

**Engineering tolerance to organic esters using
bacterial efflux systems**

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Submitted in accordance with the requirements for the degree
of Doctor of Philosophy

The University of Leeds
School of Biomedical Sciences

July 2020

The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

‘Chapter Four - Characterisation of the PACE protein Acel and its homologues’ contains work performed by the candidate that is included in the following publication: Karl A. Hassan, Varsha Naidu, **Jacob R. Edgerton**, Karla A. Mettrick, Qi Liu, Leila Fahmy, Liping Li, Scott M. Jackson, Irshad Ahmad, David Sharples, Peter J. F. Henderson, and Ian T. Paulsen (2019) Short-chain diamines are the physiological substrates of PACE family efflux pumps. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 18015–18020 (2019).

Author contributions: K.A.H., P.J.F.H., and I.T.P. designed research; K.A.H., V.N., J.R.E., K.A.M., Q.L., L.F., L.L., S.M.J., I.A., D.S., and I.T.P. performed research; K.A.H. and P.J.F.H. analyzed data; and K.A.H., P.J.F.H., and I.T.P. wrote the paper.

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Acknowledgements

I would first like to thank my supervisor, Professor Peter Henderson, whose unwavering enthusiasm for science is infectious. The knowledge and wisdom Peter has shared with me over the past four years has been invaluable and I will never forget the countless scientific and life lessons he has taught me. I am also grateful to Dr. Karl Hassan for the opportunity to contribute to the work on PACE family proteins and his guidance throughout. Professor Adrian Goldman kindly made me welcome in his Laboratory throughout the project.

I would like to thank everyone at Lucite, who funded this project, as well as those at Ingenza, the University of Nottingham and all of our other collaborators. Having the opportunity to travel to so many meetings and see this project tackled from so many different angles has been exciting and enlightening and has taught me just how important collaborative science is. I would particularly like to thank Dr. Graham Eastham, Dr. David Johnson, Dr. Jonathan Runnacles and Mark Reynolds for their input, support, guidance and valuable scientific discussion. It has been a pleasure to be part of this project and I wish the best of luck to those continuing to work on it.

Finally, I'd like to thank my family and friends. To my parents, Christine and David, as well as my grandmother Doreen, who have always supported me in everything I do and without whom I wouldn't be where I am now. Thank you to everyone on Astbury level six for all the fun, laughs, drinks, support and advice. I have learned much from all of the brilliant scientists I have met along the way and made some lifelong friends. Last but not least, to Adam, Alice and Vicky, who have put up with my constant stress levels throughout lockdown and have kept me going right to the end.

Abstract

Microbial hosts have been engineered to produce a diverse range of high-value chemicals including butyl methacrylate (BMA), an acrylic polymer precursor, from renewable feedstocks such as glucose. However, yields are often limited by end product accumulation and toxicity. Owing to their substrate promiscuity, bacterial efflux pumps have previously been exploited for their ability to export toxic end products from the cell, thus increasing productivity and yield.

In order to identify efflux pumps that export BMA, genes encoding bacterial efflux pumps were cloned and expressed in an efflux deficient *E. coli* strain and screened for efflux activity. Cell viability assays demonstrated that the resistance-nodulation-division (RND) family efflux pumps AcrAB-TolC, MexAB-OprM and MexCD-OprJ, as well as the major facilitator superfamily (MFS) transporter MdtM, conferred significantly enhanced tolerance to BMA and a range of other organic esters. Viability assays were combined with a commercially available ATP assay to show that BMA causes a rapid loss of viability, likely by partitioning into biological membranes. Transporters of the RND family were able to increase the LD₅₀ of BMA and reduce both the rate and extent of loss of viability. The efflux pumps identified here represent promising candidates for expression in the BMA production strain to enhance product export and increase productivity.

Bacterial efflux pumps also have diverse physiological functions, although the native substrates of the Proteobacterial antimicrobial compound efflux (PACE) family transporters have remained elusive. The prototypical PACE family gene, *aceI*, and eight of its homologues were expressed in *E. coli* where transport of cadaverine, a biologically and industrially relevant diamine, was identified as a conserved function of PACE proteins. AceI was purified and reconstituted into proteoliposomes, where a

cadaverine-dependent alkalisation of the proteoliposome lumen was observed. These data add to existing evidence that transport of short chain diamines, which play essential roles in a diverse range of cellular processes, is the conserved physiological function of PACE family proteins and that they function as substrate/proton antiporters.

List of abbreviations

ABC	ATP-binding cassette
ATP	Adenosine triphosphate
BIB	Butyl isobutyrate
BMA	Butyl methacrylate
DDM	n-Dodecyl β -D-maltoside
DNA	Deoxyribonucleic acid
EHIB	2-Ethylhexyl isobutyrate
EHMA	2-Ethylhexyl methacrylate
HepIB	Heptyl isobutyrate
HepMA	Heptyl methacrylate
HIB	Hexyl isobutyrate
HMA	Hexyl methacrylate
IPIB	Isopropyl isobutyrate
IPMA	Isopropyl methacrylate
IPTG	Isopropyl- β -D-thiogalactoside
LB	Lysogeny broth
LMV	Large multilamellar vesicles
MATE	Multidrug and toxic compound extrusion
MFS	Major facilitator superfamily
PACE	Proteobacterial antimicrobial compound efflux
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDB	Protein Data Bank

PIB	Pentyl isobutyrate
PMA	Pentyl methacrylate
RND	Resistance-nodulation-division
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SMR	Small multidrug resistance
SUV	Small unilamellar vesicles
Tris	Tris(hydroxymethyl)aminomethane

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Chapter 1

Introduction

1. Introduction

Metabolic engineering describes the modification of host metabolism to redirect and/or increase flux through native or engineered pathways for increased production of valuable metabolites or enhanced cellular properties. Pathways can be systematically designed and optimised, often using *Escherichia coli* as a tractable host system, to produce high value products such as biofuels, pharmaceuticals and monomers from simple sugars and other renewable feedstocks. These approaches provide alternative, sustainable routes of production for compounds typically derived from crude oil. However, intracellular accumulation of often-toxic end products remains a major bottleneck, preventing high yields and economic viability from being achieved.

E. coli has previously been engineered to produce butyl methacrylate (BMA) (Yiakoumetti, unpublished), a ubiquitous acrylic polymer precursor with high commercial value. Toxicity of highly hydrophobic solvent-like compounds such as BMA is of particular concern due to their tendency to accumulate in biological membranes (Isken and de Bont, 1998), disrupting essential cellular processes. Accumulation causes changes in membrane fluidity, integrity and porosity, leading to perturbation of membrane potential (Huffer *et al.*, 2011) and eventually causes a collapse in ATP synthesis (Ramos *et al.*, 1997), thus having a profound effect on cell productivity and survival.

Tolerance engineering is a growing field that aims to overcome these toxicity issues by identifying and exploiting natural host stress responses. Transcriptomic and mutational analyses have highlighted solvent stress responses such as lipid-modifying enzymes (Junker and Ramos, 1999; Heipieper *et al.*, 2003), altered expression of global regulators (Asako *et al.*, 1997; Asako *et al.*, 1999) and up-regulation of efflux systems (Muller *et al.*, 2007; Rojas *et al.*, 2001) as important tolerance mechanisms

and potential targets for tolerance engineering (Kell *et al.*, 2015; Mukhopadhyay, 2015). Naturally occurring efflux pumps are particularly promising solvent-tolerance factors owing to their ability to capture and export substrates from the membranes and periplasm (Tal *et al.*, 2009; Elkins and Nikaido, 2002), where these compounds typically accumulate (Sikkema *et al.*, 1995). In a large-scale industrial process, extracellular release of BMA would allow for continuous product extraction using a two-phase system to maintain a BMA concentration below its toxic threshold, simultaneously increasing yield and simplifying downstream processing (Luo and Lee, 2017; Malinowski, 2001). The present research therefore aims to identify bacterial efflux pumps involved in tolerance to organic esters, particularly BMA, in the hope that their expression in the final production strain results in a more efficient and therefore economically viable process.

1.1 Overview of protein structure

In order to understand the properties and roles of membrane proteins, a basic understanding of protein structure is necessary. Proteins are polymers consisting of individual amino acids, each of which has its own unique structure and properties (Fig. 1.1). Amino acids are covalently bound by a peptide bond between the carboxyl group of one amino acid and the amino group of another to form proteins (Fig. 1.1).

1.1.1 Primary structure

The formation of this bond is energetically unfavourable and thus requires the energy released from ATP hydrolysis to form, resulting in a condensation reaction with the release of one molecule of H₂O (Mathews *et al.*, 2000; Voet and Voet, 2004). This is typically carried out by the ribosome, the molecular machinery responsible for

translation of mRNA into its corresponding amino acid sequence. This sequence of amino acids in a polypeptide chain is referred to as protein primary structure (Mathews *et al.*, 2000; Voet and Voet, 2004).

1.1.2 Secondary structure

The primary structure of a protein will dictate how it folds once synthesised and typically one of two secondary structures will be adopted. An α helix is a right-handed helix formed when the polypeptide chain is twisted around each of its α carbon atoms (Fig. 1.2). This structure is stabilised by the formation of hydrogen bonds between the hydrogen of the N-H group and the oxygen of the C=O group of every fourth amino acid in the chain (Fig. 1.2) (Mathews *et al.*, 2000; Voet and Voet, 2004). An α helix has 3.6 residues per turn and a pitch (distance between turns) of 5.4 angstroms (Fig. 1.2) (Mathews *et al.*, 2000; Voet and Voet, 2004).

The other most commonly occurring secondary protein structure is the β pleated sheet (Fig. 1.2). Rather than forming hydrogen bonds within a single polypeptide chain as observed in α helices, β sheets form hydrogen bonds between adjacent chains running parallel or antiparallel to one another. In these chains, each amino acid is rotated 180° relative to the previous residue, resulting in the characteristic 'pleat', and are stabilised by the formation of hydrogen bonds between the hydrogen of the N-H group and the oxygen of the C=O group of adjacent parallel/antiparallel chains residues per turn and a pitch (distance between turns) of 5.4 angstroms (Fig. 1.2) (Mathews *et al.*, 2000; Voet and Voet, 2004). β sheets formed from antiparallel chains are thought to be more stable than those formed from parallel chains due to the closer packing of antiparallel sheets and therefore shorter hydrogen bond distances.

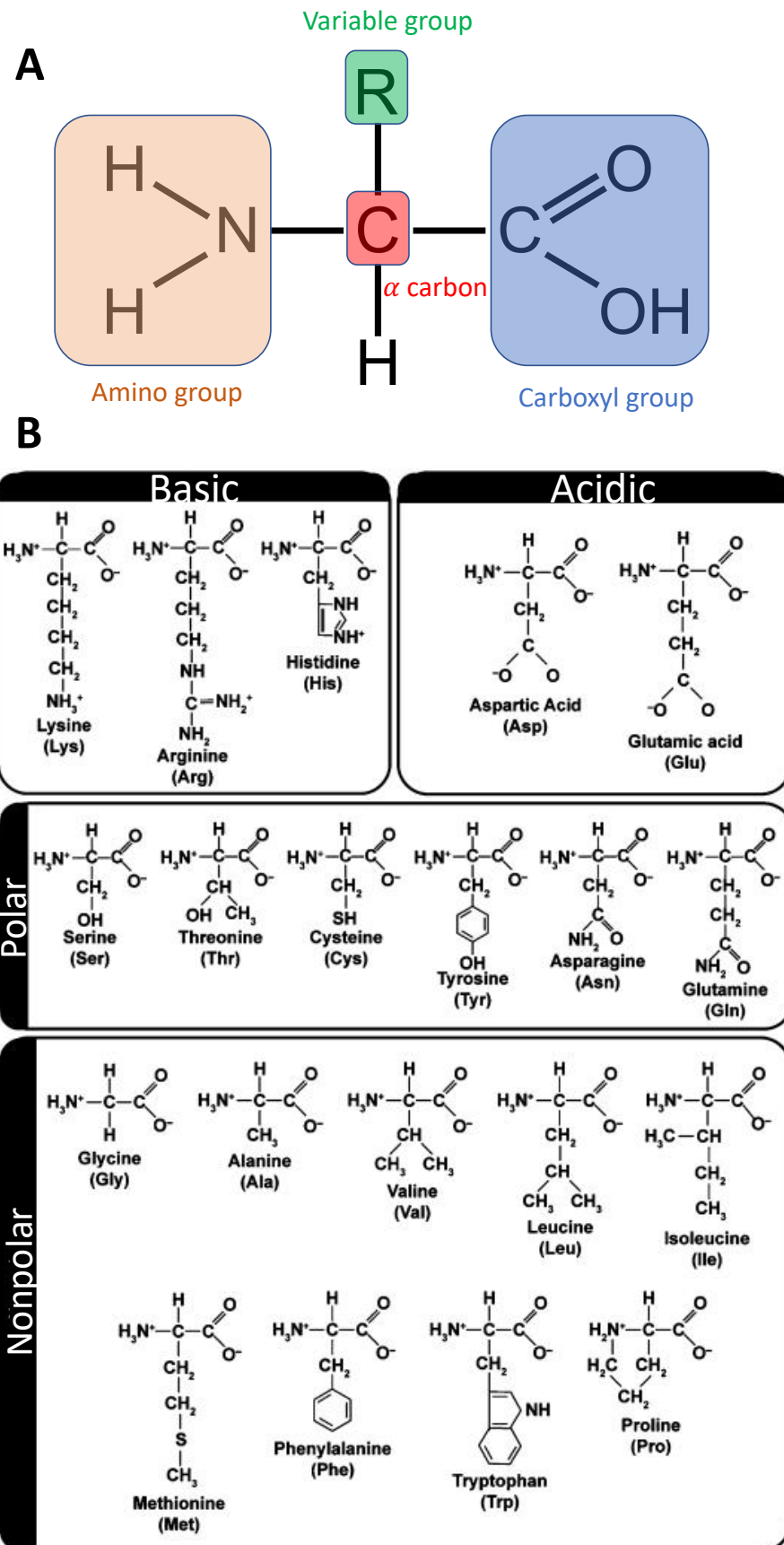


Figure 1.1. General structure of an amino acid (A) and the 20 commonly occurring amino acids (B). Image adapted from Frihart and Birkeland, 2014.

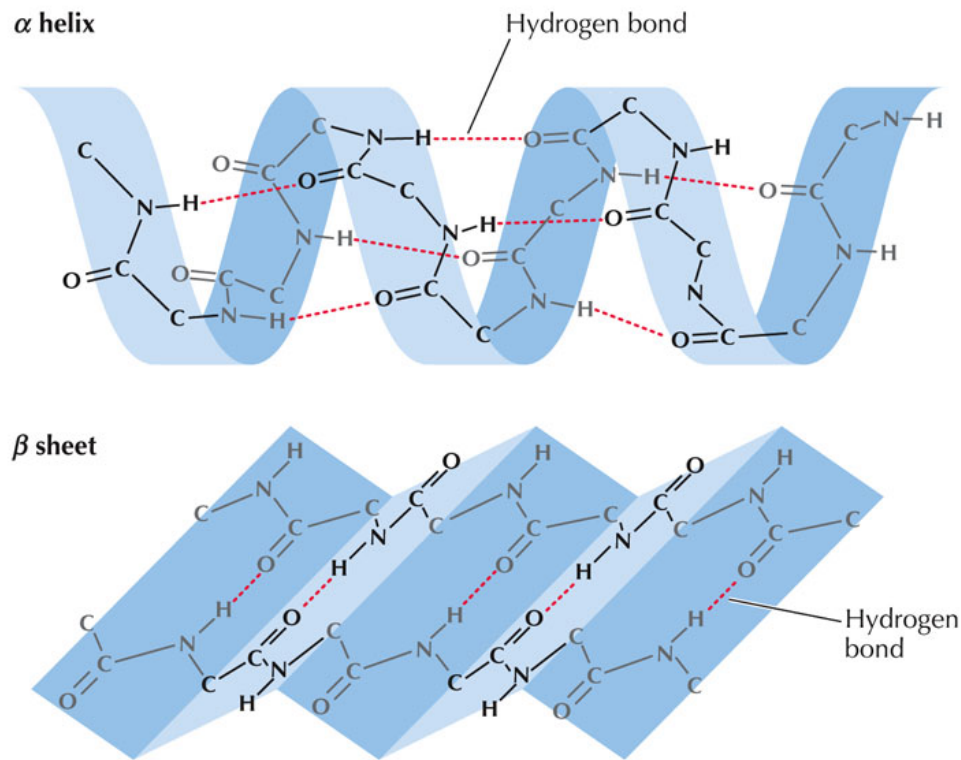


Figure 1.2. The common secondary structures of proteins. Image taken from http://dnacamp.cgrb.oregonstate.edu/w1d5_930.html

1.1.3 Tertiary structure

The tertiary structure of a protein describes the folding of secondary structures into a larger three-dimensional conformation as well as the resulting organisation of amino acid side chains. Fibrous proteins adopt elongated forms and are largely defined by their secondary structure. For example, keratin consists of two α helices coiled around one another (coiled coil) (Voet and Voet, 2004), while fibroins consist of stacked β sheets (Koh *et al.*, 2015). Globular proteins, however, fold due to a combination of hydrophobic interactions, hydrogen bonds, ionic bonds, van der Waals forces and disulphide bridges within a single protein molecule to adopt a much more compact conformation and include enzymes, receptors and transport proteins (Mathews *et al.*,

2000; Voet and Voet, 2004). Globular proteins often consist of both α helices and β sheets.

1.1.4 Quaternary structure

Quaternary structure refers to the assembly of multiple protein subunits into a larger multi-subunit assembly. Proteins interact specifically with one another at complementary interfaces via the same noncovalent interactions that stabilise tertiary structure. These interactions can be transient or result in a more stable complex either with multiple copies of the same protein (homotypic) or with various other proteins (heterotypic). For example, the AcrAB-TolC efflux pump of *E. coli* is an assembly of the AcrB, AcrA and TolC proteins in a 3:6:3 stoichiometry (Du *et al.*, 2014; Shi *et al.*, 2019).

1.2 Bacterial membranes and membrane proteins

Bacteria are broadly categorised into two groups, Gram-positive and Gram-negative. These groups get their name from the Gram stain, which was first described by Hans Christian Gram in 1884 and is still used to this day to categorise bacteria based on their membrane structures (Moyes *et al.*, 2009). Gram-positive bacteria possess a thick, highly cross-linked peptidoglycan layer at the cell surface (Fig. 1.3), which retains a crystal violet/iodine stain complex, staining cells purple (Moyes *et al.*, 2009). Gram-negative bacteria, such as *Escherichia coli*, are characterised by a thin peptidoglycan layer situated between an inner and outer membrane (Fig. 1.3). During a Gram stain, a solvent rinse dissolves this outer membrane and destains the thin peptidoglycan layer, allowing cells to then be counterstained pink using safranin (Moyes *et al.*, 2009). The outer membrane of Gram-negative bacteria acts as a

selective permeability barrier and is responsible for the higher level of intrinsic resistance to a number of toxic agents compared to Gram-positive bacteria (Silhavy *et al.*, 2010; Goessens *et al.*, 2013; Martorana *et al.*, 2014).

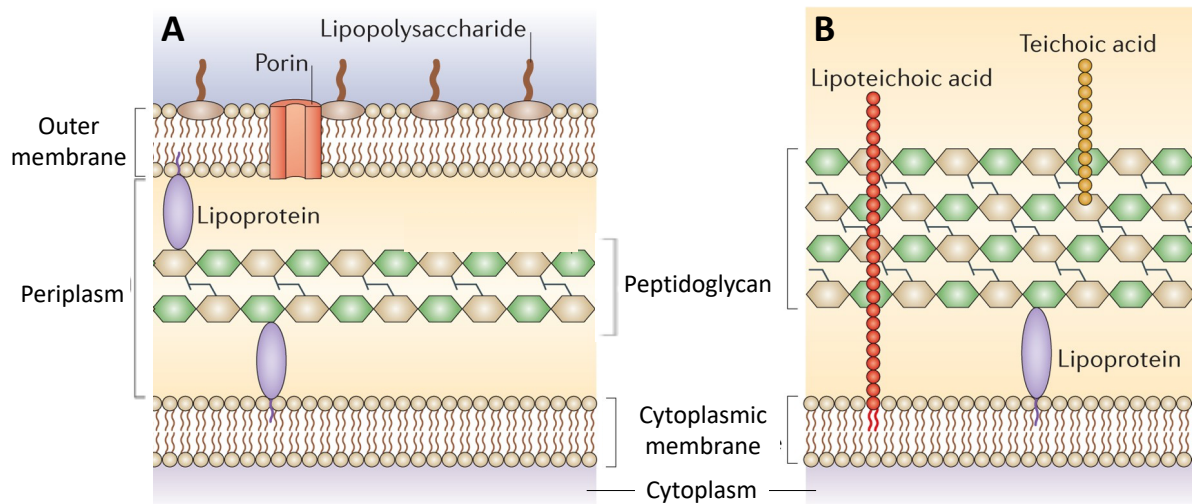


Figure 1.3. The general structure of Gram-negative (A) and Gram-positive (B) bacterial membranes. Gram-negative bacteria possess an inner and outer membrane, the latter of which is rich in lipopolysaccharide and acts as a permeability barrier. Between these two membranes is the periplasm and a thin layer of peptidoglycan. Gram-positive bacteria lack an outer membrane but instead have a much thicker peptidoglycan layer, which is anchored to the cytoplasmic membrane by lipoteichoic acids. Adapted from Brown *et al.*, 2015.

