic of candicidin found in MVs from *Streptomyces* S4 WT and *ΔantC.* These results are also in agreement with the results of the zone of inhibition plates with *C. albicans.*

6.1.3 Can we target a protein of interest to be incorporated into bacterial MVs?

6.1.3.1 FimA (*E. coli* OMVs)

GFP was successfully targeted to *E. coli* K-12 OMVs by fusion to FimA monomer. However, expression of a FimA fusion protein using a plasmid caused an unexpected hypervesiculation of the strain. Furthermore, the OMVs produced appeared to contain many proteins with no particular proteins enriched in the OMVs. The experiments showed that to target a protein of interest to *E. coli* K-12 OMVs, a chromosomal insert of the protein within the *fimA* gene was required.

6.1.3.2 Flagellin (*E. coli* and *P. aeruginosa* OMVs)

It is possible that Flagellin could be used in the same way as FimA to target cargo to *E. coli* K-12 OMVs. Additionally, B-type Flagellin was detected as one of the most heavily enriched proteins found in *P. aeruginosa* OMVs so, in theory, could be used in the same way.

6.1.3.3 Serine protease (*Streptomyces* S4 MVs)

One protein in particular appeared to be enriched in the *Streptomyces* S4 MVs compared with the protein profile of the whole cells. This protein was identified by mass spectrometry and it was found to be a serine protease. Perhaps this could be used as a target to fuse proteins of interest to or as a biomarker of *Streptomyces* S4 MVs.

6.2 Unanswered questions and further work

6.2.1 Possible improvements to the methods used to characterise purified MVs

6.2.1.1 Is there a better method for purifying OMVs than the protocol developed?

The OMV purification protocol successfully isolated OMVs from all bacterial strains tested. However, fimbriae and flagella are consistently co-purified with *E. coli* OMVs which was not resolved. One solution for this could be to purchase a specialised OMV purification kit which claims to purify OMVs quickly and with minimal contaminants from the cell. The main OMV isolation kit currently on the

market is the System Biosciences ExoBacteria™ OMV Isolation Kit (EXOBAC100A-1). However, unfortunately it was not cost effective to purify OMVs using this kit for this project.

6.2.1.2 What is the best method to quantify MVs?

In this study, MVs were quantified using a Bradford assay which measures the concentration of protein present in each MV sample. Dynamic light scattering (DLS) was also trialled to quantify OMVs. However, the DLS appeared to detect the fimbriae and flagella in the samples rather than the OMVs. This led to inaccurate quantification of the OMVs. The NanoPhotometer 50 (Implen) also was trialled to quantify MVs, however, this technique was found to not accurately detect protein concentrations under 50 µg/mL. Alternative options for MV quantification are by flow cytometry (Wieser *et al.* 2014) or Nanoparticle tracking analysis (Gerritzen *et al.* 2017).

6.2.2.3 Further characterisation of MV proteases

Further attempts to characterise proteases within *P. aeruginosa, E. coli* and *Streptomyces* S4 MVs could be beneficial. Characterisation of proteases was trialled in an attempt to couple cargo function to MV production. If MV protease activity directly correlates to MV production, a colorimetric assay could potentially be developed to quantify MVs and learn more about factors that affect vesiculation. In this study, MVs from *E. coli, P. aeruginosa* and *Streptomyces* S4 were incubated for 1 hour with the chromogenic esterase substrate 4-nitrophenyl acetate. Unfortunately, it was discovered that the substrate was unexpectedly reacting with both LB and ammonium sulphate. For this reason, the colour change could not clearly be correlated with MV protease activity. In order to characterise MV proteases further, kits such as the Sigma Protease Fluorescent Detection Kit (PF0100) or Zymogram gels (Novex) could be used.

6.2.2.4 Characterisation of the lipid and DNA components of purified MVs

The main focus of this study was to characterise the protein profile of the MVs. Unfortunately, time did not allow for characterisation of the lipid content of the MVs. Initially, purified membrane vesicles could be visualised using Nile red or FM4-64 dyes, which would provide further evidence that the OMVs purified are genuine. During the Proteinase K test in Chapter 3, the presence of 0.02% (w/v) SDS caused disruption of *E. coli* and *Streptomyces* S4 MVs. This allowed Proteinase K access to the proteins, which are usually protected within the MVs and allowed their degradation. However, this did not seem to be the case for OMVs purified from *P. aeruginosa* strains *PA01* and *PA14*, which did not appear to be disrupted after addition of 5% (w/v) SDS. This could be investigated further using a range of detergents and comparing the lipid composition of *E. coli* and *P. aeruginosa* OMVs. A study into differences in lipid composition may provide insight into the differences in OMV stability in the presence of detergents. Lastly, time did not allow characterisation of any DNA found within the OMVs. The development of a technique to amplify OMV DNA by PCR may also be useful.

6.2.2 Main unanswered questions about OMV cargo

6.2.2.1 How are FimA or Flagellin monomers delivered to the OMVs?

FimA and Flagellin are likely to be packaged into OMVs via different mechanisms due to their locations within the cell. Flagellin monomers usually reside in the cytoplasm whereas FimA monomers are found in the periplasm. During flagella biosynthesis, Flagellin monomers are transported directly from the cytoplasm into a central channel (which bypasses the periplasm) to be added to the growing filament (see Section 1.6.4). However, Western blotting in this study indicated that Flagellin destined for the OMVs was transported to the periplasm first (Figure 4.8). In order to elucidate the mechanisms of targeting proteins such as Flagellin to the periplasm, the Sec or Tat pathways could be disrupted to see the effect on the proteins packaged within the OMVs. An example of this would be inhibition of the Type I signal peptidase enzyme, which cleaves the signal peptide of proteins during translocation.

6.2.2.2 How does the cell prevent premature polymerisation of FimA or Flagellin monomers destined for OMVs?

Both FimA and Flagellin monomers have strong oligomerisation potential and bind to chaperone proteins to prevent premature polymerisation (FimC and FliS). It is also known that FimA monomers can adopt an alternative conformation which prevents polymerisation (Zyla *et al.* 2019). It is possible that the FimA and Flagellin monomers have an additional signal sequence that targets them to sites where vesiculation occurs. To identify any differences in signal sequence, FimA purified from Type 1 fimbriae can be compared to those found in OMVs by N-terminal sequencing. Similarly, Flagellin purified from flagella and OMVs can also be compared.

6.2.2.3 Is the mutually exclusive packaging of FimA and Flagellin monomers beneficial *in vivo*?

The effect of FimA monomers and Flagellin monomers on the human immune system from OMVs could be studied either *in vitro* and *in vivo.* This may give further insight into why these proteins are enriched in *E. coli* OMVs.

6.2.3 Discussion of findings about *Streptomyces* S4 MVs

Further characterisation of *Streptomyces* S4 MVs

Further MV characterisation is needed to reach the same standard of OMV characterisation from Gram-negative bacteria. Characterisation work could include application of the Proteinase K test to confirm that the MVs are intact and also confirm which proteins are protected within the MVs. *Streptomyces* S4 MVs could also be run on the DLS machine to estimate MV size and quantity. MV biogenesis could also be studied using TEM and further confocal microscopy using the WGA-FITC tag. In the literature, MV biogenesis from *Streptomyces lividans* was observed using lipid-specific dyes. They discovered that “lipid- and phospholipid-rich sites at the substrate hyphae correlate with clusters of vesicle-like

particles" (Schrempf, Merling. 2015). TEM images could also be improved by embedding the cells and MVs in resin for EM to see a cross-section inside the cells/MVs.

6.3 Wider Implications of study

6.3.1 Blocking FimA and/or Flagellin incorporation into OMVs could be a drug target for preventing or treating *E. coli* infection

In this study, six clinical *E. coli* isolates were examined to see if the OMVs produced contained FimA and/or Flagellin. One of the clinical isolates expressed Type 1 fimbriae and the OMVs produced contained FimA monomers. Another clinical isolate expressed flagella and produced OMVs containing Flagellin. The selective enrichment of these two proteins indicates that the packaging is deliberate and most likely beneficial to the pathogenicity of the *E. coli* cell. For this reason, preventing OMV production or disrupting the OMVs produced could be a potential drug target for preventing or treating *E. coli* infection. In this study, B type Flagellin monomer was also detected in OMVs from both *PA01* and *PA14* which express flagella. This indicates that this finding could be relevant to a range of other bacterial species including *P. aeruginosa*.

6.3.2 Using targeted expression of recombinant proteins and other molecules for drug delivery using OMVs

As demonstrated in Chapter 4, GFP was successfully targeted to *E. coli* K-12 OMVs by protein fusion to FimA. Other proteins of interest could be targeted to OMVs using this method which could be used for drug delivery. Other enriched proteins in OMVs such as Flagellin could also be used for protein fusions. For this application, further research on the effect of these OMVs on the host immune system is needed as well as modification of the OMV to reduce its immunogenicity.

6.3.3 Using Gram-positive MVs for targeted drug delivery

Packaging antifungal compounds (such as candicidin) within a vesicle may enhance its uptake by the target cells and enhance the killing. This is a similar method to the AmBisome where Amphotericin B is packaged in a liposome for enhanced uptake (see Section 1.9.3). Studying the natural packaging of antifungals in MVs by *Streptomyces* S4 could be useful in enhancing the delivery of anti-fungal drugs within vesicles for therapeutic purposes. The study of MVs from Gram-positive bacteria is a relatively new area of research which could have potential for use in vaccines and drug delivery in the same way as OMVs. Although the proteome analysis of the *Streptomyces* S4 MVs was limited, a serine protease was detected. Fusion of a protein of interest to the serine protease could be a potential method to target cargo to S4 MVs.

6.3.4 Vaccines and immunogenic properties

The membrane vesicles secreted by bacteria are naturally immunogenic and so have the potential to be used as vaccines against a range of diseases. One advantage of using MVs for vaccines are that they can be easily manipulated and engineered to display specific bacterial antigens either inside or on their

surface. An example of this was the targeting of GFP to *E. coli* K-12 OMVs by fusion to FimA. Purified *E. coli* OMVs are also being sold as inducers of the immune response for research purposes. For example, purified *E. coli* BL21 OMVs are currently sold as potent inducers of Caspase 11-4/5 inflammasome and activators of TLR2 and TLR4 which recognise bacterial cell walls (InvivoGen, catalogue code: tlrl-omv).

6.3.5 Packaging of recombinant proteins into OMVs impacts biotechnology

Information about *E. coli* OMV biogenesis and cargo is useful as B strains are commonly used for recombinant protein production. It is possible that some of the recombinant proteins of interest are being packaged and secreted in OMVs, decreasing the final yield of purified protein. It is also still unclear how the expression of plasmids influence OMV production and cargo. Further research is needed to quantify the yield of recombinant proteins lost through packaging into *E. coli* OMVs.

6.4 Contributions to the field and final thoughts

Throughout this project, membrane vesicles have been isolated and characterised from a wide range of bacterial strains. During the project, it became possible to separate the membrane vesicles into three distinct categories based on their different characteristics. Table 6.2 is a final summary of the findings for each membrane vesicle type. Some of the findings in this study are novel and will contribute to the current MV knowledge already in the literature. This information may be used in the development of using MVs for therapeutic purposes in the future.

Table 6.2 Final summary of MV findings which are separated into three categories

Purified vesicle characteristic	Gram-negative bacteria		Gram-positive bacteria
	Wild type OMVs	**Recombinant OMVs**	
Proposed function of vesicles	Signalling function *in vivo*: secreting OMVs that contain specific cargo to benefit the OMV-producing cell (e.g. virulence factors)	Secreted as part of a stress response. For example, to remove any accumulations of unwanted products in the cell	Produced to secrete the insoluble antifungal compound candicidin into the environment
Examples of bacterial strains studied	*PA01* , *PA14* , *E. coli* K-12, *E. coli* clinical isolates	*E. coli B* strains	*Streptomyces* S4 strains
Membrane vesicle composition	Formed from the outer membrane and contains cargo from the periplasm	Formed from the outer membrane and contains cargo from the periplasm	Formed from the cytoplasmic membrane and contains cargo from the cytoplasm
Enrichment of particular proteins within vesicles?	✓	✗	✗
Does the strain hypervesiculate?	✗	✓	✓
Co-purification of fimbriae or flagella with OMVs?	✓	✗	✗
Antifungal compounds detected in MVs?	✗	✗	✓
Protein cargo detected in vesicles	***PA01/PA14*** **:** B type flagellin, phage tail protein, elastase, aminopeptidase, chitin-binding protein ***E. coli*** **K-12:** FimA, Flagellin, OmpA, Antigen 43, Flagellar hook protein FlgE	***E. coli*** **BL21 (DE3):** OmpF, OmpA	***Streptomyces*** **S4:** Serine protease, succinate dehydrogenase

References

ACEVEDO, R., FERNANDEZ, S., ZAYAS, C., ACOSTA, A., SARMIENTO, M.E., FERRO, V.A., ROSENQVIST, E., CAMPA, C., CARDOSO, D., GARCIA, L. and PEREZ, J.L., 2014. Bacterial outer membrane vesicles and vaccine applications. *Frontiers in Immunology*, **5**, pp. 121.

ADICIPTANINGRUM, A.M., BLOMFIELD, I.C. and TANS, S.J., 2009. Direct observation of type 1 fimbrial switching. *EMBO Reports*, **10**(5), pp. 527-532.

AGUILAR, C., FRISCINA, A., DEVESCOVI, G., KOJIC, M. and VENTURI, V., 2003. Identification of quorum-sensing-regulated genes of Burkholderia cepacia. *Journal of Bacteriology*, **185**(21), pp. 6456-6462.

AL-BADR, A. and AL-SHAIKH, G., 2013. Recurrent Urinary Tract Infections Management in Women: A review. *Sultan Qaboos University medical journal*, **13**(3), pp. 359-367.

ANGERT, E.R., 2005. Alternatives to binary fission in bacteria. *Nature Reviews Microbiology*, **3**(3), pp. 214-224.

AUVRAY, F., THOMAS, J., FRASER, G.M. and HUGHES, C., 2001. Flagellin polymerisation control by a cytosolic export chaperone. *Journal of Molecular Biology*, **308**(2), pp. 221-229.

BABA, T., ARA, T., HASEGAWA, M., TAKAI, Y., OKUMURA, Y., BABA, M., DATSENKO, K.A., TOMITA, M., WANNER, B.L. and MORI, H., 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Molecular systems biology*, **2**, pp. 2006.0008.

BACHMANN, B.J., 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. *Bacteriological Reviews*, **36**(4), pp. 525-557.

BAKARE, N., RICKERTS, V., BARGON, J. and JUST-NUBLING, G., 2003. Prevalence of *Aspergillus fumigatus* and other fungal species in the sputum of adult patients with cystic fibrosis. *Mycoses*, **46**(1-2), pp. 19-23.

BAKER, J.L., CHEN, L., ROSENTHAL, J.A., PUTNAM, D. and DELISA, M.P., 2014. Microbial biosynthesis of designer outer membrane vesicles. *Current Opinion in Biotechnology*, **29**, pp. 76-84.

BAKER, M.A., HYNSON, R.M., GANUELAS, L.A., MOHAMMADI, N.S., LIEW, C.W., REY, A.A., DUFF, A.P., WHITTEN, A.E., JEFFRIES, C.M., DELALEZ, N.J., MORIMOTO, Y.V., STOCK, D., ARMITAGE, J.P., TURBERFIELD, A.J., NAMBA, K., BERRY, R.M. and LEE, L.K., 2016. Domain-swap polymerization drives the self-assembly of the bacterial flagellar motor. *Nature Structural & Molecular Biology*, **23**(3), pp. 197-203.

BARBIER, F., ANDREMONT, A., WOLFF, M. and BOUADMA, L., 2013. Hospital-acquired pneumonia and ventilator-associated pneumonia: recent advances in epidemiology and management. *Current Opinion in Pulmonary Medicine,* **19**(3), pp. 216-228.

BAUMAN, S.J. and KUEHN, M.J., 2006. Purification of outer membrane vesicles from *Pseudomonas aeruginosa* and their activation of an IL-8 response. *Microbes and infection / Institut Pasteur,* **8**(9-10), pp. 2400-2408.