1.2.1 Membrane lipids

Biological membranes are composed primarily of glycerophospholipids (GPLs), which are amphipathic molecules consisting of a hydrophilic head group and hydrophobic lipid tail (Fig. 1.4A; Fig. 1.4B). While membrane composition varies greatly across different bacterial genera, *E. coli* membranes are constituted predominantly of phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) (Sohlenkamp and Geiger, 2015). A glycerol-3-phosphate molecule forms the backbone of the GPL and is esterified with fatty acids at its C1 and C2 positions and with an alcohol at its phosphoryl group (Fig. 1.4A). The most abundant lipid species,

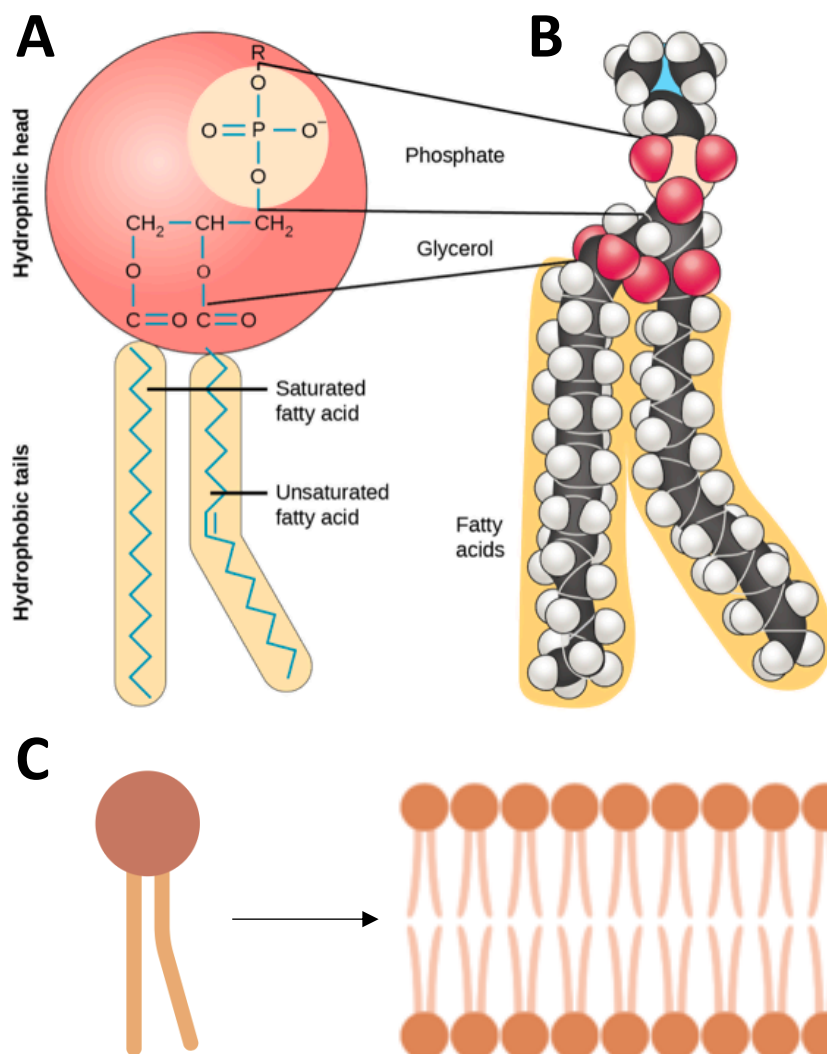


Figure. 1.4. Overview of membrane glycerophospholipids (GPLs). Structural (A) and space-filling model (B) of a GPL and organisation of the lipid bilayer (C). GPLs comprise a glycerol-3-phosphate backbone esterified to two fatty acid chains at C1 and C2 and a variable alcohol (R) at the phosphoryl group. Owing to their amphipathic nature, GPLs spontaneously form bilayers in which the hydrophobic tails pack together and the hydrophilic head groups are exposed to the aqueous environment. Image adapted from <https://alevelbiology.co.uk/notes/lipids-triglyceride-and-phospholipid-synthesis/>

PE, is zwitterionic and constitutes roughly 72-80% of the *E. coli* inner membrane (Morein *et al.*, 1996). As the predominant species, PE is essential to a number of membrane-localised processes such as the electron transfer chain, motility and assembly and function of transport proteins (Mileykovskaya and Dowhan, 1993; Shi

et al., 1993; Bogdanov *et al.*, 1999; Bogdanov *et al.*, 2010). PG and CL are both anionic and constitute approximately 17-21% and 4-7% of the *E. coli* inner membrane, respectively (Morein *et al.*, 1996), and are known to play roles in DNA replication and in the translocation of proteins to the outer membrane (Kusters *et al.*, 1991; Xia and Dowhan, 1995). Lipid modifications are also observed in *E. coli* and often occur in response to environmental stress. For example, cyclopropanation of lipids is commonly seen in cells entering stationary phase, where cell densities are high and nutrients are depleted, and are derived from *in situ* methylenation of the double bond of cis-unsaturated fatty acids (Grogan and Cronan, 1997). *In-silico* models have suggested that the presence of cyclopropanyl lipids simultaneously increases fluidity of the membrane while also promoting a more ordered, stable state of the lipid chains (Poger and Mark, 2015). As such, cyclopropanyl lipids are thought to reduce the permeability of the membrane to toxic compounds and therefore may play a role in tolerance to organic solvents.

Fatty acid tails vary and are synthesised from an acetoacetyl-CoA precursor in *E. coli*, which is derived from the central metabolite acetyl-CoA. This precursor then undergoes cyclic elongation by a series of acyl carrier proteins (ACPs), which are small soluble proteins that are covalently linked to each of the fatty acid intermediates (Lennen and Pflieger, 2012). Bacteria use predominantly fatty acids of 16 or 18 carbon atoms in length and can be saturated or unsaturated, although bacteria usually lack polyunsaturated fatty acids (Cronan and Thomas, 2009). The most abundant fatty acids in *E. coli* are palmitic acid, palmitoleic acid and cis-vaccenic acids (Cronan and Thomas, 2009). The amphipathic nature of GPLs causes them to spontaneously form a bilayer, where the hydrophobic lipid tails face inwards and the hydrophilic heads face outwards to interact with the aqueous environment (Fig. 1.4C) (Engelman, 2005).

1.2.2 Lipopolysaccharide (LPS)

The outer leaflet of the outer membrane of Gram-negative bacteria is structurally distinct as it is composed primarily of lipopolysaccharide (LPS) (Fig. 1.5). LPS is essential to the integrity of the outer membrane and consists of a lipid A anchor, which is attached to a core oligosaccharide region (Nikaido *et al.*, 2003; Zhang *et al.*, 2013; Maldonado *et al.*, 2016). A repeating O-antigen (O-polysaccharide) caps the LPS (Fig. 1.5) and is responsible for the activation of the innate immune system associated with Gram-negative infections, while also protecting the cell from immune attack (Vaara, 1992; Maldonado *et al.*, 2016). LPS is polyanionic and forms electrostatic interactions with adjacent LPS molecules via divalent cations (Mg^{2+} , Ca^{2+}), creating a dense, hydrophilic barrier that is effective in excluding a range of hydrophobic drugs (Vaara, 1992; Erridge *et al.*, 2002; Agrawal and Weisshaar, 2018).

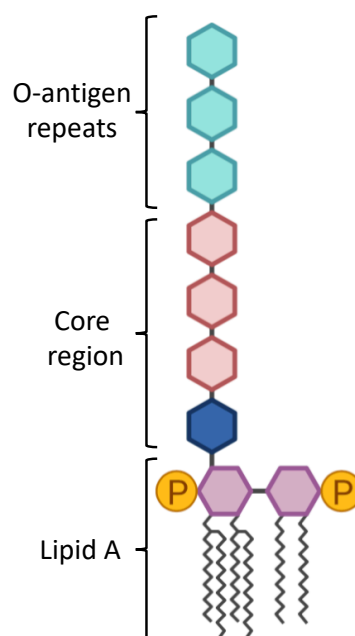


Figure. 1.5. Overview of lipopolysaccharide (LPS) structure in Gram-negative bacteria. Lipid A anchors LPS to the outer membrane and is attached to a core oligosaccharide, which is then capped by a repeating O antigen. Image created using BioRender.com.

1.2.3 Membrane proteins

Proteins are a major component of biological membranes and are thought to be roughly equal to phospholipids by weight in *E. coli* (Kadner, 1996; Strahl and Errington, 2017). Membrane proteins are a diverse group of proteins found in all forms of life, where they are responsible for a range of activities such as membrane transport, cell signalling, signal transduction, homeostasis and pathogenesis (Yeagle, 2016). It is estimated that 20-30% of all genes in both eukaryotes and prokaryotes encode membrane proteins and that this class of proteins accounts for over 50% of all known drug targets (Schlegel *et al.*, 2010; Mathieu *et al.*, 2019), indicating their importance in essential biological functions. Membrane phospholipids have no direct catalytic activity, thus the protein content of a biological membrane broadly determines its function (Yeagle, 2016). Membrane proteins can be broadly categorised into two groups.

1.2.3.1 Integral membrane proteins. Integral membrane proteins (IMPs) have hydrophobic surfaces that allow them to be accommodated within the membrane, where they can span the entire membrane (transmembrane) or are anchored to one side. These characteristic hydrophobic stretches also allow putative identification of IMPs from genome sequences (Ren *et al.*, 2007; Elbourne *et al.*, 2017). Topological predictions can also be made using the 'positive inside rule' first described by von Heijne (1989), who observed that positive residues are abundant in the cytoplasmic domains of IMPs and depleted in external/periplasmic loops (von Heijne, 1989; von Heijne, 2006). Lipids are closely associated with integral membrane proteins and protein-lipid interactions often modulate enzyme activity and stabilise quaternary protein structures (Phillips *et al.*, 2009; Contreras *et al.*, 2011; Pyle *et al.*, 2018; Pyle

et al., 2019). The lipid environment of IMPs is therefore essential for correct function, which is important to consider when engineering strains using components from heterologous hosts. As IMPs are deeply embedded in the membrane, extraction of IMPs requires dissolution of the membrane using amphipathic detergents, which will be discussed later.

In Gram-negative bacteria, inner membrane IMPs are typically α -helical, while outer membrane IMPs are predominantly β -barrels (Shulz, 2002) and are marked for translocation across the membrane(s) by the presence of an N-terminal signal sequence (Beck *et al.*, 2000; Driessen and Nouwen, 2008; Lycklama a Nijeholt and Driessen, 2012). In the case of inner membrane proteins, translocation is thought to happen co-translationally via the Sec translocation machinery and is initiated by binding of the signal recognition particle (SRP) to the N-terminal signal sequence (Valent *et al.*, 1998; Beck *et al.*, 2000; Driessen and Nouwen, 2008). The SRP then directs the nascent polypeptide chain and associated ribosome to the membrane-associated Sec machinery (Ménétret *et al.*, 2007; Frauenfeld *et al.*, 2011; Singh *et al.*, 2014). Alternatively, periplasmic or outer membrane proteins may be translocated post-translationally via the association of the cytoplasmic chaperone SecB, which stabilises the full length preprotein allowing it to reach the Sec machinery in an unfolded state (Valent *et al.*, 1998). The SecYEG heterotrimer forms a pore through which the unfolded protein or nascent polypeptide chain can pass, energised by the associated ATPase 'motor protein' SecA (Lycklama a Nijeholt and Driessen, 2012; Bauer *et al.*, 2014). Once in the periplasm, membrane-bound peptidases cleave the N-terminal signal sequence, while insertases and periplasmic chaperones ensure correct folding and/or insertion of membrane proteins into the inner membrane (Scotti *et al.*, 2000; Luirink *et al.*, 2014).

An extra step is required to ensure that outer membrane proteins are correctly inserted into the outer membrane and is carried out by the Bam (β -barrel assembly machinery) complex. BamA is itself an outer membrane β -barrel pore possessing five polypeptide transport-associated (POTRA) domains on its periplasmic side, which are the binding sites of four associated lipoproteins designated BamB-E (Bakelar *et al.*, 2016; Gu *et al.*, 2016; Han *et al.*, 2016). The mechanism by which translocation of outer membrane proteins occurs is still poorly understood, although existing data suggest multiple periplasmic chaperones, particularly SurA, are responsible for delivering outer membrane proteins to the Bam complex (Rizzitello *et al.*, 2001; Skalar *et al.*, 2007; Fardini *et al.*, 2009). Multiple mechanisms of protein translocation have been suggested (Hagan *et al.*, 2011; Konovalova *et al.*, 2017), but recent data suggest that partially folded outer membrane proteins form an asymmetrical hybrid barrel with the Bam complex, allowing entry into the outer membrane and facilitating folding to result in fully folded, membrane-inserted protein (Doyle and Bernstein, 2019).

1.2.3.2 Peripheral membrane proteins. Conversely, peripheral membrane proteins (PMPs) are weakly associated with the membrane via interactions with IMPs and/or membrane phospholipids. Unlike IMPs, it is difficult to putatively identify PMPs based on their gene sequences as they are soluble (i.e. do not possess characteristic hydrophobic regions) and are not preceded by membrane-targeting sequences (Papanastasiou *et al.*, 2013). PMPs can exist on either side of the membrane, allowing them to carry out functions in the cytoplasm, periplasm or at the cell surface and can be recruited on demand (Papanastasiou *et al.*, 2013). PMPs associate with biological membranes or IMPs through electrostatic and moderate hydrophobic interactions and thus can be dissociated easily from the membrane using high salt concentrations,

altering the pH or using chaotropes without disrupting the lipid bilayer (Papanastasiou *et al.*, 2013; Yeagle, 2016). Their simultaneous interactions with membranes and cellular compartments make them key proteins in electron transport chains, cell signalling and membrane regulation (Yeagle, 2016).

1.2.4 Challenges in membrane protein expression

The natural abundance of membrane proteins is typically too low to isolate sufficient material for functional characterisation, which has led to developments in membrane protein production in heterologous hosts in order to increase yield and simplify the purification process. *E. coli* is the most widely used expression host for bacterial membrane proteins due to its fast growth, availability of genetic tools and ease of manipulation, the ability to reach high cell densities in inexpensive media and its well-characterised genetics, metabolism and physiology (Schlegel *et al.*, 2014; Schlegel *et al.*, 2015; Snijder and Hakulinen, 2016). A number of strategies have been developed to successfully produce membrane proteins in *E. coli* expression hosts but their production, purification and characterisation remains difficult compared to soluble proteins and overexpression often results in poor yields of correctly folded, functional protein (Mathieu *et al.*, 2019). The hydrophobic surfaces of membrane proteins cause them to aggregate in the cytoplasm when produced in large quantities, resulting in large inclusion bodies consisting of the overproduced protein(s) as well as chaperones, proteases and other membrane proteins (Wagner *et al.*, 2007). This appears to happen as a result of saturation of the Sec and BAM membrane protein translocation machinery, resulting in induction of the envelope stress response and up-regulation of periplasmic proteases, eventually resulting in a strong reduction in the

levels of membrane-bound respiratory chain complexes with profound effects on the cell (Alba and Gross, 2004; Wagner *et al.*, 2007; Meuskens *et al.*, 2017).

1.2.5 Expression hosts

A number of engineered *E. coli* strains have been developed and commercialised for their ability to achieve increased yields of membrane proteins. Consistent with the findings of Wagner *et al.* (2007), deletion of genes encoding naturally abundant outer membrane proteins has been shown to improve yield of recombinant membrane proteins, due to reduced strain on cellular machinery (Meuskens *et al.*, 2017). Similarly, *E. coli* strains commonly used in membrane protein expression studies, such as the BL21 and its C43 derivative used in this study, have been shown to possess mutations that reduce levels of promoter-specific RNA polymerases in order to reduce the rate of protein production and prevent overloading of translocation machinery (Miroux and Walker, 1996; Wagner *et al.*, 2007; Wagner *et al.*, 2008; Zhang *et al.*, 2015; Baumgarten *et al.*, 2017). Although this may seem counterintuitive, preventing overloading of cellular machinery by reducing the rate of expression ultimately allows a higher yield to be achieved.

1.2.6 Expression vectors

A wide range of expression vectors have been developed for use in *E. coli* to provide control over production of recombinant proteins. The backbone of any expression vector encodes a selection marker, typically an antibiotic resistance determinant allowing for selection of cells carrying the plasmid, an origin of replication that dictates the plasmid copy number per cell and therefore gene copy number, a multiple cloning site allowing for insertion of a gene of interest and either a constitutive or inducible

promoter. Inducible promoters are particularly useful when producing membrane proteins in *E. coli* as they allow for tuneable expression of toxic genes to be ‘switched on’ on demand, preventing toxic effects of gene expression in the early stages of growth when increasing biomass is the main goal. The most commonly used inducible promoter systems are based on the p_{lac} and p_{BAD} promoters.

1.2.6.1 p_{lac} -based expression. In the p_{lac} promoter system, gene expression is induced by addition of lactose. Isopropyl β -D-1-thiogalactopyranoside (IPTG), a non-hydrolysable lactose analogue, is used in the lab as it is not metabolised by *E. coli* and thus the cellular concentration remains stable. The p_{lac} promoter is followed immediately by an operator site to which the repressor protein LacI binds and blocks transcription. IPTG binds to the LacI repressor, causing it to dissociate from the operator region and allowing transcription to occur (Yildirim and Mackey, 2003; Santillan and Mackey, 2008; Bornwing *et al.*, 2019). Systems such as the T7 expression system rely on lactose/IPTG-inducible expression of specific polymerases on the chromosome to provide tighter regulation than the classical Plac system (Rosano and Ceccarelli, 2014), while Plac fusions such as Ptac provide lower levels of basal expression making them more appropriate for the expression of toxic genes (de Boer *et al.*, 1983; Beckwith, 2013). The p_{tac} promoter was used in this study for the production of Proteobacterial antimicrobial compound efflux (PACE) family proteins and is discussed further in Chapter Four.

1.2.6.2 p_{BAD} -based expression. The p_{BAD} promoter system works in a similar manner to the Plac system but is induced by addition of L-arabinose. Consumption of L-arabinose can be circumvented by the use of *E. coli* BW25113, in which the *araBAD*

genes involved in arabinose metabolism have been deleted (Datsenko and Wanner, 2000; Fritz *et al.*, 2014). When L-arabinose is absent, the regulatory protein AraC binds to the I_1 site upstream of the p_{BAD} promoter and distal O_2 site causing looping of the DNA (Fig. 1.6), thus making the promoter physically inaccessible to RNA polymerase and resulting in little to no basal transcription (Schleif, 2000).

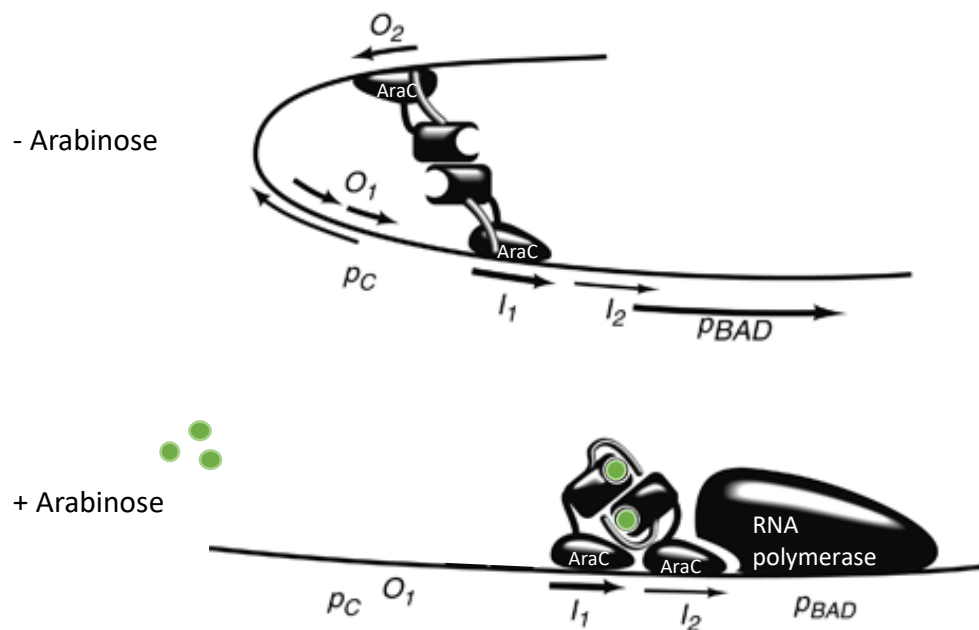


Figure 1.6. Regulation of gene expression by the p_{BAD} promoter system. In the absence of arabinose, the AraC protein binds the I_1 and distal O_2 sites causing looping of the DNA upstream of the promoter, making the promoter inaccessible to RNA polymerase. When arabinose is present, the AraC dimer undergoes a conformational change causing it to release the O_2 site and instead bind I_2 , relaxing the DNA and allowing transcription to occur. Image adapted from Schleif, 2010.

Binding of L-arabinose to the N-terminus of AraC causes conformational changes resulting in one AraC monomer being released from the O_2 site and instead binding the I_2 site adjacent to I_1 (Schleif, 2000). This relaxes the DNA looping, leaving the p_{BAD} promoter accessible to RNA polymerase resulting in markedly increased levels of transcription (Fig. 1.6) (Schleif, 2000). The p_{BAD} system has a significantly lower

basal transcription level and demonstrates an induction/repression ratio of 1,200-fold, compared to just 50-fold for the p_{tac} promoter (Guzman *et al.*, 1995), hence its use in this study.

1.2.7 Purification of membrane proteins

Once appropriate expression levels are achieved, purification is the next hurdle faced in the characterisation of membrane proteins. Owing to their hydrophobicity and incorporation into the membrane, IMPs are insoluble in aqueous buffers and require extraction from their native lipid environment prior to purification. Detergents provide a more tractable environment for the study of IMPs and have been widely used to purify and study IMPs from all manner of organisms (Seddon *et al.*, 2004; Vinothkumar and Henderson, 2010; Anandan and Vrielink, 2016; Rawson *et al.*, 2016). Detergents are amphipathic and consist of a hydrophilic headgroup and hydrophobic tail group and therefore mimic the native lipid environment of IMPs (Seddon *et al.*, 2004). Detergents used to extract membrane proteins are broadly categorised into three groups. Ionic detergents such as sodium dodecyl sulphate (SDS) possess a charged head group and are excellent at solubilising membranes but are typically harsh and denature proteins to varying extents. Zwitterionic detergents, such as 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), have both positively and negatively charged groups resulting in a neutral net charge and are generally less denaturing than ionic detergents (Seddon *et al.*, 2004; Anandan and Vrielink, 2016). Nonionic detergents are characterised by an uncharged head group and are typically milder (ie. less denaturing) than both ionic and Zwitterionic detergents as they are less likely to disrupt protein-protein interactions, resulting in better retention of protein structural and functional properties (Seddon *et al.*, 2004; Anandan and

Vrielink, 2016). An example of a nonionic detergent is n-Dodecyl β -D-maltoside (DDM), which is used widely in the field of membrane biology due to its compatibility with a broad range of membrane proteins (Seddon *et al.*, 2004; Smith, 2011; Anandan and Vrielink, 2016). The amphipathic nature of detergents means they spontaneously form spherical micelles in solution (Fig. 1.7) when above their respective critical micelle concentration (CMC). The detergent micelles have a hydrophobic core perfect for capturing hydrophobic membrane proteins and it is therefore essential that detergent is included in all purification buffers well above its CMC in order to effectively disrupt the membrane, delipidate the protein of interest and prevent precipitation of the hydrophobic protein (Helenius and Simons, 1975, Schimerlik, 1998; Lin and Guidotti, 2009).

Delipidation of membrane proteins can however lead to further loss of activity as the native lipid environment often modulates activity and stabilises structure of IMPs (Phillips *et al.*, 2009; Contreras *et al.*, 2011; Pyle *et al.*, 2018; Pyle *et al.*, 2019). Various methods have been developed to overcome this, including the employment of a styrene-maleic acid (SMA) copolymer to act as a 'molecular cookie cutter'. When used to solubilise membranes, SMA forms ~10 nm discs, termed SMA lipid particles (SMALPs), consisting of membrane proteins and their surrounding lipid bilayer encased in the SMA polymer (Postis *et al.*, 2015; Dorr *et al.*, 2016; Simon *et al.*, 2018). This retention of the local lipid environment means that membrane proteins purified this way are more thermostable and more likely to retain their native structure and function than when purified in detergent (Postis *et al.*, 2015; Dorr *et al.*, 2016; Simon *et al.*, 2018).

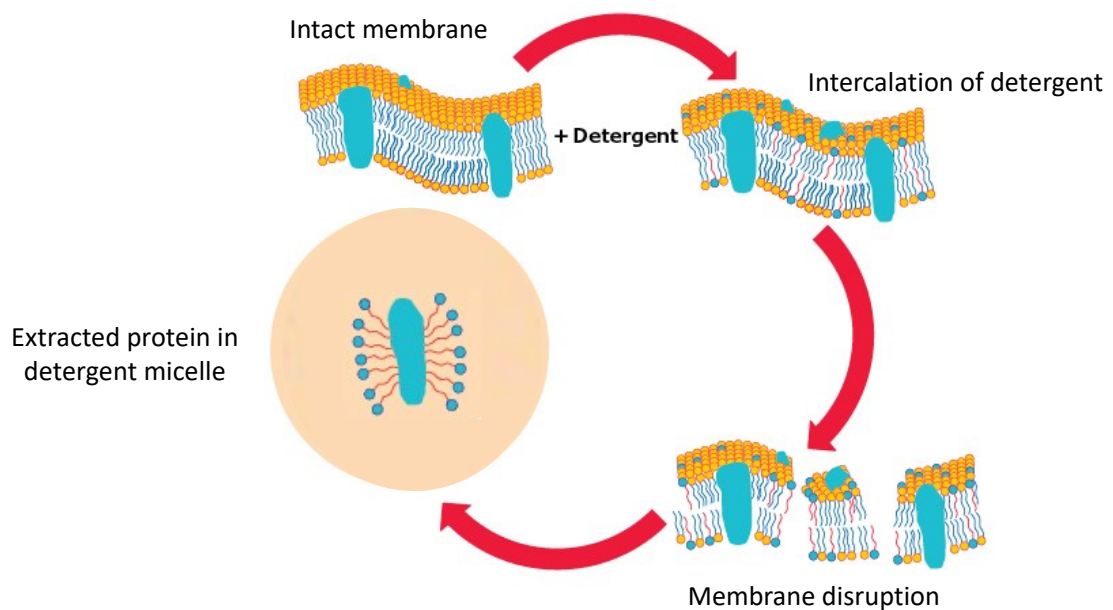


Figure 1.7. Overview of membrane protein solubilisation using detergents. Amphipathic detergents first intercalate with biological membranes and as the concentration increases, so does the level of membrane disruption. Above their respective critical micelle concentration (CMC), detergents form micelles with hydrophobic cores that solubilise and stabilise membrane proteins in aqueous buffers. Image adapted from <https://www.sigmaaldrich.com/technical-documents/articles/biology/detergent-solubilization-of-membrane-proteins.html>

Following solubilisation, membrane proteins can be purified following standard protocols with the addition of solubilising agent. Poly-histidine tags are widely used in the purification of membrane proteins as they are small and rarely disrupt IMP structure, stability or function (Bornhorst and Falke, 2000; Graslund *et al.*, 2008; Smith, 2011), hence their use in this study. Solubilising agents can however cause problems in downstream applications and it is therefore necessary to minimise their concentration following purification. Excess detergent can interfere with protein quantification assays, chromatography columns, SDS-PAGE and crystallisation efforts and can be removed using dialysis, a hydrophobic adsorption matrix, gel chromatography or ultrafiltration (Seddon *et al.*, 2004). Excess SMA can be removed

from solution by dialysis, although SMALPs are known to rapidly lose structural integrity in the presence of divalent cations (Simon *et al.*, 2018), which should be considered when designing experiments using SMA-solubilised protein.

1.2.8 Reconstitution of membrane proteins

Reconstitution of membrane proteins into lipid vesicles is a technique that has been widely used to study the function of membrane proteins in isolation from native IMPs of the expression host. While detergent-solubilised membrane proteins are limited to substrate binding studies, reconstituted systems provide a more native environment in which to study the mechanism of transport across a membrane. Proteoliposomes can be formed by mixing detergent solubilised protein and detergent solubilised lipids, then slowly reducing the detergent concentration using dialysis, hydrophobic adsorption or gradual dilution (Sejwal *et al.*, 2017). However, reconstitution of membrane proteins into preformed liposomes has become a preferred method as it is reported to result in more unidirectional insertion of proteins (Rigaud and Levy, 2003) and will be the method discussed here.

1.2.8.1 Formation of empty liposomes. The first step in liposome preparation is to rehydrate a dried lipid film using the appropriate buffer to form large multilamellar vesicles (LMVs) (Fig. 1.8). Lipid composition can be altered easily to investigate the roles of specific lipid-protein interactions. Total *E. coli* polar lipid extract is commercially available and is isolated from total biomass by solvent extraction using polar solvents such as chloroform and methanol. This polar lipid extract is commonly used as the composition roughly reflects that of whole cells and in the case of this study, the lipid composition is relevant to the final production strain.

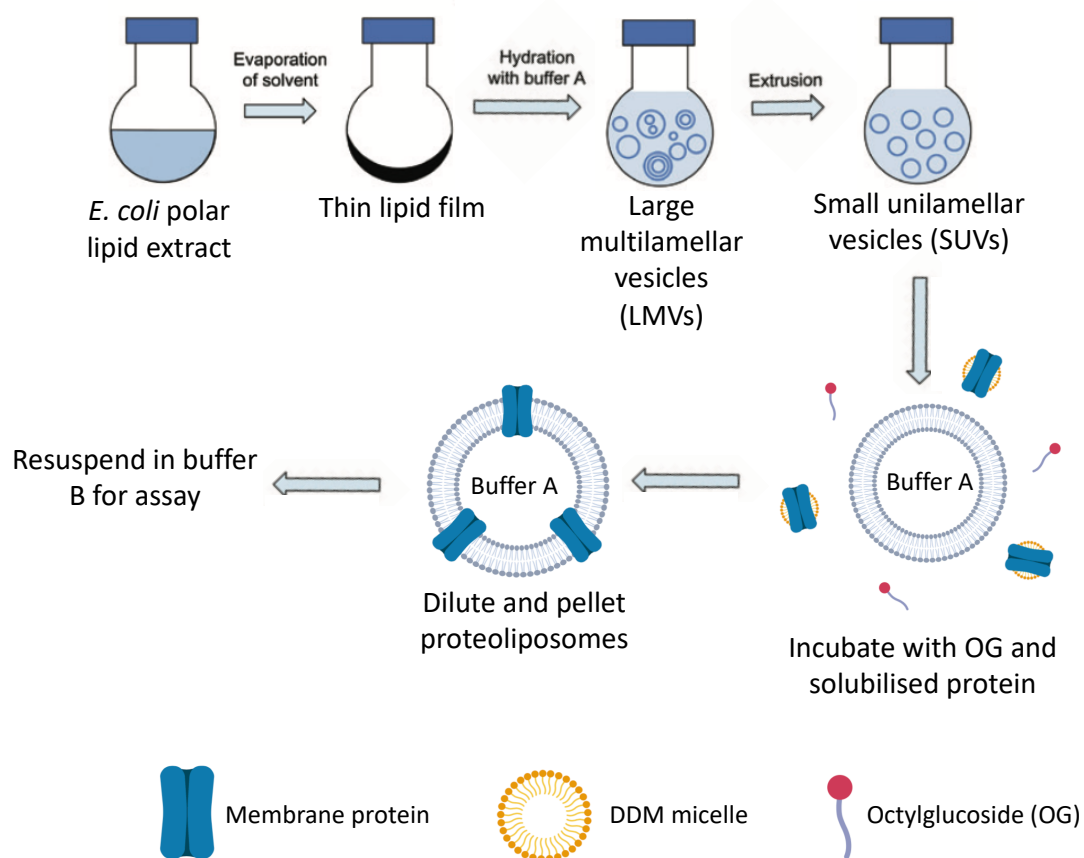


Figure 1.8. An overview of reconstitution of membrane proteins. To generate empty liposomes, *E. coli* lipid extract is dried and rehydrated using the appropriate buffer (shown here as buffer A). Rehydration causes the lipids to spontaneously form large multilamellar vesicles (LMVs), which can then be extruded through a filter membrane to generate small unilamellar vesicles (SUVs). Addition of octylglucoside (OG) destabilises the SUV membrane and allows insertion of a detergent-solubilised membrane protein. Excess OG is then removed by dilution and sedimentation of proteoliposomes, which can then be diluted in an appropriate buffer B to generate electrochemical gradients across the proteoliposome membrane for measurements of transport activity. Image adapted from Sejwal *et al.*, 2017

The chosen rehydration buffer will dictate the conditions in the (proteo)liposome lumen and can be tuned easily to most applications, for example to a desired pH or to include/exclude substrates, salts and environment sensitive dyes. Prior to addition of protein, LMVs are broken down to generate small unilamellar vesicles (SUVs) using

freeze thawing, sonication, homogenisation or extrusion through a filter membrane (Fig. 1.8). Extrusion has a number of benefits including tunability, reproducibility and homogeneous size distribution (Mui *et al.*, 2003; Ong *et al.*, 2016), while also being relatively gentle compared to the other methods listed above.

1.2.8.2 Incorporation of protein to generate proteoliposomes. Generation of proteoliposomes is achieved by addition of a membrane protein solubilised in mild detergent, such as DDM. Addition of a second detergent with a high CMC, which facilitates later removal, intercalates with SUV lipids to destabilise them and allow insertion of the detergent-solubilised protein (Fig. 1.8). Octylglucoside is typically used for this purpose and can then be removed by either hydrophobic adsorption, dialysis or by rapid dilution, then proteoliposomes harvested by ultracentrifugation (Rigaud and Levy, 2003; Sejwal *et al.*, 2017). More recently, reconstitution into lipid bilayers has been achieved following SMA solubilisation of IMPs, circumventing detergent-solubilisation and therefore delipidation altogether (Dorr *et al.*, 2014; Smirnova *et al.*, 2018).

It is also important to understand in which direction the reconstituted protein is inserted into the proteoliposome as measurements of transport are of course dependent on orientation. A range of methods have been used to determine the orientation of membrane proteins in lipid vesicles including electron microscopy, proteolytic digestion, antibody labelling, inhibitor studies and spectroscopic studies (Madden, 1988; Rigaud and Levy, 2003). Resuspension of harvested proteoliposomes in a buffer different to that of the lumen, along with addition of ionophores such as nigericin, valinomycin or CCCP, allows for manipulation of

electrochemical gradients in order to study the mechanism/energetics of transport. This is discussed further in Chapter Four.

1.3 Membrane transport

The hydrophobic phospholipid bilayer acts as a physical barrier to ionic and polar substances, necessitating the presence of membrane transport proteins that mediate uptake or removal of nutrients, signalling molecules, secretory proteins, secondary metabolites and more. Transport across biological membranes can occur down or against a concentration gradient and be either passive or active.

1.3.1 Simple and facilitated diffusion

Diffusion describes the passive movement of a solute down its concentration gradient ie. from an area of high local concentration to an area of low local concentration (Voet and Voet, 2004). Lipophilic compounds and small, uncharged molecules such as oxygen and carbon dioxide can freely cross the bilayer by simple diffusion. Facilitated diffusion is the term used to describe diffusion of a solute across the membrane via a protein channel. In both cases, the energy required for transport is derived solely from the concentration gradient itself and therefore the rate of transport is proportional to the size of the gradient (Mathews *et al.*, 2000; Voet and Voet, 2004). Porins are a well-characterised group of membrane proteins that form water-filled channels in the membrane allowing for facilitated diffusion of a range of solutes (Fig. 1.9). In *E. coli*, three major porins have been identified and display broad selectivity for cationic molecules in the case of OmpC and OmpF and for anionic molecules in the case of PhoE (Vergalli *et al.*, 2020).

1.3.2 Group translocation

In bacteria, uptake of carbohydrates can be mediated by phosphotransferase systems (PTS). These systems consist of an integral membrane protein component and multiple cytoplasmic phosphoryl transfer proteins that work to simultaneously transport the carbohydrate into the cell while also catalysing the transfer of a phosphate group from phosphoenolpyruvate, rather than ATP, to the carbohydrate (Fig. 1.9) (Erni, 1992; Deutscher *et al.*, 2014). These transporters generally have high specificity for a single carbohydrate and systems involved in the uptake of fructose, glucose, mannose, ascorbate and mannitol have been identified and characterised (Erni, 1992; Deutscher *et al.*, 2014). The lipid bilayer is impermeable to phosphorylated sugars so once inside the cell they cannot freely diffuse out (Voet and Voet, 2004).

1.3.3 Primary active transport

Primary active transport of a substrate against its concentration gradient is energetically unfavourable and therefore must be coupled to an exergonic process, such as ATP hydrolysis, to provide the required energy. A relevant example is the ATP-binding cassette (ABC) family of transporters, which are discussed further in Section 1.4.2, and consist of a transmembrane pump domain and a cytoplasmic nucleotide-binding domain responsible for the hydrolysis of ATP (Fig. 1.9) (Rees *et al.*, 2009).