BECK-SAGUE, C. and JARVIS, W.R., 1993. Secular trends in the epidemiology of nosocomial fungal infections in the United States, 1980-1990. National Nosocomial Infections Surveillance System. *Journal of Infectious Diseases,* **167**(5), pp. 1247-1251.

BIELASZEWSKA, M., RUTER, C., BAUWENS, A., GREUNE, L., JAROSCH, K.A., STEIL, D., ZHANG, W., HE, X., LLOUBES, R., FRUTH, A., KIM, K.S., SCHMIDT, M.A., DOBRINDT, U., MELLMANN, A. and KARCH, H., 2017. Host cell interactions of outer membrane vesicle-associated virulence factors of enterohemorrhagic *Escherichia coli* O157: Intracellular delivery, trafficking and mechanisms of cell injury. *PLOS pathogens,* **13**(2), pp. e1006159.

BIELIG, H., DONGRE, M., ZUREK, B., WAI, S.N. and KUFER, T.A., 2011. A role for quorum sensing in regulating innate immune responses mediated by *Vibrio cholerae* outer membrane vesicles (OMVs). *Gut Microbes,* **2**(5), pp. 274-279.

BITTO, N.J., CHAPMAN, R., PIDOT, S., COSTIN, A., LO, C., CHOI, J., D'CRUZE, T., REYNOLDS, E.C., DASHPER, S.G., TURNBULL, L., WHITCHURCH, C.B., STINEAR, T.P., STACEY, K.J. and FERRERO, R.L., 2017. Bacterial membrane vesicles transport their DNA cargo into host cells. *Scientific Reports,* **7**(1), pp. 7072-017-07288-4.

BJERRE, A., BRUSLETTO, B., MOLLNES, T.E., FRITZSONN, E., ROSENQVIST, E., WEDEGE, E., NAMORK, E., KIERULF, P. and BRANDTZAEG, P., 2002. Complement activation induced by purified *Neisseria meningitidis* lipopolysaccharide (LPS), outer membrane vesicles, whole bacteria, and an LPS-free mutant. *Journal of Infectious Diseases,* **185**(2), pp. 220-228.

BLOMFIELD, I.C., MCCLAIN, M.S. and EISENSTEIN, B.I., 1991. Type 1 fimbriae mutants of *Escherichia coli* K12: characterization of recognized afimbriate strains and construction of new fim deletion mutants. *Molecular Microbiology,* **5**(6), pp. 1439-1445.

BLOMFIELD, I.C., MCCLAIN, M.S., PRINC, J.A., CALIE, P.J. and EISENSTEIN, B.I., 1991. Type 1 fimbriation and fimE mutants of *Escherichia coli* K-12. *Journal of Bacteriology,* **173**(17), pp. 5298-5307.

BONNINGTON, K.E. and KUEHN, M.J., 2014. Protein selection and export via outer membrane vesicles. *Biochimica et Biophysica Acta,* **1843**(8), pp. 1612-1619.

BRADFORD, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, pp. 248-254.

BRANNON, J.R., BURK, D.L., LECLERC, J., THOMASSIN, J., PORTT, A., BERGHUIS, A.M., GRUENHEID, S. and LE MOUAL, H., 2015. Inhibition of Outer Membrane Proteases of the Omptin Family by Aprotinin. *Infection and Immunity*, **83**(6), pp. 2300.

BROWN, L., WOLF, J.M., PRADOS-ROSALES, R. and CASADEVALL, A., 2015. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nature Reviews Microbiology*, **13**(10), pp. 620-630.

CAHILL, B.K., SEELEY, K.W., GUTEL, D. and ELLIS, T.N., 2015. *Klebsiella pneumoniae* O antigen loss alters the outer membrane protein composition and the selective packaging of proteins into secreted outer membrane vesicles. *Microbiological Research*, **180**, pp. 1-10.

CATALDI, T.R.I., BIANCO, G., PALAZZO, L. and QUARANTA, V., 2007. Occurrence of N-acyl-l-homoserine lactones in extracts of some Gram-negative bacteria evaluated by gas chromatography–mass spectrometry. *Analytical Biochemistry*, **361**(2), pp. 226-235.

CHATER, K.F., 2016. Recent advances in understanding Streptomyces. *F1000Research*, **5**, pp. 2795-2795.

CHATTERJEE, S.N. and CHAUDHURI, K., 2012. *Outer Membrane Vesicles of Bacteria.* 1st edn. Springer.

CHATTERJEE, S.N. and DAS, J., 1966. Secretory activity of *Vibrio cholerae* as evidenced by electron microscopy. *Electron Microscopy*, **II**, pp. 0-259.

CHATTERJEE, D. and CHAUDHURI, K., 2011. Association of cholera toxin with *Vibrio cholerae* outer membrane vesicles which are internalized by human intestinal epithelial cells. *FEBS letters*, **585**(9), pp. 1357-1362.

CHATTERJEE, S.N. and DAS, J., 1967. Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. *Journal of General Microbiology*, **49**(1), pp. 1-11.

CHATTERJEE, S.N. and DAS, J., 1966. Secretory activity of *Vibrio cholerae* as evidenced by electron microscopy. In Electron Microscopy. *Electron Microscopy*, **II**, pp. 0-259.

CHOTIRMALL, S.H., O'DONOGHUE, E., BENNETT, K., GUNARATNAM, C., O'NEILL, S.J. and MCELVANEY, N.G., 2010. Sputum *Candida albicans* presages FEV(1) decline and hospital-treated exacerbations in cystic fibrosis. *Chest*, **138**(5), pp. 1186-1195.

CHUTKAN, H., MACDONALD, I., MANNING, A. and KUEHN, M.J., 2013. Quantitative and qualitative preparations of bacterial outer membrane vesicles. *Methods in Molecular Biology (Clifton, N.J.)*, **966**, pp. 259-272.

CONFER, A.W. and AYALEW, S., 2013. The OmpA family of proteins: roles in bacterial pathogenesis and immunity. *Veterinary Microbiology*, **163**(3-4), pp. 207-222.

COOPER, L.A., SIMMONS, L.A. and MOBLEY, H.L., 2012. Involvement of mismatch repair in the reciprocal control of motility and adherence of uropathogenic *Escherichia coli. Infection and immunity*, **80**(6), pp. 1969-1979.

COUTO, N., SCHOOLING, S.R., DUTCHER, J.R. and BARBER, J., 2015. Proteome Profiles of Outer Membrane Vesicles and Extracellular Matrix of *Pseudomonas aeruginosa* Biofilms. *Journal of Proteome Research*, **14**(10), pp. 4207-4222.

CROWLESMITH, I., GAMON, K. and HENNING, U., 1981. Precursor proteins are intermediates in vivo in the synthesis of two major outer membrane proteins, the OmpA and OmpF proteins, of *Escherichia coli* K12. *European journal of biochemistry*, **113**(2), pp. 375-380.

CYSTIC FIBROSIS TRUST. 2004. The Burkholderia Cepacia Complex. [Online]. [7 July 2019]. Available from: https://www.cysticfibrosis.org.uk

DARVEAU, R.P., PHAM, T.T., LEMLEY, K., REIFE, R.A., BAINBRIDGE, B.W., COATS, S.R., HOWALD, W.N., WAY, S.S. and HAJJAR, A.M., 2004. *Porphyromonas gingivalis* lipopolysaccharide contains multiple lipid A species that functionally interact with both toll-like receptors 2 and 4. *Infection and immunity*, **72**(9), pp. 5041-5051.

DE LIMA PROCÓPIO, R.E., DA SILVA, I.R., MARTINS, M.K., DE AZEVEDO, J.L. and DE ARAÚJO, J.M., 2012. *Antibiotics produced by Streptomyces*, **16**(5), pp.466-471

DEATHERAGE, B.L. and COOKSON, B.T., 2012. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. *Infection and Immunity*, **80**(6), pp. 1948-1957.

DEMUTH, D.R., JAMES, D., KOWASHI, Y. and KATO, S., 2003. Interaction of *Actinobacillus actinomycetemcomitans* outer membrane vesicles with HL60 cells does not require leukotoxin. *Cellular Microbiology*, **5**(2), pp. 111-121.

DEPLUVEREZ, S., DEVOS, S. and DEVREESE, B., 2016. The Role of Bacterial Secretion Systems in the Virulence of Gram-Negative Airway Pathogens Associated with Cystic Fibrosis. *Frontiers in Microbiology*, **7**, pp. 1336.

DREVINEK, P. and MAHENTHIRALINGAM, E., 2010. *Burkholderia cenocepacia* in cystic fibrosis: epidemiology and molecular mechanisms of virulence. *Clinical microbiology and Infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, **16**(7), pp. 821-830.

DUDLER, R. and EBERL, L., 2006. Interactions between bacteria and eukaryotes via small molecules. *Current Opinion in Biotechnology*, **17**(3), pp. 268-273.

DUTTA, S., IIDA, K., TAKADE, A., MENO, Y., NAIR, G.B. and YOSHIDA, S., 2004. Release of Shiga toxin by membrane vesicles in *Shigella dysenteriae* serotype 1 strains and in vitro effects of antimicrobials on toxin production and release. *Microbiology and Immunology*, **48**(12), pp. 965-969.

EBERL, L. and TUMMLER, B., 2004. *Pseudomonas aeruginosa* and *Burkholderia cepacia* in cystic fibrosis: genome evolution, interactions and adaptation. *International Journal of Medical Microbiology : IJMM*, **294**(2-3), pp. 123-131.

EL-LABANY, S., SOHANPAL, B.K., LAHOOTI, M., AKERMAN, R. and BLOMFIELD, I.C., 2003. Distant cis-active sequences and sialic acid control the expression of fimB in *Escherichia coli* K-12. *Molecular Microbiology*, **49**(4), pp. 1109-1118.

ELLIS, T.N. and KUEHN, M.J., 2010. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiology and Molecular Biology Reviews : MMBR*, **74**(1), pp. 81-94.

FANTAPPIE, L., DE SANTIS, M., CHIAROT, E., CARBONI, F., BENSI, G., JOUSSON, O., MARGARIT, I. and GRANDI, G., 2014. Antibody-mediated immunity induced by engineered *Escherichia coli* OMVs carrying heterologous antigens in their lumen. *Journal of Extracellular Vesicles*, **3**, pp. 10.3402/jev.v3.24015. eCollection 2014.

FERRARI, G., GARAGUSO, I., ADU-BOBIE, J., DORO, F., TADDEI, A.R., BIOLCHI, A., BRUNELLI, B., GIULIANI, M.M., PIZZA, M., NORAIS, N. and GRANDI, G., 2006. Outer membrane vesicles from group B *Neisseria meningitidis* delta gna33 mutant: proteomic and immunological comparison with detergent-derived outer membrane vesicles. *Proteomics*, **6**(6), pp. 1856-1866.

FERRAZ, T.P.L., FIÚZA, M.C., DOS SANTOS, M.L.A., PONTES DE CARVALHO, L. and SOARES, N.M., 2004. *Comparison of six methods for the extraction of lipids from serum in terms of effectiveness and protein preservation.* **58**(3), pp. 187-193.

FLORES-MIRELES, A.L., WALKER, J.N., CAPARON, M. and HULTGREN, S.J., 2015. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. *Nature reviews.Microbiology*, **13**(5), pp. 269-284.

FOX, A., 2016. BACTERIAL CELL ENVELOPE, SPORES AND MACROMOLECULAR BIOSYNTHESIS. [Online]. [7 July 2019]. Available from: https://www.microbiologybook.org/fox/cell_envelope.htm.

FU, L.M., 2002. Is *Mycobacterium tuberculosis* a closer relative to Gram-positive or Gram-negative bacterial pathogens? *Tuberculosis (Edinb)*, **82**(2-3), pp. 85-90.

GALEVA, A., MOROZ, N., YOON, Y.H., HUGHES, K.T., SAMATEY, F.A. and KOSTYUKOVA, A.S., 2014. Bacterial Flagellin-specific chaperone FliS interacts with anti-sigma factor FlgM. *Journal of Bacteriology*, **196**(6), pp. 1215-1221.

GE HEALTHCARE LIFE SCIENCES. 2019. Sephacryl S-500 HR. [Online]. [7 July 2019]. Available from: https://www.gelifesciences.com/en/ps/shop/chromatography/resins/size-exclusion/sephacryl-s-500-hr-p-0548

GELLATLY, S.L. and HANCOCK, R.E., 2013. *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. *Pathogens and Disease*, **67**(3), pp. 159-173.

GERRITZEN, M.J.H., MARTENS, D.E., WIJFFELS, R.H., VAN DER POL, L. and STORK, M., 2017. Bioengineering bacterial outer membrane vesicles as vaccine platform. *Biotechnology Advances*, **35**(5), pp. 565-574.

GILEAD SCIENCES, INC ASTELLAS. 2018. AmBisome (amphotericin B) liposome for injection. [Online]. [7 July 2019]. Available from: https://www.ambisome.com/ambisome-mechanism-of-action

GORBY, Y., MCLEAN, J., KORENEVSKY, A., ROSSO, K., EL-NAGGAR, M.Y. and BEVERIDGE, T.J., 2008. Redox-reactive membrane vesicles produced by *Shewanella*. *Geobiology*, **6**(3), pp. 232-241.

GORRINGE, A., HALLIWELL, D., MATHESON, M., REDDIN, K., FINNEY, M. and HUDSON, M., 2005. The development of a meningococcal disease vaccine based on *Neisseria lactamica* outer membrane vesicles. *Vaccine*, **23**(17-18), pp. 2210-2213.

GUJRATI, V., KIM, S., KIM, S.H., MIN, J.J., CHOY, H.E., KIM, S.C. and JON, S., 2014. Bioengineered bacterial outer membrane vesicles as cell-specific drug-delivery vehicles for cancer therapy. *ACS Nano*, **8**(2), pp. 1525-1537.

HAAS, B. and GRENIER, D., 2015. Isolation, Characterization and Biological Properties of Membrane Vesicles Produced by the Swine Pathogen *Streptococcus suis*. *PLOS one*, **10**(6), pp. e0130528.

HAJAM, I.A., DAR, P.A., SHAHNAWAZ, I., JAUME, J.C. and LEE, J.H., 2017. Bacterial Flagellin-a potent immunomodulatory agent. *Experimental & Molecular Medicine*, **49**(9), pp. e373.

HAJJAR, A.M., ERNST, R.K., TSAI, J.H., WILSON, C.B. and MILLER, S.I., 2002. Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nature Immunology*, **3**(4), pp. 354-359.

HALL, R.A., TURNER, K.J., CHALOUPKA, J., COTTIER, F., DE SORDI, L., SANGLARD, D., LEVIN, L.R., BUCK, J. and MUHLSCHLEGEL, F.A., 2011. The quorum-sensing molecules farnesol/homoserine lactone and dodecanol operate via distinct modes of action in *Candida albicans*. *Eukaryotic cell*, **10**(8), pp. 1034-1042.

HEDARI, C.P., KHINKARLY, R.W. and DBAIBO, G.S., 2014. Meningococcal serogroups A, C, W-135, and Y tetanus toxoid conjugate vaccine: a new conjugate vaccine against invasive meningococcal disease. *Infection and Drug Resistance*, **7**, pp. 85-99.

HEIMLICH, G., MCKINNON, A.D., BERNARDO, K., BRDICZKA, D., REED, J.C., KAIN, R., KRONKE, M. and JURGENSMEIER, J.M., 2004. Bax-induced cytochrome c release from mitochondria depends on alpha-helices-5 and -6. *The Biochemical Journal*, **378**(Pt 1), pp. 247-255.

HOEKSTRA, D., VAN DER LAAN, J.W., DE LEIJ, L. and WITHOLT, B., 1976. Release of outer membrane fragments from normally growing *Escherichia coli*. *Biochimica et Biophysica Acta*, **455**(3), pp. 889-899.