Bacteria also possess both P- and F-type ATPases. P-ATPases have been shown to hydrolyse ATP to pump a range of ions such as H^+ , Na^+ , K^+ , Ca^{2+} and Mg^{2+} from the cytoplasm, generating electrochemical gradients that are essential for various cellular processes including secondary active transport (Mathews *et al.*, 2000; Voet and Voet, 2004). Although F-ATPases are also able to hydrolyse ATP to transport

Na^+ or H^+ from the cytoplasm, their primary function is as an ATP synthase. The F-ATP synthases harness the energy stored in the transmembrane H^+ or Na^+ gradient by allowing movement of these ions down their concentration gradient into the

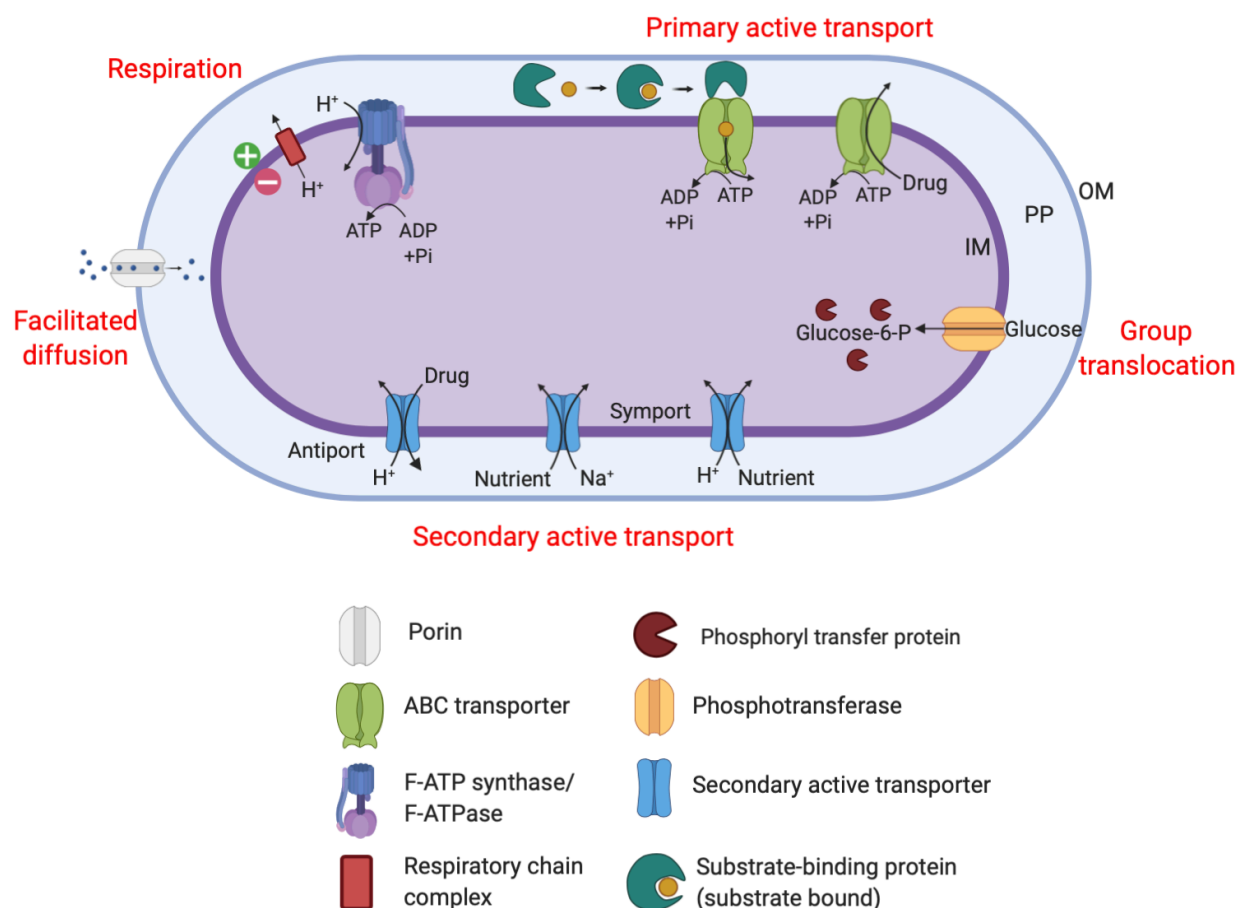


Figure 1.9. Bacterial membrane transport. A simplified view of an *E. coli* cell is shown with the outer membrane (OM) shown in blue, the inner membrane (IM) shown in purple and the periplasm (PP) shown in light blue. An electrochemical gradient of H^+ is generated by respiration, or by photosynthesis in photosynthetic bacteria, which is then used to drive ATP synthesis via F-ATP synthase. F-ATP synthase can also have ATPase activity, hydrolysing ATP to drive H^+ transport in the opposite direction (primary active transport). A simplified view of facilitated diffusion, PEP-dependent group translocation and both primary and secondary active transport are also shown. Image created using BioRender.com.

cytoplasm. This movement of ions drives the functional rotation of the F-ATP synthase complex to generate ATP from ADP and inorganic phosphate (Pi) (Mathews *et al.*, 2000; Voet and Voet, 2004) (Fig. 1.9).

1.3.4 Secondary active transport

Secondary active transporters derive their energy from electrochemical gradients generated during respiration by respiratory chain complexes (Fig. 1.9). These transporters use the movement of ions down a concentration gradient to energise the transport of a substrate against its concentration gradient and can function as antiporters or symporters. An antiport mechanism involves the simultaneous movement of substrate and the driving ion (for example H⁺) in opposite directions across a membrane, while symporters move both in the same direction (Fig. 1.9) (Boudka and Verdon, 2010; Forrest *et al.*, 2011). Secondary active transporters have a range of physiological functions including but not limited to nutrient uptake, cell signalling, homeostasis and extrusion of toxic compounds (Voet and Voet, 2004; Boudka and Verdon, 2010; Forrest *et al.*, 2011). Other than the ABC family, all other efflux pumps found in bacteria are secondary active transporters.

1.4 Bacterial efflux pumps

Bacterial efflux pumps have been widely studied for their diverse physiological roles in virulence, pathogenicity, homeostasis, quorum sensing, heavy metal extrusion and particularly for their roles in antibiotic resistance (Piddock, 2006; Sun *et al.*, 2014; Li *et al.*, 2015; Blanco *et al.*, 2016; Alav *et al.*, 2018). These pumps can be categorised into six major families based on their sequence and structure.

1.4.1 Resistance-nodulation-division (RND)

RND family transporters are the largest of the bacterial efflux pumps and consist of 12 transmembrane helices as well as a large periplasmic domain and typically assemble to form functional trimers that act as substrate/proton antiporters (Fig. 1.10) (Murakami *et al.*, 2002; Sennhauser *et al.*, 2009; Tsutsumi *et al.*, 2019). The trimeric RND pump then also associates with an outer membrane channel via a periplasmic linker protein to allow transport of substrates across the outer membrane of Gram-negative bacteria (Fig. 1.10) (Du *et al.*, 2014; Daury *et al.*, 2016; Tsutsumi *et al.*, 2019). The prototypical member of the RND family is the AcrAB-TolC pump of *E. coli*, which has been studied extensively for its notable substrate promiscuity and has been shown to confer tolerance to diverse antibiotics, organic solvents, detergents, uncoupling agents, dyes, biocides and more (Cha *et al.*, 2014; Ramaswamy *et al.*, 2017; Du *et al.*, 2018). It is widely accepted that RND pumps recruit their substrates from the periplasm and inner membrane (Fig. 1.10) (Elkins and Nikaido, 2002; Lomovskaya and Totrov, 2005; Nikaido and Takatsuka, 2009), making RND pumps particularly important in the removal of hydrophobic/lipophilic agents. In AcrB, at least 2 substrate binding sites have been identified in the periplasmic domains (Yu *et al.*, 2005; Cha *et al.*, 2014) and a possible third site recently proposed (Zwama *et al.*, 2018), while mutational analyses also confirmed that substrate specificity is defined by these periplasmic domains (Elkins and Nikaido, 2002). When no substrate is bound, the AcrB pump exists in an apo state in which all three protomers adopt a loose conformation (Murakami *et al.*, 2006; Wang *et al.*, 2017). The RND trimer is thought to use a peristaltic mechanism to export substrates, in which each protomer cycles through loose, tight and open states upon substrate binding (Murakami *et al.*, 2006; Seeger *et al.*, 2006). Substrates are thought to first interact with AcrB in the loose conformation via a periplasmic

access site and are then moved into the deep substrate pocket in the tight conformation, before finally being extruded in the open conformation via the associated outer membrane channel (Murakami *et al.*, 2006; Seeger *et al.*, 2006; Nikaido, 2010).

1.4.2 ATP binding cassette (ABC)

The ABC family of efflux pumps are primary active transporters that hydrolyse ATP to catalyse transport of substrates across the membrane (Fig. 1.9). ABC transporters are hetero- or homodimeric, with each subunit possessing one nucleotide binding domain (NBD) and one α helical transmembrane domain (TMD) (Dawson and Locher, 2006; Davidson *et al.*, 2008; Wilkens, 2015). Structural data suggest an alternating access mechanism for these pumps, in which transporters shifts between inward open, occluded and outward open conformations driven by hydrolysis of ATP (Jones and George, 2002; Wilkens, 2015). In bacteria, ABC transporters for both uptake and efflux have been identified with some utilising extracytoplasmic solute-binding proteins (SBPs) to bind substrate molecules with high affinity and 'deliver' them to the ABC transporter (Fig. 1.9) (Hosie *et al.*, 2001; Yadava *et al.*, 2016). Examples include MsbA, a lipid 'flippase' in *E. coli* that is responsible for transport of lipid A and lipopolysaccharide from the cytoplasmic leaflet to the periplasmic leaflet of the inner membrane (Fig. 1.10) (Zhou *et al.*, 1998; Doerrler and Raetz, 2002; Ward *et al.*, 2007). The MacB pump of *E. coli* has also been shown to form a large tripartite complex, similar to an RND pump, with the MacA periplasmic linker protein and TolC outer membrane channel to transport substrates across the outer membrane (Fitzpatrick *et al.*, 2017). A periplasmic opening was also identified in MacB, suggesting that this

member of the ABC family captures substrates from the periplasm rather than the cytoplasm (Fitzpatrick *et al.*, 2017).

1.4.3 Major facilitator superfamily (MFS)

MFS transporters have been identified in all domains of life and represent the largest, most diverse group of transporters. They possess 12-14 transmembrane helices organised into two structurally similar domains and typically function as monomers (Fig. 1.10) (Quistgaard *et al.*, 2013; Kumar *et al.*, 2014). Members of the MFS derive their energy from the movement of protons across the membrane and while both symporters and antiporters have been identified, all MFS transporters known to be involved in multidrug resistance are drug/proton antiporters, including EmrD from *E. coli* (Fig. 1.10) (Yin *et al.*, 2006; Yan *et al.*, 2013). Much like ABC transporters, those of the MFS are also thought to function via an alternating access mechanism that is driven by substrate binding and protonation/deprotonation of conserved glutamate and aspartate residues (Quistgaard *et al.*, 2013; Kumar *et al.*, 2014). EmrB of the MFS also forms tripartite assemblies with the outer membrane channel TolC and the periplasmic linker protein EmrA (Lewis, 2000; Tanabe *et al.*, 2009; Tikhonova *et al.*, 2009), similar to an RND assembly. Capture of substrate from the cytoplasm and transport across both the inner and outer membrane of Gram-negative bacteria has been suggested for EmrAB-TolC (Tanabe *et al.*, 2009), although no clear evidence exists to support this mechanism.

1.4.4 Multidrug and toxic compound extrusion (MATE)

In bacteria, MATE transporters are proton and/or sodium-driven antiporters (Fig.1.10). MATE transporters in bacteria are best characterised for their role in export and

therefore tolerance to a number of polyaromatic and cationic antibiotics such as fluoroquinolones and aminoglycosides (Kuroda and Tsuchiya, 2009; Kusakizako *et al.*, 2020). MATE transporters were originally thought to belong to the MFS family due to their predicted 12 transmembrane helices arranged into two transmembrane domains (He *et al.*, 2010). While structural data have revealed MATE family members share a number of common structural features with the MFS and also function as a monomer, the two transmembrane domains appear to be topologically distinct from MFS transporters (He *et al.*, 2010; Radchenko *et al.*, 2015). Examples include the NorM multidrug efflux pump from *Vibrio cholerae*, which has been shown to confer resistance to fluoroquinolones, aminoglycosides and norfloxacin (Morita *et al.*, 1998; Singh *et al.*, 2006; Kitaoka *et al.*, 2011).

1.4.5 Small multidrug resistance (SMR)

Members of the SMR family are known to transport quaternary ammonium compounds and other small cationic antimicrobials in a substrate/proton antiport mechanism and are characterised by their small size, possessing just four transmembrane helices which function as homo- or heterodimers (Fig. 1.10) (Bay *et al.*, 2008). The EmrE transporter from *E. coli* has been the focus of much study and has been shown to function as a dimer in which the two protomers sit antiparallel to one another and exchange conformations in order to alternate between inward open and outward open states (Fig. 1.10) (Morrison *et al.*, 2012; Du *et al.*, 2015). This switching of conformations means that both functional states are in fact identical and differ only in their orientation in the membrane (Morrison *et al.*, 2012; Du *et al.*, 2015). A conserved glutamate residue in transmembrane helix one (TM1) has also been shown to be essential for function of EmrE, indicating a role in proton translocation across the

membrane (Muth and Schuldiner, 2000; Du *et al.*, 2015). Recent research has identified guanidinium, a small cationic metabolic byproduct, as the physiological substrate of SMR family transporters (Kermani *et al.*, 2018).

1.4.6 Proteobacterial antimicrobial compound efflux (PACE)

The most recently described family of efflux pumps is the PACE family. The prototypical PACE family protein, Acel, was first identified as a chlorhexidine resistance determinant in *Acinetobacter baumannii* and sequence homology has since identified numerous other PACE transporters in a range of Gram-negative pathogens,

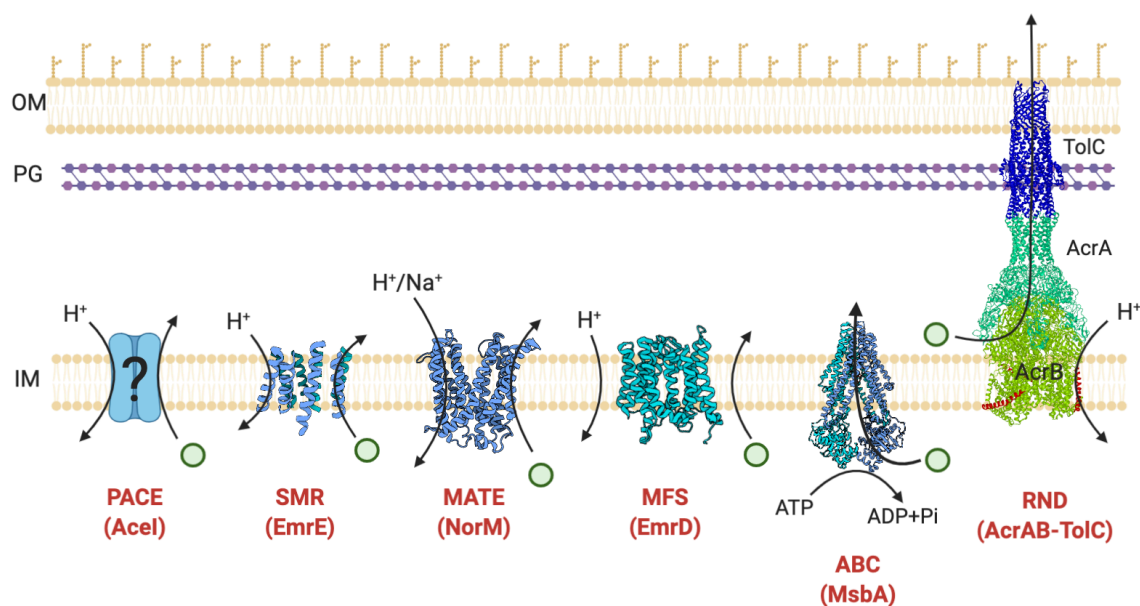


Figure 1.10. Efflux pump families found in bacteria. Members of the Proteobacterial antimicrobial compound efflux (PACE) family, small multidrug resistance (SMR) family, multidrug and toxic compound extrusion (MATE) family, major facilitator superfamily (MFS), ATP-binding cassette (ABC) and resistance-nodulation-division (RND) family are shown. Efflux pumps transport substrates (green circles) across the inner membrane (IM), peptidoglycan layer (PG) and outer membrane (OM) by hydrolysing ATP or in exchange for cations. Example members of each family are shown in brackets and structures from the Protein Databank (PDB) are shown for EmrE (PDB ID 2I68, Fleishman *et al.*, 2006), NorM (3MKU, He *et al.*, 2010), EmrD (2GFP, Yin *et al.*, 2006), MsbA (5TV4, Mi *et al.*, 2017) and AcrAB-TolC (5O66, Wang *et al.*, 2017). Image created using BioRender.com.

although they are not found in the core genome of *E. coli* (Hassan *et al.*, 2013; Hassan *et al.*, 2018). Much like SMR transporters, PACE family proteins are known to transport small cationic antimicrobials such as benzalkonium and acriflavine (Hassan *et al.*, 2015) and are predicted to have four transmembrane helices (Hassan *et al.*, 2018). Experimental data suggest that PACE proteins are proton-driven antiporters (Fig. 1.10) (Hassan *et al.*, 2019) and, like the SMR transporter EmrE, neutralisation of the highly conserved glutamate residue in TM1 has been shown to abolish transport activity (Hassan *et al.*, 2013; Hassan *et al.*, 2019). The oligomeric state of functional PACE transporters is as yet unknown. Acel and other PACE family proteins are the subject of Chapter Four and are discussed further there.

1.5 Microbial production of renewable chemical feedstocks

The development of improved, sustainable bioprocesses in recent years offers exciting new routes of production for a range of biofuels and commodity chemicals and promises to reduce reliance on fossil fuels and crude oil derivatives in these industries (Chen *et al.*, 2013; Adkins *et al.*, 2012; Liao *et al.*, 2016). The Biotechnology and Biological Sciences Research Council (BBSRC) lists industrial biotechnology and bioenergy as their second strategic research priority to ‘reduce dependence on fossil fuels and help drive the UK bioeconomy’ (BBSRC, 2014). For this purpose, microorganisms have been engineered to produce high value commodity chemicals, pharmaceuticals and fuels from renewable feedstocks such as sugars, atmospheric CO₂ and plant biomass and have been reviewed extensively (Rojo, 2008; Liao *et al.*, 2016; Sela *et al.*, 2018; Pham *et al.*, 2019; Jiang *et al.*, 2019). However, production of these compounds is often hindered by their toxicity, inhibiting cell growth and therefore limiting yield.

1.5.1 Methacrylate monomers

Methacrylate esters, particularly methyl methacrylate (MMA), are industrially relevant monomers used in the manufacture of acrylic glass, resins, paints, coatings and more. One major application of these monomers is in the manufacture of acrylic glass (polymethyl methacrylate), which is used in vehicle lights, television and computer displays, commercial signage, work surfaces and medical equipment, to name just a few, due to its excellent durability, UV and abrasion resistance and strength (Lucite International, 2016; Gantrade, 2018).

1.5.2 Chemical synthesis of methacrylate esters

Production of acrylic monomers is traditionally an energy and resource-intensive process, relying on crude oil derivatives and generating large volumes of chemical waste. The current dominant route of MMA synthesis uses acetone and hydrogen cyanide (ACH route) as the two main feedstocks. Acetone cyanohydrin is generated from the reaction between acetone and hydrogen cyanide, which then reacts with excess concentrated sulfuric acid to generate methacrylamide sulphate (Mahboub *et al.*, 2018). Methacrylamide sulphate is then hydrolysed and esterified with excess aqueous methanol to yield MMA and ammonium bisulphate. This process has a number of disadvantages, including the use of the highly toxic reactant hydrogen cyanide and the production of ~1.2 kg of the ammonium bisulphate by-product per 1 kg of MMA (Nagai, 2001; Mahboub *et al.*, 2018). These considerations make the development of an alternative, more sustainable route to manufacture an attractive proposition.

1.5.3 Production of butyl methacrylate in *E. coli*

E. coli has previously been engineered to produce methacrylic acid from glucose, which could then be esterified with an alcohol co-feed to generate methacrylate esters (Eastham *et al.*, 2016). This process offers a renewable, more sustainable route of methacrylate ester production. Butyl methacrylate was chosen as the desired target product due to its reduced toxicity compared to MMA and the reduced toxicity of the butanol co-feed compared to methanol.

The engineered biosynthetic pathway for BMA can be divided into two key steps. The first step in the pathway aims to increase flux through the valine biosynthetic pathway in order to increase production of ketoisovalerate (Fig. 1.11A). Acetolactate synthase (AlsS) from *Bacillus subtilis* converts two molecules of pyruvate, a central metabolite derived from a chosen carbon source such as glucose, into acetolactic acid (Fig. 1.11A) (Aristidou *et al.*, 1994). Acetolactate is then converted to 2-ketoisovaleric acid in a two-step reaction catalysed by acetohydroxy acid isomeroreductase (IlvC) and dihydroxy acid dehydratase (IlvD) from *E. coli* (Park *et al.*, 2007), which is then fed into the second part of the process, the engineered BMA production pathway (Fig. 1.11A).

A branched-chain alpha-ketoacid dehydrogenase (BCKD) from *P. aeruginosa* is responsible for the dehydrogenation of KIV and addition of coenzyme A (CoA) to yield isobutyryl-CoA (Fig. 1.11B) (Sokatch *et al.*, 1981; Sykes *et al.*, 1987). Acyl-CoA oxidase (Acx4) from *Arabidopsis thaliana* then carries out the oxidation of isobutyryl-CoA to methacrylyl-CoA (Rylott *et al.*, 2003), which in the final step of the pathway is esterified with butanol by alcohol acyltransferase (AAT) from *Malus pumila* (apple) to yield butyl methacrylate (Fig. 1.11B) (Souleyre *et al.*, 2005).