HOEN, E.N.M.N., VAN DER VLIST, E.J., AALBERTS, M., MERTENS, H.C.H., BOSCH, B.J., BARTELINK, W., MASTROBATTISTA, E., VAN GAAL, E.V.B., STOORVOGEL, W., ARKESTEIJN, G.J.A. and WAUBEN, M.H.M., 2012. Quantitative and qualitative flow cytometric analysis of nanosized cell-derived membrane vesicles. *Nanomedicine: Nanotechnology, Biology and Medicine*, **8**(5), pp. 712-720.

HORNBY, J.M., JENSEN, E.C., LISEC, A.D., TASTO, J.J., JAHNKE, B., SHOEMAKER, R., DUSSAULT, P. and NICKERSON, K.W., 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Applied and Environmental Microbiology*, **67**(7), pp. 2982-2992.

HORSTMAN, A.L. and KUEHN, M.J., 2002. Bacterial surface association of heat-labile enterotoxin through lipopolysaccharide after secretion via the general secretory pathway. *Journal of Biological Chemistry*, **277**(36), pp. 32538-32545.

HU, Q., LIU, P., YU, Z., ZHAO, G., LI, J., TENG, L., ZHOU, M., BEI, W., CHEN, H. and JIN, M., 2010. Identification of a cell wall-associated subtilisin-like serine protease involved in the pathogenesis of Streptococcus suis serotype 2. *Microbial Pathogenesis*, **43**(3-4), pp. 103-109.

HUANG, L.S., COBESSI, D., TUNG, E.Y. and BERRY, E.A., 2005. Binding of the respiratory chain inhibitor antimycin to the mitochondrial bc1 complex: a new crystal structure reveals an altered intramolecular hydrogen-bonding pattern. *Journal of Molecular Biology*, **351**(3), pp. 573-597.

HYNES, S.O., KEENAN, J.I., FERRIS, J.A., ANNUK, H. and MORAN, A.P., 2005. Lewis epitopes on outer membrane vesicles of relevance to *Helicobacter pylori* pathogenesis. *Helicobacter*, **10**(2), pp. 146-156.

ISLES, A., MACLUSKY, I., COREY, M., GOLD, R., PROBER, C., FLEMING, P. and LEVISON, H., 1984. *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. *Journal of Pediatrics*, **104**(2), pp. 206-210.

KADURUGAMUWA, J.L. and BEVERIDGE, T.J., 1997. Natural release of virulence factors in membrane vesicles by *Pseudomonas aeruginosa* and the effect of aminoglycoside antibiotics on their release. *Journal of Antimicrobial Chemotherapy*, **40**(5), pp. 615-621.

KADURUGAMUWA, J.L. and BEVERIDGE, T.J., 1996. Bacteriolytic effect of membrane vesicles from *Pseudomonas aeruginosa* on other bacteria including pathogens: conceptually new antibiotics. *Journal of Bacteriology*, **178**(10), pp. 2767-2774.

KADURUGAMUWA, J.L. and BEVERIDGE, T.J., 1995. Virulence factors are released from *Pseudomonas aeruginosa* in association with membrane vesicles during normal growth and exposure to gentamicin: a novel mechanism of enzyme secretion. *Journal of Bacteriology*, **177**(14), pp. 3998-4008.

KAMIO, Y. and NIKAIDO, H., 1976. Outer membrane of *Salmonella typhimurium*: accessibility of phospholipid head groups to phospholipase c and cyanogen bromide activated dextran in the external medium. *Biochemistry*, **15**(12), pp. 2561-2570.

KATO, S., KOWASHI, Y. and DEMUTH, D.R., 2002. Outer membrane-like vesicles secreted by *Actinobacillus actinomycetemcomitans* are enriched in leukotoxin. *Microbial Pathogenesis*, **32**(1), pp. 1-13.

KATO, Y., MISRA, S., PUERTOLLANO, R., HURLEY, J.H. and BONIFACINO, J.S., 2002. Phosphoregulation of sorting signal-VHS domain interactions by a direct electrostatic mechanism. *Nature Structural Biology*, **9**(7), pp. 532-536.

KESTY, N.C. and KUEHN, M.J., 2004. Incorporation of heterologous outer membrane and periplasmic proteins into *Escherichia coli* outer membrane vesicles. *Journal of Biological Chemistry*, **279**(3), pp. 2069-2076.

KIM, J. and SUDBERY, P., 2011. *Candida albicans*, a major human fungal pathogen. *Journal of Microbiology (Seoul, Korea)*, **49**(2), pp. 171-177.

KIM, S., KIM, K., LEE, S., KIM, E., KIM, M., LEE, E., GHO, Y.S., KIM, J., BISHOP, R.E. and CHANG, K., 2009. Structural modifications of outer membrane vesicles to refine them as vaccine delivery vehicles. *Biochimica et Biophysica Acta (BBA) – Biomembranes*, **1788**(10), pp. 2150-2159.

KLIMENTOVÁ, J. and STULÍK, J., 2015. Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiological Research*, **170**, pp. 1-9.

KNIGHT, S.D. and BOUCKAERT, J., 2009. Structure, function, and assembly of type 1 fimbriae. *Topics in Current Chemistry*, **288**, pp. 67-107.

KNOX, K.W., VESK, M. and WORK, E., 1966. Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of *Escherichia coli*. *Journal of Bacteriology*, **92**(4), pp. 1206-1217.

KOEPPEN, K., HAMPTON, T.H., JAREK, M., SCHARFE, M., GERBER, S.A., MIELCARZ, D.W., DEMERS, E.G., DOLBEN, E.L., HAMMOND, J.H., HOGAN, D.A. and STANTON, B.A., 2016. A Novel Mechanism of Host-Pathogen Interaction through sRNA in Bacterial Outer Membrane Vesicles. *PLOS pathogens*, **12**(6), pp. e1005672.

KOLLING, G.L. and MATTHEWS, K.R., 1999. Export of virulence genes and Shiga toxin by membrane vesicles of *Escherichia coli* O157:H7. *Applied and Environmental Microbiology*, **65**(5), pp. 1843-1848.

KUEHN, M.J. and KESTY, N.C., 2005. Bacterial outer membrane vesicles and the host-pathogen interaction. *Genes & Development*, **19**(22), pp. 2645-2655.

KULP, A. and KUEHN, M.J., 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annual Review of Microbiology*, **64**, pp. 163-184.

KULP, A.J., SUN, B., AI, T., MANNING, A.J., ORENCH-RIVERA, N., SCHMID, A.K. and KUEHN, M.J., 2015. Genome-Wide Assessment of Outer Membrane Vesicle Production in *Escherichia coli*. *PLOS one*, **10**(9), pp. e0139200.

KWON, S.O., GHO, Y.S., LEE, J.C. and KIM, S.I., 2009. Proteome analysis of outer membrane vesicles from a clinical *Acinetobacter baumannii* isolate. *FEMS Microbiology Letters*, **297**(2), pp. 150-156.

LEE, E.Y., BANG, J.Y., PARK, G.W., CHOI, D.S., KANG, J.S., KIM, H.J., PARK, K.S., LEE, J.O., KIM, Y.K., KWON, K.H., KIM, K.P. and GHO, Y.S., 2007. Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli*. *Proteomics*, **7**(17), pp. 3143-3153.

LEE, E.Y., CHOI, D.Y., KIM, D.K., KIM, J.W., PARK, J.O., KIM, S., KIM, S.H., DESIDERIO, D.M., KIM, Y.K., KIM, K.P. and GHO, Y.S., 2009. Gram-positive bacteria produce membrane vesicles: proteomics-based characterization of *Staphylococcus aureus*-derived membrane vesicles. *Proteomics*, **9**(24), pp. 5425-5436.

LEE, E.Y., CHOI, D.S., KIM, K.P. and GHO, Y.S., 2008. Proteomics in gram-negative bacterial outer membrane vesicles. *Mass Spectrometry Reviews*, **27**(6), pp. 535-555.

LEE, J.C. and TIMASHEFF, S.N., 1981. The stabilization of proteins by sucrose. *Journal of Biological Chemistry*, **256**(14), pp. 7193-7201.

LI, Z., CLARKE, A.J. and BEVERIDGE, T.J., 1998. Gram-negative bacteria produce membrane vesicles which are capable of killing other bacteria. *Journal of Bacteriology*, **180**(20), pp. 5478-5483.