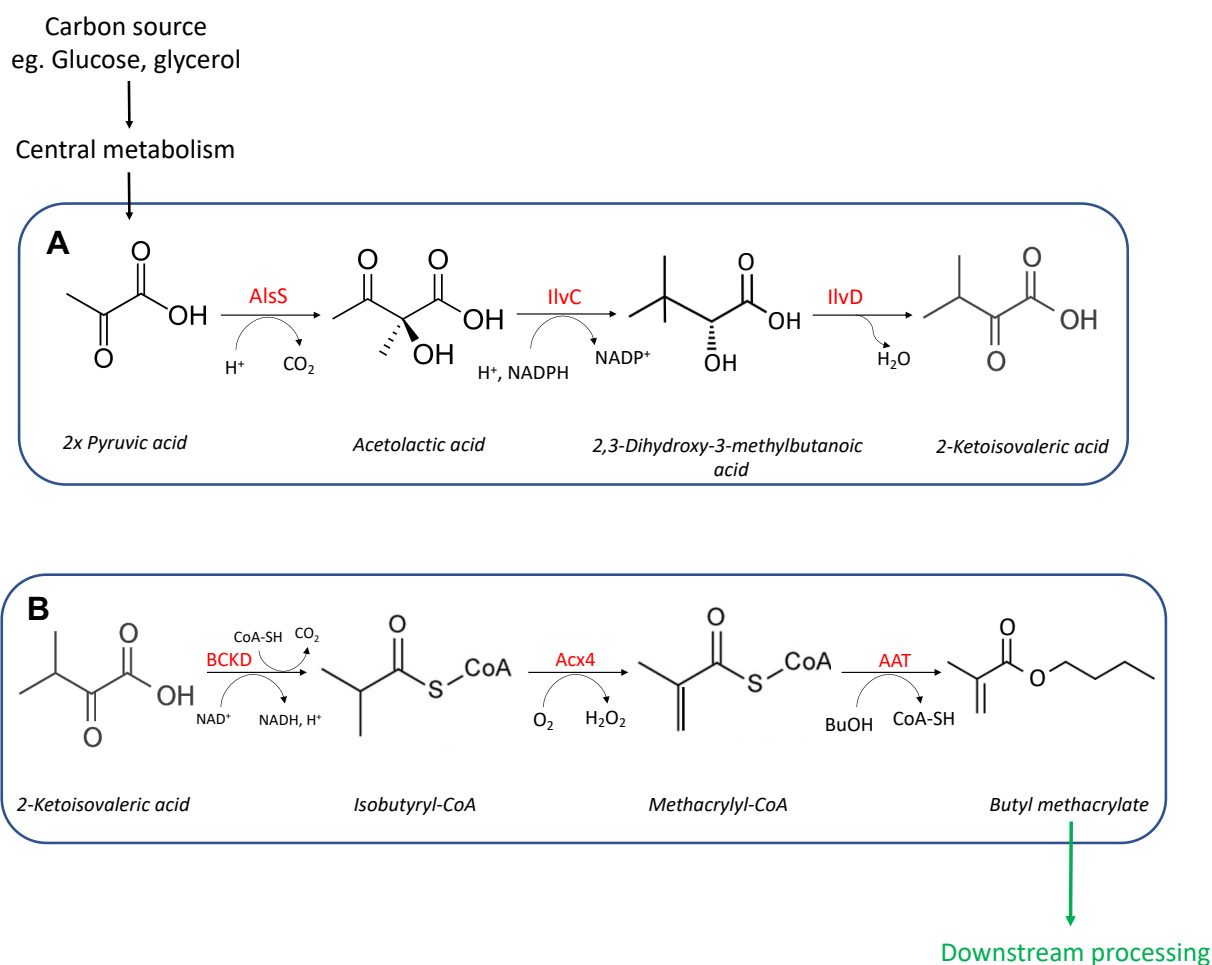


Figure 1.11. Overview of the engineered butyl methacrylate synthetic pathway in *E. coli*. Pyruvate from central metabolism is converted to 2-ketoisovaleric acid by acetolactate synthase (AlsS), acetohydroxy acid isomeroreductase (IlvC) and dihydroxy acid dehydratase (IlvD) (A), which is then converted to butyl methacrylate in a three-step reaction using branched-chain ketoacid dehydrogenase (BCKD), acyl-CoA oxidase (Acx4) and alcohol acyltransferase (AAT) (B).

1.6 Aims and objectives

In order to improve production of BMA in an engineered *E. coli* production strain, this study aims to identify bacterial efflux pumps that may transport BMA out of the cell, thereby maintaining lower intracellular concentrations and increasing cell viability. In the final industrial process, increased cell viability means a greater population of productive cells and therefore higher product yields. Export of BMA from the cell also

allows for continuous product removal, significantly simplifying downstream processing and reducing equipment down-time. These process improvements will result in a more economically viable process and contribute towards the development of a sustainable route to acrylic polymer production from renewable feedstocks such as glucose. Ultimately, this research aims to reduce reliance on crude oil and its derivatives in the production of acrylic polymers, thus greatly reducing the environmental impact of this industry. Research toward this goal was conducted as follows:

1. Identification, using published literature as well as mutational and transcriptomic analyses performed previously, of efflux pumps thought to be involved in tolerance to BMA and other organic solvents.
2. Cloning and expression of these efflux pumps in *E. coli* laboratory strains to facilitate further characterisation.
3. Using the fluorescent substrate Nile Red to measure the activity of cloned efflux pumps and attempt to measure competitive transport between Nile Red and BMA.
4. Using traditional culture-based techniques to measure changes in cell viability upon exposure to BMA and other organic esters and the effect of efflux pump expression on tolerance to these compounds.
5. Measuring cellular ATP content upon exposure to BMA in order to further characterise the effect of BMA on cells, the ability of efflux pumps to enhance tolerance to BMA and the metabolic burden of efflux pump expression and function.

An additional aim of this research was to investigate the physiological roles of the most recently described family of efflux pumps, the Proteobacterial antimicrobial compound efflux (PACE) family. Transport of cadaverine, an industrially relevant diamine used in polyamide production, was measured using radiolabelled uptake assays and a reconstituted proteoliposome system. Acel and its homologues serve as valuable model systems for the study of transport across biological membranes, while also being relevant to the field of biotechnology.

Chapter 2

Materials and Methods

2.1 Sources of materials

Chemicals of the highest available purity were used throughout the project. All solutions were prepared using MilliQ™ water. Media and solutions were sterilised by autoclaving at 121°C for 20 minutes at 15 psi. Heat labile solutions were sterilised using 0.2 µM nitrocellulose filters.

2.1.1 General laboratory reagents

General laboratory reagents were purchased from:

Sigma Chemicals Co., Poole, Dorset, UK

Merck Chemicals Ltd., Chilworth, Southampton, UK

Thermo Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK

Melford Laboratories Ltd., Chelsworth, Suffolk, UK

BDH Chemical Supplies (distributed by VWR international Ltd., Poole, Dorset, UK)

2.1.2 Bacteriological media and additives

Bacteriological yeast extract, tryptone and agar were purchased from Melford Laboratories Ltd.

Carbenicillin disodium salt, kanamycin monosulfate and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were also purchased from Melford Laboratories Ltd.

L(+)-Arabinose was sourced from Acros Organics, distributed by Fisher Scientific, Loughborough, Leicestershire, UK.

2.1.3 Recombinant DNA reagents

Molecular biology grade agarose was purchased from Melford Laboratories Ltd.

All other recombinant DNA reagents, enzymes, molecular weight markers, purification kits and buffers were purchased from New England Biolabs (NEB), distributed by CP Laboratories, Bishop Storton, Herts, UK.

2.1.4 Equipment for membrane isolation and protein purification

n-Dodecyl β -D-maltoside (DDM) was purchased from Anatrace, Maumee, OH, USA.

Ni-NTA agarose resin was purchased from Qiagen Ltd., Crawley, West Sussex, UK.

HisPur cobalt resin was purchased from Thermo Fisher Scientific UK.

Sucrose was purchased from Merck Chemicals Ltd.

Lysozyme was purchased Sigma Chemicals Co.

Econo-Pac disposable columns and Econo-Pac10DG desalting columns were purchased from BioRad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK.

VivaSpin protein concentrators of various volumes and molecular weight cut offs were purchased from Sartorius AG, Göttingen, Germany.

The Pierce BCA protein assay kit was purchased from Thermo Fisher Scientific Ltd.

2.1.5 SDS-PAGE and Western Blotting

40% acrylamide, 2% bis-acrylamide were purchased from BioRad Laboratories Ltd.

Polyvinylidene difluoride (PVDF) membranes of 0.2 μ M pore size were purchased from Roche Holding AG, Basel, Switzerland.

HisProbe™-HRP Conjugate was purchased from Thermo Fisher Scientific UK Ltd.

Clarity Western ECL Substrate was purchased from BioRad Laboratories Ltd.

2.1.6 Reconstitution of membrane proteins

E. coli polar lipid extract and Mini-Extruder set were obtained from Avanti Polar Lipids Inc., Alabaster, AL, USA.

Dithiothreitol was purchased from Melford Laboratories Ltd.

Pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt) was purchased from Sigma Chemicals Co.

n-Octyl-d17- β -D-glucopyranoside-d7 (OG) was purchased from Anatrace.

2.1.7 Assays for transport of radiolabelled substrates

Cadaverine [1,5- ^{14}C] dihydrochloride was purchased from American Radiolabeled Chemicals, St Louis, MO, USA.

n-Butyl [1- ^{14}C]methacrylate was purchased from Pharmaron, Cardiff, Wales, UK.

Emulsifier-Safe liquid scintillation cocktail was purchased from Perkin Elmer, Waltham, MA, USA.

All membrane filters were purchased from Merck Chemicals Limited.

2.1.8 Efflux substrates and dyes

Acriflavine, ethidium bromide, Hoechst 33342 and Nile Red were purchased from Sigma Chemicals Co.

2.1.9 CPM thermostability assay

7-Diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) was purchased from Sigma Chemicals Co.

2.2 General Microbiology

2.2.1 Bacterial strains

The bacterial strains used in this study are detailed in Table 2.1.

Table 2.1. Bacterial strains used throughout this study

Strain/Plasmid	Genotype	Source
<i>E. coli</i> BW25113	F ⁻ , Δ (<i>araD-araB</i>)567, <i>lacZ</i> 4787(<i>del</i>):: <i>rrnB</i> -3, <i>LAM</i> ^r , <i>rph</i> -1, Δ (<i>rhaD-rhaB</i>)568, <i>hsdR</i> 514	Datsenko and Wanner, 2000
<i>E. coli</i> BL21	F ⁻ <i>ompT gal dcm lon hsdS_B</i> (<i>r_B⁻m_B⁻</i>) [<i>malB</i> ⁺] _{K-12} (λ^S)	Studier and Moffatt, 1986
<i>E. coli</i> Omnimax	F' { <i>proAB lacI^q lacZ</i> Δ M15 <i>Tn10</i> (<i>Tet</i> ^R) Δ (<i>ccdAB</i>)} <i>mcrA</i> Δ (<i>mrr hsdRMS-mcrBC</i>) Φ 80(<i>lacZ</i>) Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD</i>	ThermoFisher
<i>E. coli</i> C43 (DE3) Δ <i>acrAB</i>	<i>ompT hsdSB</i> (<i>rB⁻ mB⁻</i>) <i>gal dcm</i> (DE3) Δ <i>acrAB</i>	Du <i>et al.</i> , 2014

2.2.2 Plasmids

pTTQ18 was a gift from Michael Stark (Addgene plasmid # 69122 ; <http://n2t.net/addgene:69122> ; RRID:Addgene_69122). The pBAD expression vector used throughout this study was a gift from Dr. Vincent Postis of Leeds Beckett University. pCR3 and pCG3 (the progenitor of pGNTK constructs) were gifts from Ingenza Ltd., Roslin, Edinburgh. The pTTQ18 *aceI* constructs were created by Hassan *et al.*, (2013), as were the PACE family *aceI* homologue constructs (Hassan *et al.*, 2015). Full details of each plasmid can be found in Table 2.2.

Table 2.2 Plasmids used throughout this study

Strain/Plasmid	Plasmid Description	Source
pBAD- <i>arcABHis₈-tolC</i> Strep	<i>acrA, acrB, tolC</i> . C-terminal His ₈ -tag on <i>acrB</i> and C-terminal StrepII-tag on <i>tolC</i> . p _{BAD} arabinose-inducible promoter	Du <i>et al.</i> , 2014
pBAD- <i>mexAB-oprMHis₈</i>	<i>mexA, mexB, oprM</i> . C-terminal His ₈ -tag on <i>oprM</i> . p _{BAD} arabinose-inducible promoter	This study
pBAD- <i>mexCD-oprJHis₈</i>	<i>mexC, mexD, oprJ</i> . C-terminal His ₈ -tag on <i>oprJ</i> . p _{BAD} arabinose-inducible promoter	This study
pBAD- <i>acrABtolCHis₈</i>	<i>acrA, acrB, tolC</i> . C-terminal His ₈ -tag on <i>tolC</i> . p _{BAD} arabinose-inducible promoter	This study
pBAD- <i>emrABtolCHis₈</i>	<i>emrA, emrB, tolC</i> . C-terminal His ₈ -tag on <i>tolC</i> . p _{BAD} arabinose-inducible promoter	This study
pCR3- <i>tolCacrAB</i>	<i>tolC, acrA, acrB</i> . Constitutive expression.	Ingenza Ltd., Edinburgh
pBAD- <i>emrDHis₈</i>	<i>emrD</i> , C-terminal His ₈ -tag. p _{BAD} arabinose-inducible promoter	This study
pBAD- <i>mdlBHis₈</i>	<i>mdlB</i> , C-terminal His ₈ -tag. p _{BAD} arabinose-inducible promoter	This study
pBAD- <i>msbAHis₈</i>	<i>msbA</i> , C-terminal His ₈ -tag. p _{BAD} arabinose-inducible promoter	This study
pBAD- <i>mdtLHis₈</i>	<i>mdtL</i> , C-terminal His ₈ -tag. p _{BAD} arabinose-inducible promoter	This study
pBAD- <i>mdtMHis₈</i>	<i>mdtM</i> , C-terminal His ₈ -tag. p _{BAD} arabinose-inducible promoter	This study
pBAD- <i>acrAHis₈</i>	<i>acrA</i> , C-terminal His ₈ -tag. p _{BAD} arabinose-inducible promoter	This study
pET24 <i>acrBHis₆</i>	<i>acrB</i> , C-terminal His ₆ -tag, T7 IPTG-inducible promoter	Pos and Diederichs, 2002
pGNTK- <i>acrABtolCHis₈</i>	<i>acrA, acrB, tolC</i> . C-terminal His ₈ -tag on <i>tolC</i> . p _{gntK} promoter	This study
pGNTK- <i>mexAB-oprMHis₈</i>	<i>mexA, mexB, oprM</i> . C-terminal His ₈ -tag on <i>oprM</i> . p _{gntK} promoter	This study
pGNTK- <i>mexCD-oprJHis₈</i>	<i>mexC, mexD, oprJ</i> . C-terminal His ₈ -tag on <i>oprJ</i> . p _{gntK} promoter	This study
pGNTK- <i>emrABtolCHis₈</i>	<i>emrA, emrB, tolC</i> . C-terminal His ₈ -tag on <i>tolC</i> . p _{gntK} promoter	This study

Table 2.2 Plasmids used throughout this study

Plasmid	Plasmid Description	Source
pTTQ18- <i>ace</i> /His ₈	<i>ace</i> / wild-type, C-terminal His ₈ -tag, p _{tac} IPTG inducible promoter	Hassan <i>et al.</i> , 2013
pTTQ18- <i>ace</i> E15QHis ₈	<i>ace</i> / E15Q mutant, C-terminal His ₈ -tag, p _{tac} IPTG inducible promoter	Hassan <i>et al.</i> , 2013
pTTQ18-A1S_1503His ₈	A1S_1503 PACE family gene, C-terminal His ₈ -tag, p _{tac} IPTG inducible promoter	Hassan <i>et al.</i> , 2015
pTTQ18-FbaI_3166His ₈	FbaI_3166 PACE family gene, C-terminal His ₈ -tag, p _{tac} IPTG inducible promoter	Hassan <i>et al.</i> , 2015
pTTQ18-Mlut_15630His ₈	Mlut_15630 PACE family gene, C-terminal His ₈ -tag, p _{tac} IPTG inducible promoter	Hassan <i>et al.</i> , 2015
pTTQ18-PFL_4558His ₈	PFL_4558 PACE family gene, C-terminal His ₈ -tag, p _{tac} IPTG inducible promoter	Hassan <i>et al.</i> , 2015
pTTQ18-PSPTO_3587His ₈	PSPTO_3587 PACE family gene, C-terminal His ₈ -tag, p _{tac} IPTG inducible promoter	Hassan <i>et al.</i> , 2015
pTTQ18-STY3166His ₈	STY3166 PACE family gene, C-terminal His ₈ -tag, p _{tac} IPTG inducible promoter	Hassan <i>et al.</i> , 2015
pTTQ18-TmargHis ₈	Tmarg PACE family gene, C-terminal His ₈ -tag, p _{tac} IPTG inducible promoter	Hassan <i>et al.</i> , 2015
pTTQ18-VP1155His ₈	VP1155 PACE family gene, C-terminal His ₈ -tag, p _{tac} IPTG inducible promoter	Hassan <i>et al.</i> , 2015

2.2.3 Bacterial growth and storage conditions

All *E. coli* strains were grown in lysogeny broth (LB) and on LB agar. All agar plates were incubated at 37°C in a static incubator while all broths were incubated at 37°C with shaking at 200 RPM. Starter cultures of 10 ml in 50 ml Falcon tubes were inoculated with a single *E. coli* colony taken from LB agar and incubated overnight (maximum of 16 hours) at 37°C with shaking at 200 RPM. For long term storage, *E. coli* strains were grown on LB agar overnight then harvested with a sterile cotton swab and suspended in 1 ml volumes of freezing mixture (see Table 2.3). These stock cultures were snap frozen in liquid nitrogen and stored at -80°C. To revive stock

cultures, a small amount of cell material was scraped from the surface of the stock culture using a sterile inoculation loop and streaked onto LB agar with the appropriate antibiotic and incubated overnight at 37°C.

Table 2.3 Growth and storage media composition

Media	Composition for 1 litre
Lysogeny broth	10g granulated tryptone, 5 g granulated yeast extract, 10 g NaCl, pH adjusted to 7.5
Lysogeny broth agar	10 g granulated tryptone, 5 g granulated yeast extract, 10 g NaCl, 15 g bacteriological agar, pH adjusted to 7.5
Super Optimal Broth with Catabolite repression (SOC)	20g granulated tryptone, 5g yeast extract, 0.58 g NaCl, 0.19 g KCl, 2.4 g MgSO ₄ . Sterile glucose solution was added to a final concentration of 0.4% after autoclaving and cooling.
Freezing mixture	12.6 g K ₂ HPO ₄ , 0.9 g Na ₃ C ₆ H ₅ O ₇ ·2H ₂ O, 0.18 g MgSO ₄ ·7H ₂ O, 1.8 g (NH ₄) ₂ SO ₄ , 3.6g KH ₂ PO ₄ , 96 g glycerol, made up to 1 L with MilliQ water

2.2.4 Preparation and use of antibiotics and inducing agents

Carbenicillin disodium and kanamycin monosulphate were dissolved in MilliQ water to 100 mg/ml and 50 mg/ml, respectively. L(+)-Arabinose was dissolved in MilliQ water to 100 mg/ml while IPTG was dissolved to 1 M, also in MilliQ water. All antibiotic and inducer stocks were filter sterilised using a 0.2 µm syringe filter and stored at -20°C, except for L(+)-arabinose which was stored at 4°C. Carbenicillin and kanamycin were added to LB or cooled LB agar to 100 µg/ml and 50 µg/ml, respectively. L(+)-Arabinose was added to cultures to a final concentration of 1 mg/ml while IPTG was added to 0.2 mM.

2.2.5 Small scale growth of cells to test gene expression

A single *E. coli* colony was taken from LB agar containing the appropriate antibiotic and used to inoculate a 10 ml LB starter culture in a 50 ml Falcon tube. After incubating overnight, this starter culture was diluted 1/100 in 50 ml LB containing the appropriate antibiotic in a 250 ml baffled flask. Cells were grown at 37°C with shaking at 200 RPM until an OD₆₀₀ of 0.8 ± 0.05 was reached. Gene expression was induced by addition of either L(+)-arabinose to 1 mg/ml or IPTG to 0.2 mM and cells reincubated for 2 hours. Cells were then harvested by centrifugation at 5,000 x g for 5 minutes and washed twice with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ pH 7.2). The cell pellet was resuspended to an OD₆₀₀ of 1.0 and divided into 500 µl aliquots. Aliquots were centrifuged at 13,000 x g for 1 minute and the supernatant discarded, then the pellet gently resuspended in 1x SDS loading dye (50 mM Tris pH 6.8, 2% w/v SDS, 10% v/v glycerol, 5% v/v mercaptoethanol, 0.01% w/v bromophenol blue). Resuspended samples were incubated at room temperature for 20 minutes and then centrifuged for 20 minutes at 13,000 x g. Samples of 10 µl were then taken from the surface of the sample tube and loaded onto an SDS-PAGE gel for protein detection by Western blotting (see Section 2.6.1).