LI, Z., CLARKE, A.J. and BEVERIDGE, T.J., 1996. A major autolysin of *Pseudomonas aeruginosa*: subcellular distribution, potential role in cell growth and division and secretion in surface membrane vesicles. *Journal of Bacteriology*, **178**(9), pp. 2479-2488.

LIM, S., WYKER, B., BARTLEY, K. and EISENHOWER, D., 2015. Lim *et al.* respond to "Measurement error and physical activity". *American Journal of Epidemiology*, **181**(9), pp. 659-660.

LIMOLI, D.H., JONES, C.J. and WOZNIAK, D.J., 2015. Bacterial Extracellular Polysaccharides in Biofilm Formation and Function. *Microbiology Spectrum*, **3**(3), DOI:10.1128/microbiolspec.MB-0011-2014.

LO, H., KÖHLER, J.R., DIDOMENICO, B., LOEBENBERG, D., CACCIAPUOTI, A. and FINK, G.R., 1997. Nonfilamentous *C. albicans* Mutants Are Avirulent. *Cell*, **90**(5), pp. 939-949.

LOUTET, S.A. and VALVANO, M.A., 2010. A decade of *Burkholderia cenocepacia* virulence determinant research. *Infection and immunity*, **78**(10), pp. 4088-4100.

JONES, M. 2019. Secretion. [Online]. [7 July 2019]. Available from: https://en.wikipedia.org/wiki/Secretion

KEGG. 2017. Flagellar assembly. [Online]. [7 July 2019]. Available from: https://www.genome.jp/kegg-bin/show_pathway?ko02040

MAI-PROCHNOW, A., CLAUSON, M., HONG, J. and MURPHY, A.B., 2016. Gram positive and Gram negative bacteria differ in their sensitivity to cold plasma. *Scientific Reports*, **6**, pp. 38610.

MANABE, T., KATO, M., UENO, T. and KAWASAKI, K., 2013. Flagella proteins contribute to the production of outer membrane vesicles from *Escherichia coli* W3110. *Biochemical and Biophysical Research Communications*, **441**(1), pp. 151-156.

MANTRI, C.K., CHEN, C.H., DONG, X., GOODWIN, J.S., PRATAP, S., PAROMOV, V. and XIE, H., 2015. Fimbriae-mediated outer membrane vesicle production and invasion of *Porphyromonas gingivalis*. *MicrobiologyOpen*, **4**(1), pp. 53-65.

MAYER, H., THARANATHAN, R.N. and WECKESSER, J., 1985. Analysis of Lipopolysaccharides of Gram-Negative Bacteria. *Methods in Microbiology*, **18**, pp. 157-207.

MCBROOM, A.J., JOHNSON, A.P., VEMULAPALLI, S. and KUEHN, M.J., 2006. Outer membrane vesicle production by *Escherichia coli* is independent of membrane instability. *Journal of Bacteriology*, **188**(15), pp. 5385-5392.

MCBROOM, A.J. and KUEHN, M.J., 2007. Release of outer membrane vesicles by Gram-negative bacteria is a novel envelope stress response. *Molecular Microbiology*, **63**(2), pp. 545-558.

MCCAIG, W.D., KOLLER, A. and THANASSI, D.G., 2013. Production of outer membrane vesicles and outer membrane tubes by *Francisella novicida*. *Journal of Bacteriology*, **195**(6), pp. 1120-1132.

MCCLAIN, M.S., BLOMFIELD, I.C., EBERHARDT, K.J. and EISENSTEIN, B.I., 1993. Inversion-independent phase variation of type 1 fimbriae in *Escherichia coli*. *Journal of Bacteriology*, **175**(14), pp. 4335-4344.

MCCLEAN, S. and CALLAGHAN, M., 2009. *Burkholderia cepacia* complex: epithelial cell-pathogen confrontations and potential for therapeutic intervention. *Journal of Medical Microbiology*, **58**(Pt 1), pp. 1-12.

MERCK. 2019. Lectin from Triticum vulgaris (wheat). [Online]. [7 July 2019]. Available from: https://www.sigmaaldrich.com/catalog/product/sigma/l4895?lang=en®ion=GB

METRUCCIO, M.M., EVANS, D.J., GABRIEL, M.M., KADURUGAMUWA, J.L. and FLEISZIG, S.M., 2016. *Pseudomonas aeruginosa* Outer Membrane Vesicles Triggered by Human Mucosal Fluid and Lysozyme Can Prime Host Tissue Surfaces for Bacterial Adhesion. *Frontiers in Microbiology*, **7**, pp. 871.

MILLER, L. 2010. Analyzing gels and western blots with ImageJ. [Online]. [7 July 2019]. Available from: https://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/

MILLER, M.B. and BASSLER, B.L., 2001. Quorum sensing in bacteria. *Annual Review of Microbiology*, **55**, pp. 165-199.

MIYADAI, H., TANAKA-MASUDA, K., MATSUYAMA, S. and TOKUDA, H., 2004. Effects of lipoprotein overproduction on the induction of DegP (HtrA) involved in quality control in the *Escherichia coli* periplasm. *Journal of Biological Chemistry*, **279**(38), pp. 39807-39813.

MULCAHY, L.A., PINK, R.C. and CARTER, D.R., 2014. Routes and mechanisms of extracellular vesicle uptake. *Journal of extracellular vesicles*, **3**, pp. 10.3402/jev.v3.24641. eCollection 2014.

MURPHY, K., TRAVERS, P. and WALPORT, M., 2007. *Janeway's Immunobiology.* 7th edn. Garland Science.

NAGLIK, J., ALBRECHT, A., BADER, O. and HUBE, B., 2004. *Candida albicans proteinases and host/pathogen interactions.* https://onlinelibrary.wiley.com/doi/full/10.1111/j.1462-5822.2004.00439.x edn. Wiley Online Library: Cellular Microbiology.

NANTEL, A., DIGNARD, D., BACHEWICH, C., HARCUS, D., MARCIL, A., BOUIN, A.P., SENSEN, C.W., HOGUES, H., VAN HET HOOG, M., GORDON, P., RIGBY, T., BENOIT, F., TESSIER, D.C., THOMAS, D.Y. and WHITEWAY, M., 2002. Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Molecular Biology of the Cell,* **13**(10), pp. 3452-3465.

NATALE, P., BRÜSER, T. and DRIESSEN, A.J.M., 2008. Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane—Distinct translocases and mechanisms. *Biochimica et Biophysica Acta (BBA) - Biomembranes,* **1778**(9), pp. 1735-1756.

NEDERGAARD PEDERSEN, J., SKOV PEDERSEN, J. and OTZEN, D.E., 2017. Liprotides assist in folding of outer membrane proteins. *Protein Science,* **27**(2), pp. 451-462.

NEW ENGLAND BIOLABS. 2019. BL21 Competent E coli. [Online]. [7 July 2019]. Available from: https://www.neb.com/products/c2530-bl21-competent-e-coli.

NEW ENGLAND BIOLABS. 2019. FAQ: What is the difference between BL21 and BL21(DE3) competent Ecoli cells?. [Online]. [7 July 2019]. Available from: https://international.neb.com/faqs/2016/01/21/what-is-the-difference-between-bl21-and-bl21-de3-competent-e-coli-cells2.

NISHIYAMA, M., HORST, R., EIDAM, O., HERRMANN, T., IGNATOV, O., VETSCH, M., BETTENDORFF, P., JELESAROV, I., GRUTTER, M.G., WUTHRICH, K., GLOCKSHUBER, R. and CAPITANI, G., 2005. Structural basis of chaperone-subunit complex recognition by the type 1 pilus assembly platform FimD. *The EMBO Journal,* **24**(12), pp. 2075-2086.

O'DONOGHUE, E.J. and KRACHLER, A.M., 2016. Mechanisms of outer membrane vesicle entry into host cells. *Cellular Microbiology,* **18**(11), pp. 1508-1517.

OKUDA, S., SHERMAN, D.J., SILHAVY, T.J., RUIZ, N. and KAHNE, D., 2016. Lipopolysaccharide transport and assembly at the outer membrane: the PEZ model. *Nature Reviews Microbiology,* **14**(6), pp. 337-345.

OLSEN, I. and AMANO, A., 2015. Outer membrane vesicles - offensive weapons or good Samaritans? *Journal of Oral Microbiology,* **7**, pp. 27468-27468.

PFALLER, M.A. and DIEKEMA, D.J., 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clinical Microbiology Reviews,* **20**(1), pp. 133-163.

PHAN, Q.T., MYERS, C.L., FU, Y., SHEPPARD, D.C., YEAMAN, M.R., WELCH, W.H., IBRAHIM, A.S., EDWARDS, J.E.,JR and FILLER, S.G., 2007. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLOS biology,* **5**(3), pp. e64.

POIREL, L., MADEC, J.Y., LUPO, A., SCHINK, A.K., KIEFFER, N., NORDMANN, P. and SCHWARZ, S., 2018. Antimicrobial Resistance in *Escherichia coli*. *Microbiology Spectrum,* **6**(4), DOI: 10.1128/microbiolspec.ARBA-0026-2017.

POST, D.M., ZHANG, D., EASTVOLD, J.S., TEGHANEMT, A., GIBSON, B.W. and WEISS, J.P., 2005. Biochemical and functional characterization of membrane blebs purified from *Neisseria meningitidis* serogroup B. *Journal of Biological Chemistry,* **280**(46), pp. 38383-38394.

PRANGISHVILI, D., HOLZ, I., STIEGER, E., NICKELL, S., KRISTJANSSON, J.K. and ZILLIG, W., 2000. Sulfolobicins, specific proteinaceous toxins produced by strains of the extremely thermophilic archaeal genus *Sulfolobus*. *Journal of Bacteriology,* **182**(10), pp. 2985-2988.

PREMJANI, V., TILLEY, D., GRUENHEID, S., LE MOUAL, H. and SAMIS, J.A., 2014. Enterohemorrhagic *Escherichia coli* OmpT regulates outer membrane vesicle biogenesis. *FEMS Microbiology Letters,* **355**(2), pp. 185-192.

QUAN, S., HINIKER, A., COLLET, J.F. and BARDWELL, J.C., 2013. *Isolation of Bacteria Envelope Proteins.* 1st edn. Humana Press.

RAETZ, C.R. and WHITFIELD, C., 2002. Lipopolysaccharide endotoxins. *Annual Review of Biochemistry,* **71**, pp. 635-700.

RAMSEY, M.M., KORGAONKAR, A.K. and WHITELEY, M., 2009. Quorum-Sensing in Bacteria. In: M. SCHAECHTER, ed, *Encyclopedia of Microbiology (Third Edition)*. Oxford: Academic Press, pp. 357-374.

RASHEED, M.U., THAJUDDIN, N., AHAMED, P., TEKLEMARIAM, Z. and JAMIL, K., 2014. Antimicrobial drug resistance in strains of *Escherichia coli* isolated from food sources. *Revista do Instituto de Medicina Tropical de Sao Paulo,* **56**(4), pp. 341-346.

RECORDS, A.R., 2011. The type VI secretion system: a multipurpose delivery system with a phage-like machinery. *Molecular Plant-Microbe Interactions : MPMI,* **24**(7), pp. 751-757.

REYES-ROBLES, T., DILLARD, R.S., CAIRNS, L.S., SILVA-VALENZUELA, C., HOUSMAN, M., ALI, A., WRIGHT, E.R. and CAMILLI, A., 2018. *Vibrio cholerae* Outer Membrane Vesicles Inhibit Bacteriophage Infection. *Journal of Bacteriology,* **200**(15), DOI: 10.1128/JB.00792-17.

RIVERA, J., CORDERO, R.J.B., NAKOUZI, A.S., FRASES, S., NICOLA, A. and CASADEVALL, A., 2010. *Bacillus anthracis* produces membrane-derived vesicles containing biologically active toxins. *Proceedings of the National Academy of Sciences*, **107**(44), pp. 19002-19007.

RODERER, D., BENKE, S., SCHULER, B. and GLOCKSHUBER, R., 2016. Soluble Oligomers of the Pore-forming Toxin Cytolysin A from *Escherichia coli* Are Off-pathway Products of Pore Assembly. *Journal of Biological Chemistry*, **291**, pp. 5652-5663.

ROY, K., BARTELS, S., QADRI, F. and FLECKENSTEIN, J.M., 2010. Enterotoxigenic *Escherichia coli* elicits immune responses to multiple surface proteins. *Infection and Immunity*, **78**(7), pp. 3027-3035.

SABATE, M., PRATS, G., MORENO, E., BALLESTE, E., BLANCH, A.R. and ANDREU, A., 2008. Virulence and antimicrobial resistance profiles among *Escherichia coli* strains isolated from human and animal wastewater. *Research in Microbiology*, **159**(4), pp. 288-293.

SANTOS, S., ARAUZ, L.J., BARUQUE-RAMOS, J., LEBRUN, I., CARNEIRO, S.M., BARRETO, S.A. and SCHENKMAN, R.P., 2012. Outer membrane vesicles (OMV) production of *Neisseria meningitidis* serogroup B in batch process. *Vaccine*, **30**(42), pp. 6064-6069.

SATO, K., 2011. Por Secretion System of *Porphyromonas gingivalis*. *Journal of Oral Biosciences*, **53**(3), pp. 187-196.

SCHREMPF, H., KOEBSCH, I., WALTER, S., ENGELHARDT, H. and MESCHKE, H., 2011. Extracellular *Streptomyces* vesicles: amphorae for survival and defence. *Microbial Biotechnology*, **4**(2), pp. 286-299.

SCHREMPF, H. and MERLING, P., 2015. Extracellular *Streptomyces lividans* vesicles: composition, biogenesis and antimicrobial activity. *Microbial Biotechnology*, **8**(4), pp. 644-658.

SCHWECHHEIMER, C. and KUEHN, M.J., 2015. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. *Nature Reviews Microbiology*, **13**(10), pp. 605-619.

SCHWECHHEIMER, C., KULP, A. and KUEHN, M.J., 2014. Modulation of bacterial outer membrane vesicle production by envelope structure and content. *BMC Microbiology*, **14**, pp. 324-014-0324-1.

SEIPKE, R.F., BARKE, J., BREARLEY, C., HILL, L., YU, D.W., GOSS, R.J. and HUTCHINGS, M.I., 2011. A single *Streptomyces* symbiont makes multiple antifungals to support the fungus farming ant *Acromyrmex octospinosus*. *PLOS one*, **6**(8), pp. e22028.

SHAN, Q., DWYER, M., RAHMAN, S., GADJEVA, M., 2014. Distinct Susceptibilities of Corneal *Pseudomonas aeruginosa* Clinical Isolates to Neutrophil Extracellular Trap-Mediated Immunity. *Infection and Immunity*, **87**(12), pp. e00383-19.

SHEVCHENKO, A., WILM, M., VORM, O. and MANN, M., 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Analytical Chemistry,* **68**(5), pp. 850-858.

SIGMA-ALDRICH. 2019. Trichloroacetic acid SigmaUltra. [Online]. [7 July 2019]. Available from: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma-Aldrich/Product_Information_Sheet/t9159pis.pdf

SILHAVY, T.J., KAHNE, D. and WALKER, S., 2010. The bacterial cell envelope. *Cold Spring Harbor perspectives in biology,* **2**(5), pp. a000414.

SIMBAC. 2013. Cell wall. [Online]. [7 July 2019]. Available from: http://simbac.gatech.edu/wordpress/wp-content/uploads/2013/04/cellwall-1.png

SIMPANYA, M.F., ANSARI, R.R., LEVERENZ, V. and GIBLIN, F.J., 2008. Measurement of lens protein aggregation *in vivo* using dynamic light scattering in a guinea pig/UVA model for nuclear cataract. *Photochemistry and Photobiology,* **84**(6), pp. 1589-1595.

SINNOTT, C.R. and TEALL, A.J., 1987. Persistent gallbladder carriage of *Salmonella typhi. Lancet (London, England),* **1**(8539), pp. 976.