2.2.6 Large scale growth of cells for protein purification

E. coli stocks were revived and grown in 50 ml starter cultures as described in Section 2.2.5. The starter culture was diluted 1/100 in fresh LB with the appropriate antibiotic (500 ml in a 2 L baffled flask) and incubated at 37°C with shaking at 200 RPM then induced as described in Section 2.2.5. Cells were harvested by centrifugation using

a Beckman Avanti 8.100 rotor at 14,000 x g for 30 minutes then resuspended in 20 mM Tris, 0.5 mM EDTA, 10% glycerol, pH 7.5 and stored at -80°C.

2.2.7 Growth of cells for transport assays

Cells were revived and starter cultures prepared exactly as described in Section 2.2.5. Starter cultures were used to inoculate 10 ml LB with appropriate antibiotic in 50 ml Falcon tubes, which were then incubated at 37°C with shaking at 200 RPM. Cells were grown and induced as described in Section 2.2.5. Following induction, cells were washed 3 times and resuspended in 5 ml of the appropriate transport buffer (see Section 2.4). After incubating cells for a further 10 minutes to ensure any remaining carbon sources were consumed, the resuspended cells were diluted to an OD₆₀₀ of 1.0 or 2.0, depending on the assay being performed.

2.3 Recombinant DNA Work

2.3.1 Plasmid DNA preparation

Cultures of 10 ml LB with appropriate antibiotic were grown in 50 ml Falcon tubes overnight at 37°C with shaking at 200 RPM. DNA was then isolated using the Monarch Plasmid MiniPrep kit (NEB) following the manufacturer's instructions using an Eppendorf MiniSpin microcentrifuge (Eppendorf, Hamburg, Germany).

2.3.2 Quantification of DNA

DNA concentration was determined using a DeNovix DS-11 spectrophotometer.

2.3.3 Agarose gel electrophoresis

Agarose gels were prepared by dissolving agarose to a final concentration of 1% in Tris acetate EDTA buffer (TAE, 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3). Agarose was added to TAE buffer and heated in a microwave oven until fully dissolved. After cooling to approximately 50°C, SYBR Safe DNA gel stain (ThermoFisher) was added at a dilution of 1 in 10,000. Samples were prepared in 6x DNA gel loading dye (NEB), loaded into wells and run at 100 volts for 15 to 30 minutes. Quick-Load® Purple 2-log DNA Ladder (NEB) was used as a standard.

2.3.4 Isolation of DNA fragments from agarose gels

After separation of DNA fragments by electrophoresis (see Section 2.3.3), fragments were visualised on a Safe Imager Transilluminator (ThermoFisher) and excised from the gel using a clean razor blade. DNA was then extracted from the gel using a Monarch DNA Gel Extraction Kit (NEB) following the manufacturer's instructions.

2.3.5 Restriction digestion

Restriction digests were performed at 37°C with shaking at 1000 RPM for 1 hour using the reaction composition detailed in Table 2.4. Double digests were performed in the same way with 1 µl of each restriction enzyme added per 50 µl reaction. All restriction enzymes and buffers were purchased from New England BioLabs (NEB).

Table 2.4 Restriction digest reaction components

Component	50 µl reaction
DNA	1 µg total DNA
10x CutSmart buffer (NEB)	5 µl
Restriction enzyme	1 µl (20 units)
Nuclease-free water	to 50 µl

2.3.6 DNA Ligation

Ligation reactions were prepared at room temperature using T4 DNA ligase and the supplied buffer (NEB) as detailed in Table 2.5 before briefly chilling on ice to aid sticky end annealing. Reactions were then incubated overnight at 16°C. DNA was ligated at a range of plasmid:insert molar ratios (typically 1:3, 1:5 and 1:10) which were calculated using NEB Bio Calculator (NEB).

Table 2.5 Ligation reaction composition

Component	20 µl reaction
DNA	50 ng vector DNA, variable insert based on molar ratio
10x T4 DNA Ligase Buffer	2 µl
Nuclease-free water	To 20 µl
T4 DNA ligase	1 µl (400 units)

2.3.7 DNA purification

Following PCR or other enzymatic reactions, the resulting DNA was purified from the reaction mixture using the Monarch PCR Cleanup Kit (NEB) following the manufacturer's instructions.

2.3.8 Polymerase chain reaction (PCR)

Target genes were amplified by PCR from *Escherichia coli* MG1655 or *Pseudomonas aeruginosa* PAO1 genomic DNA using Q5 High-Fidelity DNA Polymerase (NEB). For reaction compositions see Table 2.6. For cycling programmes, see Table 2.7. Primer melting temperatures (T_m) were calculated using NEB T_m Calculator (NEB). Cycling was carried out in a BioRad T100 thermal cycler (BioRad Laboratories Ltd.).

Table 2.6 Composition of PCR reactions

Component	50 μ l reaction	Final concentration
5X Q5 Reaction Buffer	10 μ l	1X
10 mM dNTPs	1 μ l	200 μ M
10 μ M Forward Primer	2.5 μ l	0.5 μ M
10 μ M Reverse Primer	2.5 μ l	0.5 μ M
Template DNA	variable	1 ng-1 μ g gDNA
Q5 Polymerase	0.5 μ l (1 unit)	0.02 units/ μ l
5X Q5 High GC Enhancer (optional)	(10 μ l)	(1X)
Nuclease-Free Water	to 50 μ l	

Table 2.7 PCR cycling conditions

Step	Temperature	Time
Initial Denaturation	98°C	30 seconds
Amplification 35-40 Cycles	98°C Lowest primer T_m +3°C 72°C	5–10 seconds 10–30 seconds 20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	12°C	-

2.3.9 Primers used for cloning of target genes

Cloning primers were designed using Benchling Biology Software and synthesised by Eurofins Genomics (Eurofins Scientific, Luxembourg). Primers used in this study are detailed in Table 2.8.

Table 2.8 Primers used for cloning of target genes. Endonuclease restriction sites are shown in red. Bases preceding restriction sites were added to aid endonuclease sequence recognition and binding.

Target sequence	Primer pair sequences (5' - 3')	Restriction sites introduced
<i>acrAB</i>	ATAACCTAGGAACAAAAACAGAGGGTTTACGCCTC AAATGAGCTCTCAATGATGATCGACAGTATGGCTG	AvrII, SacI
<i>tolC</i>	TTTAGAGCTCATGAAGAAATTGCTCCCCATTC TTTTCTGCAGGGTTACGGAAAGGGTTATGACC	SacI, SbfI
<i>mexAB- oprM</i>	CCGGCTAGCCAACGAACGCCAGCCATGCGTGTAC AAAAATGCATGAGCCTGGGGATCTTCCTTCTTCGCG	NheI, NsiI
<i>mexCD- oprJ</i>	AAAAATGCATGACTCCTGCCGCCTCG CCGGCTAGCGCTGATTTGCGTGCAATA	NsiI, NheI
<i>emrAB</i>	AAATCCTAGGTCTCAGGTGTCTGGCAGCGTG TTTTGAGCTCTTAGTGCGCACCGCCTCC	AvrII, SacI
<i>emrD</i>	AAAAGCTAGCAAAAGGCCAAAGAAACGTCAATTG AAAAATGCATTAACGGGCTGCCCCTGATG	NheI, NsiI
<i>mdlB</i>	AAAAGCTAGCCGTAGTTTTAGCCAACCTGTG AATTATGCATAGGCGCTCAATGATTCCTC	NheI, NsiI
<i>mdtL</i>	TTTTGCTAGCTCCCGCTTTTTGATTTGTAGTTTTG TTTTATGCATAAGCGTGGTGATGGATTTCTTC	NheI, NsiI
<i>mdtM</i>	AAAAGCTAGCCACGTTTTTTTACCCGCCATGC AAAAATGCATACTGCTCCTCCACTAGCTCGGCTG	NheI, NsiI
<i>msbA</i>	TTTTGCTAGCCATAACGACAAAGATCTCTCTACG TTTTATGCATAACATTGGCCAACTGCATTTTGTG	NheI, NsiI
<i>acrA</i>	ATAACCTAGGAACAAAAACAGAGGGTTTACGCCTC AAAACCTGCAGGAGACTTGGAAGTTTCAGGCTGAG	AvrII, SbfI
pGNTK backbone	AAAACCTAGGCAAGACGTAAATGTGGTGATCATGG AAAAAGCTTGCCTGCTAACAAAGCCCGAAAG	AvrII, HindIII

2.3.10 Preparation of chemically competent cells

Cultures of *E. coli* (10 ml in 50 ml Falcon tubes) were grown overnight in LB at 37°C with shaking at 200 RPM then used to inoculate 1 L of fresh LB, as described in Section 2.2.5. Cells were grown at 37°C with shaking at 200 RPM in 2 L baffled shaker flasks to an OD₆₀₀ of 0.35-0.4, at which point they were immediately placed on ice and kept chilled for the remainder of the procedure. Cells were sedimented at 3000 x g for

15 minutes and resuspended in approximately 400 ml of ice cold 100 mM MgCl_2 . Cells were sedimented at 2000 x g for 15 minutes and resuspended in 200 ml of 100 mM CaCl_2 , incubated on ice for 20 minutes and sedimented again. A final wash in 50 ml of 85 mM CaCl_2 with 15% glycerol was performed before finally resuspending the cell pellet in 2 ml of the same solution. Aliquots of 50 μl were prepared and snap frozen in liquid nitrogen before storing at -80°C .

2.3.11 Transformation of chemically competent cells

Chemically competent cells were thawed on ice and used immediately by adding 1 pg-100 ng of plasmid DNA and gently mixing before re-incubating on ice for 30 minutes. Cells were then heat shocked at 42°C for 30 seconds and returned to ice for 5 minutes, after which 450 μl of SOC medium (see Table 2.3) was added and cells incubated at 37°C with shaking for one hour. Finally, transformation mixtures were diluted in LB broth and spread onto agar plates containing the appropriate antibiotic then incubated overnight at 37°C .

2.3.12 Colony PCR

Following transformation, colonies that grew in the presence of appropriate antibiotic were screened for the correct plasmid insert by colony PCR (cPCR). A cPCR mastermix was prepared using Taq DNA polymerase (NEB) according to Table 2.9 and aliquoted into 10 μl volumes. Individual transformant colonies were carefully picked using a sterile toothpick and used to inoculate a fresh LB plate with appropriate antibiotic. The same toothpick was then briefly dipped in a 10 μl reaction. In order to select for plasmids with the correct insert in the correct orientation, one vector-specific and one insert-specific primer was used for each cPCR. Cycling programmes are

detailed in Table 2.10. DNA loading dye was added directly to the PCR mixtures and each reaction loaded onto an agarose gel (see Section 2.3.3). Colonies yielding a PCR product of the expected size were subcultured and tested for protein expression (see Section 2.2.5).

Table 2.9 Colony PCR mastermix composition

Component	For 100 µl (10 reactions)
Nuclease-free water	83.5 µl
10x ThermoPol buffer	10.0 µl
10 mM dNTPs	2.0 µl
10 µM Forward primer	2.0 µl
10 µM Reverse primer	2.0 µl
Taq DNA polymerase	0.5 µl (2.5 units)

Table 2.10 Colony PCR cycling conditions

Step	Temperature	Time
Initial denaturation	95°C	2 minutes
Amplification 30-35 cycles	95°C Lowest primer T _m -5°C 68°C	20 seconds 30 seconds 1 minute/kb
Final extension	68°C	5 minutes
Hold	12°C	-

2.3.13 Plasmid sequencing

Plasmids were sequenced by Eurofins Genomics using a Mix2Seq kit, which uses the Sanger chain termination method for DNA sequencing. Sequencing results were analysed and aligned to the template plasmid sequence using Benchling Biology Software.

2.4 Measurement of Efflux Activity

2.4.1 Preparation and use of stock solutions

Carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was dissolved in 100% ethanol to a concentration of 1 mg/ml and stored at -20°C protected from light. This stock was diluted in 1 mM NaOH immediately before use and used only on the same day. Nile Red and Hoechst 33342 were both dissolved to 3 mM in DMSO and MilliQ water, respectively, and stored at room temperature protected from light. Both of these substrates were added to cells at a final concentration of 5 μ M. Ethidium bromide was purchased as a solution in water and further diluted to 2.55 mM in MilliQ water and stored at room temperature protected from light. Ethidium bromide was added to cells at a final concentration of 130 μ M. For Nile Red, excitation and emission wavelengths were 552 nm and 636 nm, respectively. Ethidium transport was measured using excitation and emission wavelengths of 526 and 588, respectively. Transport of Hoechst 33342 was monitored at excitation and emission wavelengths of 355 and 457, respectively. All fluorescence measurements were made at a rate of 1/s⁻¹ with slit widths of 0.6 mm and a path length of 1 cm. Sample cuvettes were maintained at 25°C throughout.

2.4.2 Measuring uptake and efflux of fluorescent substrates

Cells were prepared as described in Section 2.2.7 using PBS supplemented with 1 mM MgCl₂ to wash and resuspend cells to OD₆₀₀=1. For efflux assays, CCCP was added to a final concentration of 10 μ M to dissipate the proton motive force (PMF) and cells incubated at room temperature for 20 minutes. Fluorescent efflux substrates were then added to cells at the appropriate concentration (see Section 2.4.1) and cells incubated for 30 minutes at 37°C with shaking. Preloading of cells with Nile Red was

always performed in glass test tubes as Nile Red adsorbs to plastics. After loading with dye, cells were dispensed into 1 ml volumes and centrifuged at 5,000 x g for 5 minutes. The supernatant was removed as thoroughly as possible and discarded; then cell pellets were stored on ice until needed. Measurements were taken by resuspending cells in 1 ml PBS with 1 mM MgCl₂ and immediately transferring to a quartz cuvette with a magnetic stirrer in a Quantamaster spectrophotofluorometer (Photon Technology International) and data acquisition started immediately. After 50-60 seconds, efflux was initiated by addition of sterile glucose solution at a range of concentrations.

2.4.2.1 Efflux competition assays To measure competition between BMA and fluorescent efflux substrates, BMA was added to cells at a range of concentrations immediately after CCCP addition, then the protocol followed with addition of glucose as described in Section 2.4.2.

2.4.3 Monitoring uptake of fluorescent substrates

To measure uptake of fluorescent substrates, cells were prepared and diluted as described in Section 2.4.2 and the suspension kept on ice. For each measurement, 1 ml of cell suspension was transferred directly to a quartz cuvette and allowed to equilibrate to 25°C in the fluorimeter for 1 minute. Glucose was added to the cell suspension at a range of concentrations and data acquisition started immediately. After 1 minute, fluorescent substrate was added to the appropriate final concentration (see Section 2.4.1) and fluorescence monitored continuously.

2.4.4 Radiochemical accumulation assays

2.4.4.1 Preparation of cells and sampling procedure Cells were prepared as described in Section 2.2.7 then washed 3 times in the appropriate buffer before resuspending to OD₆₀₀=2.0. Cells were kept on ice until required and warmed to room temperature for a minimum of 15 minutes before use. Glucose was added to a final concentration of 26 mM and cells incubated at room temperature for 1 minute. After precisely 1 minute, the appropriate radiolabelled substrate was added and the sample mixed by vortexing. Samples of 100 µl were filtered at defined time points through 0.45 µm pore size membrane filters then washed with 2.5 ml of appropriate uptake buffer. Filters were then placed in 20 ml plastic scintillation vials and 10 ml Emulsifier-Safe scintillation fluid added. After vigorous mixing, samples were left overnight then direct disintegrations per minute (DPM) counted in a Tri-Carb liquid scintillation counter with a count time of 5 minutes per sample. Rate of uptake was calculated using the following formula:

$$\text{Uptake (nmol/mg of cells)} = (\text{DPM-blank}) \times \frac{[\text{total volume}]}{[\text{sample volume}]} \times \frac{1}{[\text{mg of cells}]} \times \frac{1}{[\text{DPM std/nmol std}]}$$

mg of cells = Volume of cell suspension x A₆₈₀ x 0.68

DPM – disintegrations per minute

2.4.4.2 [1,5-¹⁴C]-Cadaverine uptake assay

For cadaverine uptake experiments, cells were washed and resuspended in 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 145 mM NaCl and 5 mM KCl at pH 7.0. The assay began with 740 µl of OD₆₀₀=2.0 cells to which 40 µl of 20% glucose solution was added, followed by 20 µl of 2 mM [¹⁴C]-cadaverine to give a final

concentration of 50 μM . Samples of 100 μl were filtered at 0.5, 2.0, 5.0, 10.0, 20.0, 30.0 and 40.0 minutes through nitrocellulose 0.45 μM filters.

2.4.4.3 [^{14}C]-BMA uptake assay

For BMA uptake experiments, cells were washed and resuspended in 20 mM 4-morpholinepropanesulfonic acid (MOPS), 140 mM KCl and 1 mM MgCl_2 at pH 6.6. PVDF filters (0.45 μM pore size) were briefly wet in 100% ethanol then equilibrated in uptake buffer before use. Samples of 100 μl were filtered at 0.5, 2.0, 5.0 and 10.0 minutes.

2.5 Measuring the Effect of Esters on Cell Viability

2.5.1 Measuring the effect of BMA exposure on cell viability

Cells were prepared exactly as described in Section 2.2.7, then resuspended to an OD_{600} of 2.0 in PBS with 1 mM MgCl_2 . Glucose was added at a final concentration of 22 mM and cells incubated at room temperature for 3 minutes. For cell viability assays, samples of 20 μl were taken as pre-exposure controls, then BMA added to a final concentration of 2 mM and incubated at room temperature for one hour with regular mixing. Further samples of 20 μl were taken after 10 and 60 minutes. All samples were serially diluted in 180 μl volumes of PBS (10-fold dilution series) in a 96 well polypropylene plate. Multiple dilutions of each sample were plated onto LB agar containing the appropriate antibiotic by inoculating 20 μl onto the surface of well-dried plates in triplicate (9 drops per plate) and allowing the samples to dry thoroughly. Plates were incubated overnight at 37°C then colonies counted to calculate the number of colony forming units per ml of cell suspension (CFU/ml) using the following equation:

(Mean no. of colonies per drop x 50) x dilution factor

2.5.2 Measuring the effect of BMA exposure on the abundance of cellular ATP

Cellular ATP was quantified following exposure to BMA as a proxy for measuring the PMF. Cells were prepared and treated with BMA exactly as described in Section 2.5.1. After 1 hour, 20 µl samples were taken and diluted in 180 µl of cold PBS at pH 7.2 in order to dilute the BMA to a sub-toxic concentration. Samples of 100 µl were transferred to a white polypropylene 96-well plate for luminescence measurements and gently mixed 1:1 with rehydrated BacTiter-Glo reagent (Promega Corporation, WI, USA) then incubated at room temperature for 5 minutes. Luminescence was read using a Tecan Spark 10M plate reader with an integration time of 0.1 seconds per well. The remaining diluted samples were used to perform viability counts, as described in Section 2.5.1.

2.6 Protein Expression, Purification and Analysis

2.6.1 SDS-PAGE and Western blotting procedure

2.6.1.1 Separation and analysis of proteins by SDS-PAGE Samples for SDS-PAGE were prepared in 1x SDS loading dye (50 mM Tris pH 6.8, 2% w/v SDS, 10% v/v glycerol, 5% v/v mercaptoethanol, 0.01% w/v bromophenol blue), mixed gently by pipetting and incubated at 37°C for at least 30 minutes. Polyacrylamide (15%) gels were prepared at 0.75 mM thickness according to Table 2.11. Ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) were added immediately before casting gels. Samples were run at 120 volts for 90 minutes in SDS-PAGE buffer (250 mM Tris pH 7.2, 1.9M glycine, 0.5% SDS). Gels to be stained for protein visualisation

were placed in fixing solution for a minimum of 30 minutes on an orbital shaker, then transferred to stain 1 for 30 minutes, stain 2 for 1 hour and finally destain solution until background staining was eliminated (see Table 2.12).

Table 2.11 Polyacrylamide gel preparation

Component	Running gel	Stacking gel
40% acrylamide	2.11 ml	385 μ l
2% bis-acrylamide	245 μ l	195 μ l
Tris 1.5 M pH 8.8	1.41 ml	-
Tris 1.5 M pH 6.8	-	375 μ l
10% SDS	50 μ l	25 μ l
MilliQ water	1.74 μ l	1.6 μ l
10% APS	18.5 μ l	15 μ l
TEMED	6 μ l	4.5 μ l

Table 2.12 Gel staining solutions

Component	Fixing	Stain 1	Stain 2	Destain
MilliQ water	400 ml	1635 ml	2000 ml	2250 ml
Acetic acid	100 ml	250 ml	250 ml	250 ml
Isopropanol	500 ml	625 ml	250 ml	-
Coomassie R250	-	625 mg	62.5 mg	-

2.6.1.2 Detection of affinity-tagged proteins by Western blotting. SDS-PAGE gels were transferred to a PVDF membrane (0.45 μ M, Merck) by first equilibrating the gel in Towbin buffer (25 mM Tris, 192 mM glycine, 15% v/v ethanol, 0.04% SDS) for 15 minutes. PVDF membranes were wet in 100% ethanol for 1 minute then equilibrated in Towbin buffer for 15 minutes. The transfer stack was assembled in the following order from the bottom up in a TransBlot Turbo (BioRad) transfer cell: 6 sheets of thin filter paper soaked in Towbin buffer, PVDF membrane, polyacrylamide gel, 6 more sheets of thin filter paper soaked in Towbin buffer. Transfer was carried out at 15 volts for 30 minutes then the membrane blocked in Tris-buffered saline with Tween-20

(TBS-T, 50 mM Tris, 150 mM NaCl, 0.1% v/v Tween-20, pH 7.6) with 3% bovine serum albumin (BSA) overnight at 4 °C. The membrane was washed twice in TBS-T for 10 minutes per wash before incubating with HisProbe-HRP conjugate (ThermoFisher) or StrepMAB-Classic HRP Conjugate (IBA Life Sciences) in TBS-T or PBS-T, respectively, for 1 hour at room temperature, followed by 4 more washes. Membranes were then incubated with SuperSignal West Pico substrate (ThermoFisher) for 5 minutes and visualised using a G:BOX (Syngene).

2.6.2 Small scale isolation of mixed membranes by water lysis method

Mixed *E. coli* membranes were prepared following the water lysis method (Ward et al., 2000). Cell pellets from 50 ml cultures (grown and induced as described in Section 2.2.5) were resuspended in 10 ml of 200 mM Tris at pH 8.0 and mixed on rollers at room temperature for 20 minutes. 4.85 ml lysis buffer (1M sucrose, 0.2M Tris, 1 mM EDTA, pH 8.0) was added and mixed on rollers for 90 seconds, followed by 65 µl of 10 mg/ml lysozyme solution and a further 30 seconds of mixing. Finally, 9.6 ml MilliQ water was added before mixing for a further 20 minutes. Tubes were centrifuged at 25,000 x g for 20 minutes to sediment spheroplasts, which were then resuspended in MilliQ water using a glass homogeniser and incubated at room temperature for 30 minutes. Membranes were washed 3 times in 0.1 M sodium phosphate (NaPi) buffer pH 7.2 with 1 mM mercaptoethanol (MBE), then resuspended in a minimal volume of NaPi/MBE using a syringe and needle. Membranes were aliquoted and snap frozen in liquid nitrogen before storage at -80°C. Total protein concentration was determined using the Pierce BCA protein assay kit (see Section 2.6.5) according to the manufacturer's instructions. Membranes were resuspended in 1x SDS loading dye to give 4 µg total protein per lane for Western blotting, and 16 µg per lane for Coomassie

stained gels (see Section 2.2.7.1). Densitometry was performed using the Fiji software package (Schindelin et al., 2012).

2.6.3 Large-scale isolation of mixed membranes using a cell disrupter

Frozen *E. coli* cell pellet was thawed at 4°C and diluted 1:3 in ice cold cell disruption buffer (20 mM Tris HCl, 0.5 mM EDTA, pH 7.6). All suspensions were handled on ice and all equipment maintained at 4°C throughout. The cell suspension was homogenised to remove any lumps using a T18 ULTRA-TURRAX homogeniser (Ika-Werke, Staufen, Germany) at medium power. The Z Plus Series cell disrupter was flushed with 200 ml of water followed by 100 ml of cell disruption buffer. The cell suspension was passed through the disruptor twice at 30,000 PSI. The crude cell lysate was then centrifuged at 12,000 x g for 45 minutes to sediment cell debris. The supernatant (containing mixed membranes) was centrifuged for 2 hours at 131,000 x g to sediment membranes. The membrane pellet was resuspended using a syringe in a minimal volume of 20 mM Tris-HCl pH 7.6, then snap frozen and stored at -80°C. Alternatively, if separation of inner and outer membranes was required, the membrane pellet was resuspended in cell disruption buffer with the addition of 25% sucrose.

2.6.4 Separation of inner and outer membrane fractions

All samples were handled on ice at all times and equipment maintained at 4°C throughout. Using a syringe and blunt needle, a sucrose gradient was prepared in a type 45 Ti ultracentrifuge tube (Beckman Coulter Inc.) by very gently layering 10 ml of each of the following sucrose concentrations dissolved in cell disruption buffer: 55%, 50%, 45%, 40%, 35% and 30%. The membrane fraction suspended in cell disruption buffer with 25% sucrose was then gently layered on top. Tubes were centrifuged at

96,000 x g for 16 hours and the rotor allowed to stop without braking. The inner membrane fraction was then harvested using a syringe and needle from the 35-40% sucrose interface and the outer membrane fraction from the 45-50% interface. Each fraction was then diluted in 20 mM Tris-HCl pH 7.6 and sedimented at 131,000 x g for 2 hours then this process repeated two more times to remove excess sucrose. Washed membranes were suspended in a minimal volume of the same buffer and snap frozen in liquid nitrogen before storing at -80°C.

2.6.5 Determination of total protein concentration

Membranes were diluted 1:39, 1:19, 1:9, 1:4 and 1:1 in 20 mM Tris-HCl pH 7.6 and protein concentration determined using the Pierce BCA protein assay kit (Thermo Fisher) following the manufacturer's instructions, using bovine serum albumin (BSA) standards to generate a standard curve. Sample absorbance was measured in a Tecan Spark 10M plate reader (Tecan Group Ltd., Männedorf, Switzerland).

2.6.6 Protein purification using immobilised metal affinity chromatography

2.6.6.1 Membrane solubilisation Frozen membranes were slowly thawed on ice and diluted to a total protein concentration of 2 mg/ml in solubilisation buffer containing 1% DDM (see tables 2.13 and 2.14). Samples were left at 4°C for 1 to 2 hours on a blood wheel set to low speed in order to minimise foaming. Following solubilisation, samples were centrifuged at 130,000 x g for 1 hour to sediment the insoluble fraction and the supernatant retained. Before binding to IMAC resin, the supernatant was diluted 1:1 with solubilisation buffer without DDM in order to reduce the final DDM concentration and aid binding of protein to the resin.

2.6.6.2 Purification of AcrA/AcrB All purification steps were performed at 4°C and samples kept on ice at all times. Fresh HisPur cobalt resin was thoroughly resuspended (2 ml of slurry per 15 ml of diluted binding mixture) and immediately transferred to a chromatography column and the storage buffer run off. The resin was washed with 10 column bed volumes (CV) of wash buffer 1 then left to equilibrate in wash buffer 1 until needed. The diluted soluble fraction was then used to resuspend the equilibrated resin and transferred back to a sealed tube. The resin/soluble fraction mixture was left overnight on rollers to allow protein binding then transferred back to a chromatography column and the unbound fraction allowed to run off. The resin was then washed with 10 CVs of wash buffer 1 to elute unwanted proteins from the resin. AcrA/B was eluted from the resin using 15 ml elution buffer and collected in 1 ml fractions. Fractions were analysed by SDS-PAGE and Western blotting (see Section 2.6.1). Fractions containing high concentrations of AcrA or AcrB were pooled and concentrated to 3.5 ml using a Vivaspin 20 protein concentrator with 10 kDa or 100 kDa molecular weight cut-off (MWCO), respectively. The concentrated protein was then applied to an EconoPac DG10 desalting column and eluted in 4 ml wash buffer 2. Finally, the protein was concentrated to approximately 500 µl using a Vivaspin 6 protein concentrator with appropriate MWCO, aliquoted, snap frozen and stored at -80°C.

Table 2.13 AcrA/B purification buffers

Buffer	Composition
Solubilisation/binding	50 mM Tris 10% v/v glycerol 300 mM NaCl 20 mM imidazole 1% w/v DDM pH 7.4
Wash 1	50 mM Tris 10% v/v glycerol 300 mM NaCl 30 mM imidazole 0.05% w/v DDM pH 7.4
Elution	50 mM Tris 10% v/v glycerol 300 mM NaCl 250 mM imidazole 0.05% w/v DDM pH 7.4
Wash 2	50 mM Tris 2.5% v/v glycerol 1% w/v DDM pH 7.4

2.6.6.3 Purification of Acel All purification steps were performed at 4°C and samples kept on ice at all times. Fresh Ni-NTA resin was thoroughly resuspended (1 ml of slurry per 1 ml of membranes) and immediately transferred to a chromatography column and the storage buffer run off. The resin was washed with 10 column bed volumes (CV) of wash buffer 1 (see Table 2.14) then left to equilibrate in wash buffer 1 until needed. The diluted soluble fraction was used to resuspend the resin and transferred to a sealed tube before mixing on rollers for 45 minutes, then the resin was sedimented at 500 x g for 2 minutes and 75% of the supernatant discarded. The remaining supernatant was used to resuspend the resin and transfer it back to a column and the resin run dry. The resin was washed with 20 CVs wash buffer 1 then Acel eluted in 10 fractions of 250 µl using elution buffer. Fractions were analysed by

SDS-PAGE and Western blotting (see Section 2.6.1). The fraction containing the highest Acel concentration was used for reconstitution experiments (see Section 2.9).

Table 2.14 Acel purification buffers

Buffer	Composition
Solubilisation/binding	10 mM Tris 20% v/v glycerol 300 mM NaCl 20 mM imidazole 1% w/v DDM pH 8.0
Wash	10 mM Tris 10% v/v glycerol 20 mM imidazole 0.05% w/v DDM pH 8.0
Elution	10 mM Tris 2.5% v/v glycerol 200 mM imidazole 0.05% w/v DDM pH 8.0

2.6.7 Determination of purified protein concentration

The concentration of purified protein samples was determined using a Denovix DS-11 spectrophotometer by inputting the relevant molecular weight and extinction coefficient. These values were calculated from the protein amino acid sequence using Benchling Biology Software.

2.7 Characterisation of purified efflux proteins

2.7.1 CPM thermostability assay

2.7.1.1 Preparation and use of 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) CPM was dissolved in 100% DMSO to a concentration of 5 mg/ml, protected from light and stored at -80°C. Before use, CPM was thawed and

diluted to 0.1 mg/ml in wash buffer 2 (Table 2.12) and equilibrated at room temperature for 10 minutes.

2.7.1.2 Assessing protein thermostability using the CPM assay Purified protein was diluted in wash buffer 2 at a range of concentrations. Diluted protein was aliquoted into 45 µl volumes in optically clear PCR tubes and 5 µl of the diluted CPM stock added before incubating on ice in the dark for 10 minutes. Samples were transferred to an Agilent Mx3005P QPCR system and after a 3 minute equilibration step at 25°C, sample temperature was increased by 1°C every 20 seconds (3°C per minute) up to 90°C. Fluorescence endpoint measurements were recorded after every temperature step at excitation and emission wavelengths of 460 nm and 510 nm, respectively. To investigate if BMA binding had a stabilising effect on AcrB, BMA was added to wash buffer 2 at a range of concentrations before adding protein. To assess the stability of the AcrAB complex, AcrA and AcrB were mixed in a 2:1 molar ratio then the assay performed as described above.

2.8 Reconstitution of efflux proteins into proteoliposomes

2.8.1 Formation of large multilamellar vesicles (LMVs)

In order to form LMVs, 600 µl of *E. coli* polar lipid extract in chloroform (approximately 15mg total) was transferred to a 20 ml glass vial and dried under a stream of oxygen-free nitrogen while constantly turning the vial to obtain an evenly thin lipid film. Lipids were rehydrated with 1 ml reconstitution buffer (25 mM HEPES, 200 mM NaCl, 1 mM DTT, pH 7.0) and left on rollers at room temperature for at least 30 minutes with regular vortexing, until a homogeneous milky suspension was obtained and no lipid film remained on the walls of the vial.

2.8.2 Formation of small unilamellar vesicles (SUVs) by extrusion

LMVs were extruded to produce SUVs of uniform size distribution. LMVs were collected in a 1 ml glass syringe and passed 11 times through a 0.45 μ M filter, followed by another 11 passes through a 0.2 μ M filter all at room temperature in an Avanti mini-extruder. The 0.2 μ M SUVs were kept on ice until required.

2.8.3 Reconstitution of protein into membrane vesicles

To 330 μ l of SUVs, extra reconstitution buffer was added to result in a final reconstitution volume of 500 μ l, followed by 5 μ l of 100 mM pyranine, 55 μ l of 10% OG and 12.5 μ g of protein. Components were always added in this order to ensure liposomes were never exposed to critically high concentrations of detergent. Reconstitution mixtures were then incubated in the dark at room temperature for 15 minutes before rapidly diluting with 9.5 ml of ice cold reconstitution buffer. Proteoliposomes were sedimented at 60,000 x g for 1 hour at 4°C and resuspended in 100 μ l reconstitution buffer and stored at 4°C.

2.8.4 Measurement of cadaverine transport into (proteo)liposomes using the pH sensitive dye pyranine

Proteoliposomes were diluted 100-fold in assay buffer (25 mM HEPES, 200 mM KCl, pH 8.0) in a quartz cuvette and immediately transferred to a fluorimeter maintained at 25°C with a magnetic stirrer. Fluorescence was monitored at excitation wavelengths of 400 and 450 nm and an emission wavelength of 509 nm. An increase in the F509 (ex. 450 nm)/F509 (ex. 400 nm) ratio indicates an increase in proteoliposome luminal pH, indicative of proton-coupled transport. Samples were allowed to equilibrate for approximately 50 seconds in the fluorimeter, at which point valinomycin was added to

5 nM. After a further 50 s, cadaverine was added to a final concentration of 1 mM and fluorescence monitored with a frequency of 1 measurement per second. Cuvettes were washed with potassium saturated ethanol to remove residual valinomycin.

Chapter 3

Cloning of bacterial efflux pumps

3.1 Introduction

3.1.1 Identification of target efflux pumps

Bioinformatic databases have become powerful tools for consolidating sequence and experimental data. Using these tools to focus our search on solvent-tolerant organisms or transport proteins known/predicted to export organic solvents is a valuable approach to identify potential routes for BMA efflux in a time when traditional lab-based research is unable to keep up with the rapid generation of new sequence data. EcoCyc provides an *E. coli* specific collection of pathway and genome databases to include metabolites, enzymes, pathways, predicted operons and transport systems (Karp et al., 2014), while BioCyc utilises the same approach but across a broad range of organisms (Caspi et al., 2016). TransportDB 2.0 provides access to an increasingly large collection of data output from the Transporter Automatic Annotation Pipeline (TransAAP), which identifies putative membrane transport proteins based on homology to sequences deposited in databases such as the Transporter Classification Database (TCDB) and the Protein family (Pfam) database. These tools were utilised to complement literature searches and allowed us to narrow our search to those systems known/hypothesised to confer tolerance to organic solvents (Table 3.1). Since membrane proteins not only rely on successful expression but also insertion into, and correct function inside the *E. coli* membrane(s), searches were limited to Gram-negative bacteria to increase the likelihood of successful production of functional, membrane-localised protein in a Gram-negative expression host.

Table 3.1. Identification of target efflux pumps. Systems cloned in this study are underlined.

System	Family	Organism	Known substrate(s)	Reference(s)
<u>AcrAB-TolC</u>	RND	<i>E. coli</i>	Extremely promiscuous substrate specificity, range of organic solvents, multidrug, toxins etc. Increased isoprenoid yield by up to 2-fold in <i>E. coli</i> production strain. Up-regulated in response to BMA stress	Tsukagoshi and Aono, 2000; Wang <i>et al.</i> , 2013; Martins, unpublished; Menchavez, unpublished
<u>MexAB-OprM</u>	RND	<i>P. aeruginosa</i>	Up-regulated in response to solvent stress in <i>P. aeruginosa</i> . Tested against hexane and xylene. Expression of <i>mexB</i> increased isoprenoid yield by up to 70% in <i>E. coli</i> production strain. Disruption of genes results in dramatically reduced tolerance to BMA in <i>P. aeruginosa</i> .	Li and Poole, 1999; Wang <i>et al.</i> , 2013; El Bestawy, unpublished
<u>MexCD-OprJ</u>	RND	<i>P. aeruginosa</i>	Up-regulated in response to solvent stress in <i>P. aeruginosa</i> . Tested against hexane, xylene and ethanol. Disruption of genes results in dramatically reduced tolerance to BMA in <i>P. aeruginosa</i> .	Li <i>et al.</i> , 1998; El Bestawy, unpublished
<u>SrpABC</u>	RND	<i>P. putida</i>	Restores tolerance to mutant strains. Hexane, cyclohexane, ethylbenzene, p-xylene, octane, toluene, dimethylphthalate. Specific to solvents.	Fukumori <i>et al.</i> , 1998; Kieboom <i>et al.</i> , 1998
<u>AcrEF-TolC</u>	RND	<i>E. coli</i>	Confers/increases tolerance to n-nonane, n-octane, cyclooctane, diphenyl ether, n-hexane, cyclohexane	Kobayashi <i>et al.</i> , 2001
<u>MdlB</u>	ABC	<i>E. coli</i>	Increased isopentenol production by 12% in engineered <i>E. coli</i> production strain.	Foo <i>et al.</i> , 2014
<u>TtgABC</u> <u>TtgGHI</u> <u>TtgDEF</u>	RND	<i>P. putida</i>	Sequential inactivation of 3 systems has additive effect on solvent susceptibility. Expression induced by solvents. DEF specific to toluene and styrene. Possibly only aromatic solvents.	Rojas <i>et al.</i> , 2001
<u>EmrD</u>	MFS	<i>E. coli</i>	Initial studies suggest expression may enhance BMA tolerance	Ingenza Ltd., unpublished
<u>MdtL</u>				
<u>MdtM</u>				
<u>MsbA</u>	ABC	<i>E. coli</i>	Gives up to 5-fold increase in secreted isoprenoids in <i>E. coli</i> producer strains. Increased amorphadiene production >3 fold in <i>E. coli</i> . Initial studies suggest expression may enhance BMA tolerance.	Doshi <i>et al.</i> , 2013; Zhang <i>et al.</i> , 2016; Ingenza Ltd., unpublished
<u>EmrAB-TolC</u>	MFS	<i>E. coli</i>	Enhanced tolerance to octane and nonane in <i>E. coli</i> . Enhanced amorphadiene production in <i>E. coli</i> production strain. Initial studies suggest expression may enhance BMA tolerance.	Tsukagoshi and Aono, 2000; Zhang <i>et al.</i> , 2016; Ingenza Ltd., unpublished
<u>EmrKY-TolC</u>	MFS	<i>E. coli</i>	Enhanced amorphadiene production >3-fold in <i>E. coli</i> production strain.	Zhang <i>et al.</i> , 2016
<u>MacAB-TolC</u>	ABC	<i>E. coli</i>	Enhanced amorphadiene production >3-fold in <i>E. coli</i> production strain.	Zhang <i>et al.</i> , 2016

3.1.2 Tripartite efflux pumps

Pumps of the resistance nodulation division family (RND) featured heavily in these searches (Table 3.1), perhaps due to their mechanism of action. RND pumps form large tripartite complexes that span both the inner and outer membrane in Gram-negative bacteria, including an inner membrane pump, a periplasmic adapter protein

and an outer membrane channel. These large assemblies are generally thought to recruit their substrates from the periplasm to transport compounds across the outer membrane (Elkins and Nikaido, 2002; Lomovskaya and Totrov, 2005; Nikaido and Takatsuka, 2009). Hydrophobic solvents, such as BMA, are likely to accumulate in the hydrophobic environment of the membranes/periplasm upon external addition, making them the perfect target for RND-mediated transport.

In addition to bioinformatic/literature searches, data from the University of Nottingham (Martins, unpublished) found that components of the AcrAB-TolC efflux pump were upregulated upon external addition of BMA. Directed evolution studies (Menchavez, unpublished) also identified genes encoding regulators of AcrAB-TolC expression (eg. *soxR*, *rob*) as mutational hot-spots following multiple passages in the presence of BMA. Research carried out at the University of Strathclyde (El Bestawy, unpublished) also used *Pseudomonas aeruginosa* transposon mutant libraries to identify MexAB-OprM and MexCD-OprJ as key BMA resistance determinants. These data complemented our in-silico searches, making RND efflux pumps a main focus of this research.