SOUSA, S.A., RAMOS, C.G. and LEITAO, J.H., 2011. *Burkholderia cepacia* Complex: Emerging Multihost Pathogens Equipped with a Wide Range of Virulence Factors and Determinants. *International Journal of Microbiology,* DOI: 10.1155/2011/607575.

STEIMLE, A., AUTENRIETH, I.B. and FRICK, J., 2016. Structure and function: Lipid A modifications in commensals and pathogens. *International Journal of Medical Microbiology,* **306**(5), pp. 290-301.

STONE, N.R., BICANIC, T., SALIM, R. and HOPE, W., 2016. Liposomal Amphotericin B (AmBisome((R))): A Review of the Pharmacokinetics, Pharmacodynamics, Clinical Experience and Future Directions. *Drugs,* **76**(4), pp. 485-500.

STRAUSS, G. and HAUSER, H., 1986. Stabilization of lipid bilayer vesicles by sucrose during freezing. *Proceedings of the National Academy of Sciences of the United States of America,* **83**(8), pp. 2422-2426.

STUMPE, S., SCHMID, R., STEPHENS, D.L., GEORGIOU, G. and BAKKER, E.P., 1998. Identification of OmpT as the protease that hydrolyzes the antimicrobial peptide protamine before it enters growing cells of *Escherichia coli*. *Journal of Bacteriology,* **180**(15), pp. 4002-4006.

SUDBERY, P.E., 2011. Growth of *Candida albicans* hyphae. *Nature Reviews Microbiology,* **9**(10), pp. 737-748.

TABLAN, O.C., CHORBA, T.L., SCHIDLOW, D.V., WHITE, J.W., HARDY, K.A., GILLIGAN, P.H., MORGAN, W.M., CARSON, L.A., MARTONE, W.J. and JASON, J.M., 1985. *Pseudomonas cepacia* colonization in patients with cystic fibrosis: risk factors and clinical outcome. *Journal of Pediatrics,* **107**(3), pp. 382-387.

TAN, T.T., MORGELIN, M., FORSGREN, A. and RIESBECK, K., 2007. *Haemophilus influenzae* survival during complement-mediated attacks is promoted by *Moraxella catarrhalis* outer membrane vesicles. *Journal of Infectious Diseases,* **195**(11), pp. 1661-1670.

THE FREE DICTIONARY. 2019. Ambisome. [Online]. [7 July 2019]. Available from: https://medical-dictionary.thefreedictionary.com/Ambisome

UNIVERSITY OF NOTTINGHAM. 2019. What is quorum sensing?. [Online]. [7 July 2019]. Available from: https://www.nottingham.ac.uk/quorum/what2.htm

VANDEPUTTE-RUTTEN, L., KRAMER, R.A., KROON, J., DEKKER, N., EGMOND, M.R. and GROS, P., 2001. Crystal structure of the outer membrane protease OmpT from *Escherichia coli* suggests a novel catalytic site. *The EMBO journal,* **20**(18), pp. 5033-5039.

VAUGHAN, A.T., BRACKENBURY, L.S., MASSARI, P., DAVENPORT, V., GORRINGE, A., HEYDERMAN, R.S. and WILLIAMS, N.A., 2010. *Neisseria lactamica* selectively induces mitogenic proliferation of the naive B cell pool via cell surface Ig. *Journal of Immunology (Baltimore, Md.: 1950),* **185**(6), pp. 3652-3660.

VELLA, B.D. and SCHERTZER, J.W., 2014. Understanding and Exploiting Bacterial Outer Membrane Vesicles. In: J. RAMOS, J. GOLDBERG and A. FILLOUX, eds, *Pseudomonas.* 7 edn. Dordrecht: Springer, Dordrecht, pp. 217-250.

WAI, S.N., LINDMARK, B., SODERBLOM, T., TAKADE, A., WESTERMARK, M., OSCARSSON, J., JASS, J., RICHTER-DAHLFORS, A., MIZUNOE, Y. and UHLIN, B.E., 2003. Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. *Cell,* **115**(1), pp. 25-35.

WALCZAK, M.J., PUORGER, C., GLOCKSHUBER, R. and WIDER, G., 2014. Intramolecular donor strand complementation in the *E. coli* type 1 pilus subunit FimA explains the existence of FimA monomers as off-pathway products of pilus assembly that inhibit host cell apoptosis. *Journal of Molecular Biology,* **426**(3), pp. 542-549.

WALKER, L., SOOD, P., LENARDON, M.D., MILNE, G., OLSON, J., JENSEN, G., WOLF, J., CASADEVALL, A., ADLER-MOORE, J. and GOW, N.A.R., 2018. The Viscoelastic Properties of the Fungal Cell Wall Allow Traffic of AmBisome as Intact Liposome Vesicles. *mBio,* **9**(1), pp. 10.1128/mBio.02383-17.

WAN, B., ZHANG, Q., TAO, J., ZHOU, A., YAO, Y. and NI, J., 2016. Global transcriptional regulation by H-NS and its biological influence on the virulence of Enterohemorrhagic *Escherichia coli*. *Gene*, **588**(2), pp. 115-123.

WANG, Y., 2002. The function of OmpA in Escherichia coli. *Biochemical and Biophysical Research Communications*, **292**(2), pp. 396-401.

WEINER, K.S., BARNETT, M.A., LORENZ, S., CASPERS, J., STIGLIANI, A., AMUNTS, K., ZILLES, K., FISCHL, B. and GRILL-SPECTOR, K., 2017. The Cytoarchitecture of Domain-specific Regions in Human High-level Visual Cortex. *Cerebral cortex (New York, N.Y.: 1991)*, **27**(1), pp. 146-161.

WELCH, R.A., BURLAND, V., PLUNKETT, G.,3RD, REDFORD, P., ROESCH, P., RASKO, D., BUCKLES, E.L., LIOU, S.R., BOUTIN, A., HACKETT, J., STROUD, D., MAYHEW, G.F., ROSE, D.J., ZHOU, S., SCHWARTZ, D.C., PERNA, N.T., MOBLEY, H.L., DONNENBERG, M.S. and BLATTNER, F.R., 2002. Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America*, **99**(26), pp. 17020-17024.

XU, X.L., LEE, R.T., FANG, H.M., WANG, Y.M., LI, R., ZOU, H., ZHU, Y. and WANG, Y., 2008. Bacterial peptidoglycan triggers *Candida albicans* hyphal growth by directly activating the adenylyl cyclase Cyr1p. *Cell Host & Microbe*, **4**(1), pp. 28-39.

YARON, S., KOLLING, G.L., SIMON, L. and MATTHEWS, K.R., 2000. Vesicle-mediated transfer of virulence genes from *Escherichia coli* O157:H7 to other enteric bacteria. *Applied and Environmental Microbiology*, **66**(10), pp. 4414-4420.

YOON, S.I., KURNASOV, O., NATARAJAN, V., HONG, M., GUDKOV, A.V., OSTERMAN, A.L. and WILSON, I.A., 2012. Structural basis of TLR5-Flagellin recognition and signaling. *Science (New York, N.Y.)*, **335**(6070), pp. 859-864.

ZGAIR, A.K., 2012. *Escherichia coli* Flagellin stimulates pro-inflammatory immune response. *World Journal of Microbiology & Biotechnology*, **28**(5), pp. 2139-2146.

ZHOU, X. and LI, Y., 2015. *From Healthy Microflora to Disease.* 1st edn. Academic Press.

ZHOU, L., SRISATJALUK, R., JUSTUS, D.E. and DOYLE, R.J., 1998. On the origin of membrane vesicles in gram-negative bacteria. *FEMS Microbiology Letters*, **163**(2), pp. 223-228.

ZHU, W. and FILLER, S.G., 2010. Interactions of *Candida albicans* with epithelial cells. *Cellular Microbiology*, **12**(3), pp. 273-282.

ŻYŁA, D.S., PROTA, A.E., CAPITANI, G. and GLOCKSHUBER, R., 2019. Alternative folding to a monomer or homopolymer is a common feature of the type 1 pilus subunit FimA from enteroinvasive bacteria. *Journal of Biological Chemistry*, **294**(27), pp. 10553-10563.