Alternatively, the major facilitator superfamily (MFS) pump EmrB is also thought to form a tripartite complex with the periplasmic linker protein EmrA and the outer membrane channel TolC that spans both membranes (Neuberger *et al.*, 2018; Puertolas-Balint *et al.*, 2019). However, unlike RND pumps, EmrAB-TolC is hypothesised to export substrates from the cytoplasm directly across both the inner and outer membranes, which would be extremely valuable in the context of internal BMA production. However, this has only been inferred from homology to other efflux systems such as HlyBD-TolC (Lewis, 2000; Tanabe *et al.*, 2009; Thanabalu *et al.*, 1998) and there are currently no experimental data to support this mechanism of

transport. Initial experiments performed by Ingenza Ltd. have also suggested EmrAB-TolC may play a role in enhancing tolerance to BMA (R. Cortis, personal communication, June 2018), hence its investigation in this research.

3.1.3 Inner membrane efflux pumps

It is also important to consider inner membrane transporters in the context of a production strain. As mentioned previously, in order to export internally produced BMA from the cell, a multi-step efflux mechanism may be necessary to transport BMA across the inner membrane into the periplasm, where it can then be recruited by an RND pump for complete removal from the cell. Five different inner membrane efflux pumps were identified for this purpose based on preliminary data gathered by Ingenza Ltd. and are summarised in Table 3.1.

3.1.4 The cloning strategy

The cloning strategy described here was based on a traditional ‘digest and ligate’ approach, involving restriction endonuclease digestion of both the expression vector backbone and a gene insert, generated by PCR from appropriate template DNA, to generate complementary ‘sticky ends’ that will anneal to one another when combined. A ligation reaction can then be performed to covalently bond the DNA phosphodiester backbone creating a stable plasmid construct.

3.1.4.1 The pBAD expression vector. A pBAD expression vector (provided by Dr. Vincent Postis) (Fig. 3.2) was chosen due to its tight regulation (Guzman *et al.*, 1995), allowing expression of cloned efflux pumps to remain under tightly regulated control. A full description of the p_{BAD} expression system can be found in Section 1.2.6.2. The

pBAD plasmid used here is maintained in *E. coli* through the introduction of an ampicillin resistance gene under the constitutive *p_{bla}* promoter (Fig. 3.2). The vector backbone also encodes the AraC protein, which regulates arabinose-dependent gene expression through interactions with the *p_{BAD}* promoter and adjacent sites (see Section 1.2.6.2). Immediately downstream of the *p_{BAD}* promoter is a multiple cloning site, consisting of 14 unique restriction sites (Fig. 3.2), allowing digestion and insertion of a gene(s) of interest. An RGSHis₈ region downstream of the multiple cloning (Fig. 3.2) site allows addition of a polyhistidine tag to the C-terminus of cloned genes, allowing for both detection and purification of recombinant protein (Bornhorst and Falke, 2000; Terpe, 2003). The C-terminus tag was initially chosen for expression of tripartite efflux pumps in particular as these would require the simultaneous expression of three separate genes under a single promoter (Fig.). Since all 3 genes will be transcribed as a polycistronic mRNA, detection of a His₈-tag on the final protein encoded on the transcript can be used to infer successful transcription and translation of all three genes (Fig. 3.1).

3.1.4.2 The pGNTK expression vector. The pGNTK plasmid utilises the *p_{gntK}* promoter. Although this expression system is poorly characterised, experimental evidence suggests that a negative feedback mechanism is used to regulate expression of membrane proteins automatically and in response to membrane stress (Boyarskiy *et al.*, 2016). This promoter has previously been successfully utilised in *E. coli* production strains to optimise expression of efflux pumps involved in tolerance to organic solvents (Boyarskiy *et al.*, 2016), hence its utility in this study. The pGNTK plasmid carries a kanamycin resistance gene, allowing it to be maintained in *E. coli* (Fig. 3.3). PCR was used to introduce a multiple cloning site immediately downstream

of the p_{gntK} promoter (Section 3.8.2), which was designed to allow simple amplification of genes from the relevant pBAD construct, followed by digestion and ligation into the pGNTK vector without loss of the His₈ affinity tag.

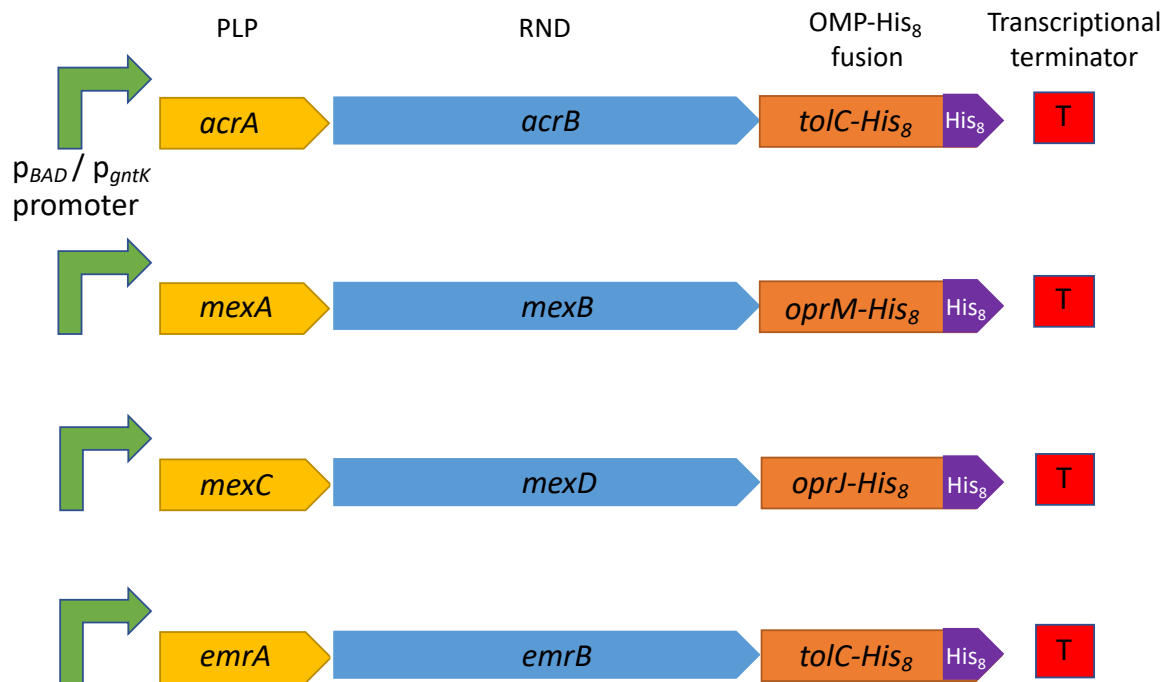


Figure 3.1. Design of tripartite efflux pump expression constructs. All three genes encoding the periplasmic linker protein (PLP), RND pump component and outer membrane protein (OMP) are inserted into the vector backbone under a single promoter, with the OMP being fused to a His₈ affinity tag allowing for protein detection and/or purification.

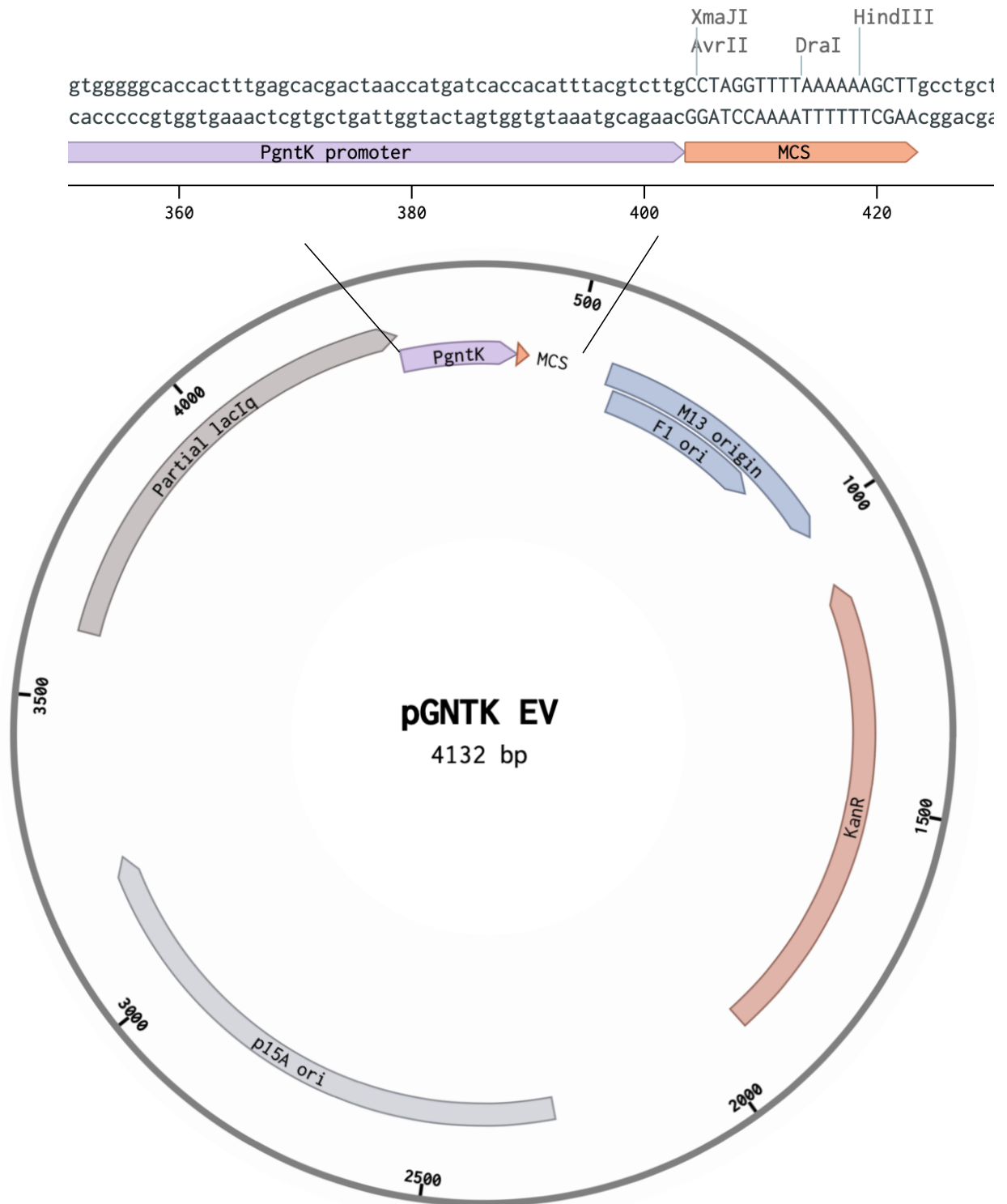


Figure 3.3. Plasmid map of the pGNTK expression vector and multiple cloning site (MCS)

3.2 Cloning the *acrAB**tolC* genes under control of the *p_{BAD}* promoter

3.2.1 Amplification of genes using PCR

The genes encoding AcrA and AcrB are located adjacent to each other in the *E. coli* chromosome, allowing them to be amplified by PCR (see Section 2.3.8) as a single 4372 bp fragment (Fig. 3.4A) from *E. coli* MG1655 (K12) genomic DNA (gDNA). The gene encoding TolC (1527 bp) was successfully amplified separately (Fig. 3.4B) from the same gDNA template. All PCR and colony PCR primers are detailed in Table 2.8.

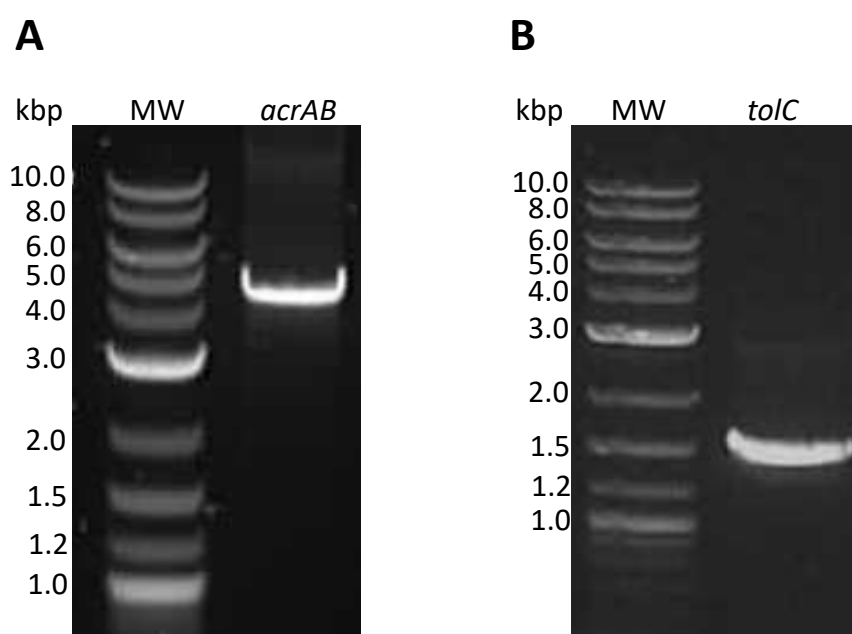


Figure 3.4. Amplification of the *acrAB* (A) and *tolC* (B) genes using PCR. PCR products were analysed on a 1% agarose gel. Lanes designated 'MW' were loaded with a molecular weight marker.

3.2.2 Digestion, ligation and transformation

The *acrAB* and *tolC* fragments were digested using *SacI* (Section 2.3.5) and ligated using T4 DNA ligase (Section 2.3.6) to generate a single *acrAB-tolC* fragment of 5905 bp, which was then purified from an agarose gel (Section 2.3.4). A second digestion was then performed using *AvrII* and *SbfI* to generate 3' and 5' overhangs. The *pBAD*

empty vector was also digested using *AvrII* and *SbfI* (Fig. 3.5), purified using a spin-column (Section 2.3.7) and stored at -20°C to be used for all inserts. The digested *acrABtoI/C* and pBAD fragments were ligated using T4 DNA ligase (Section 2.3.6) and the reaction mixture used to transform *E. coli* C43 Δ *acrAB* cells (Section 2.3.11), which were then grown overnight at 37°C on LB agar with 100 µg/ml carbenicillin.

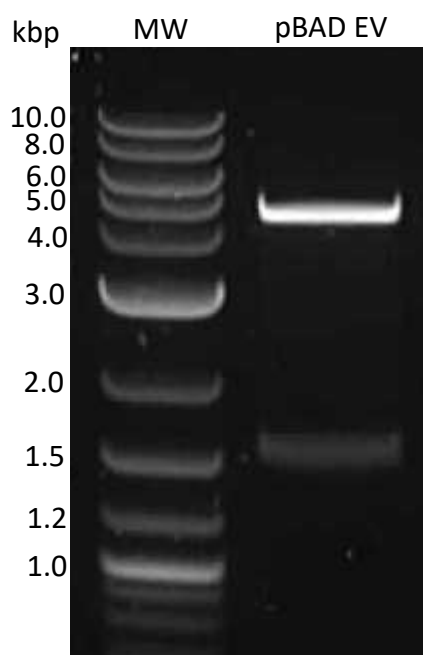


Figure 3.5. Digestion of the pBAD empty vector. The pBAD plasmid was digested with *AvrII* and *SbfI* to generate a linear 4691 bp and visualised on a 1% agarose gel. The digested fragment was purified using a spin-column (Section 2.3.7) to remove undigested, supercoiled plasmid DNA visible at ~2 kb. Lanes designated ‘MW’ were loaded with a molecular weight marker.

3.2.3 Confirming target plasmids using colony PCR and DNA sequencing

Colonies grown on LB agar with carbenicillin (Section 2.3.11) were picked with a sterile toothpick and quickly dipped in a 10 µl PCR mixture containing an *acrA* forward primer and pBAD backbone reverse primer to select for the full insert in the correct orientation. Colony PCR composition and cycle conditions are described in full in Section 2.3.12. Positive clones were identified by the presence of a band at ~6069 bp

(predicted size of PCR product) on a 1% agarose gel (Fig. 3.6) (Section 2.3.3). Three positive colonies were randomly chosen and their plasmid DNA isolated using a MiniPrep kit (Section 2.3.1) then sent for DNA sequencing by Eurofins Genomics to identify an error-free clone (Section 2.3.13).

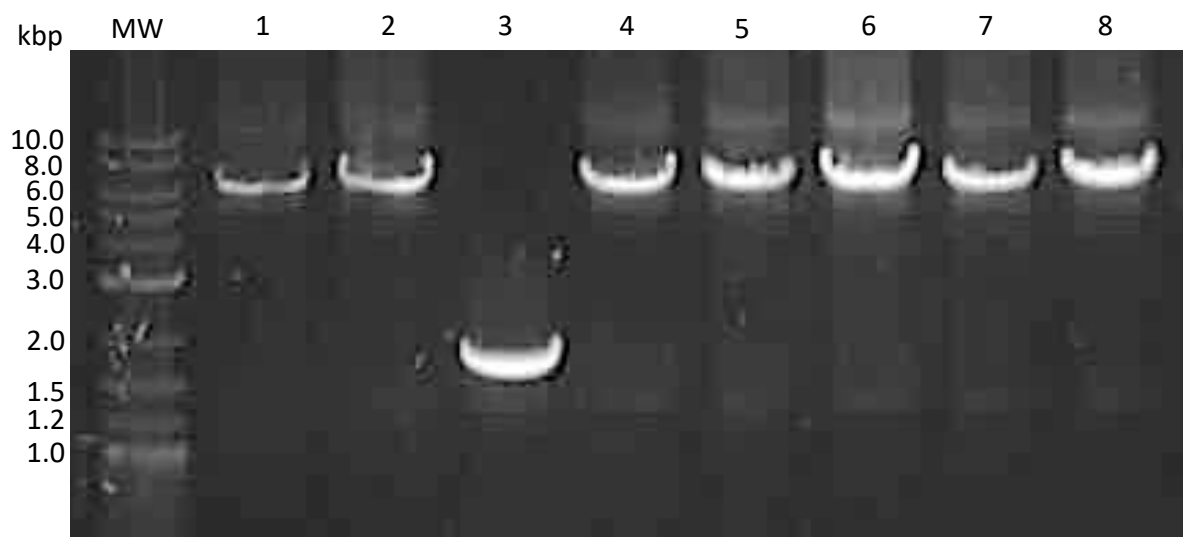


Figure 3.6. Colony PCR to identify pBAD plasmids with an *acrABtoIC* insert. PCR was performed using *acrAB* forward and pBAD reverse primers and analysed on a 1% agarose gel. Positive clones were identified by the presence of a band at ~6 kb. Lanes designated 'MW' were loaded with a molecular weight marker.

3.3 Cloning the *mexABoprM* genes under control of the p_{BAD} promoter

3.3.1 Amplification of genes using PCR

The *mexABoprM* genes exist as a cluster in the *Pseudomonas aeruginosa* genome, allowing amplification as a single fragment from *P. aeruginosa* PAO1 genomic DNA. Due to the high GC content of this region (65.9%), GC enhancer was added to the reaction mixture according to the manufacturer's instructions (Section 2.3.8), resulting in successful amplification of the 5767 bp *mexABoprM* fragment (Fig. 3.7).

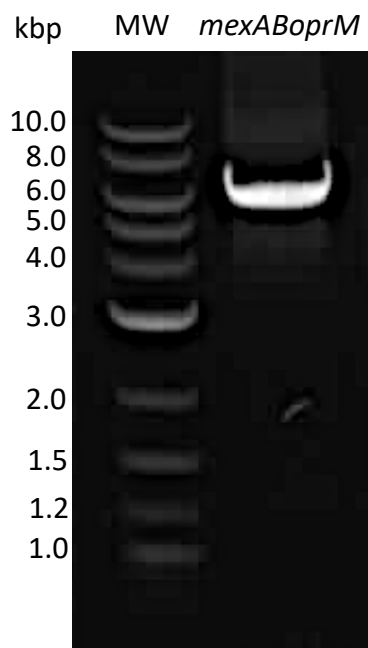


Figure 3.7. Amplification of the *mexABoprM* genes using PCR. PCR products were analysed on a 1% agarose gel. Lanes designated 'MW' were loaded with a molecular weight marker.

3.3.2 Digestion, ligation and transformation

The amplified fragment was digested with *NheI* and *NsiI* and ligated to the pBAD vector, which had been digested with *AvrII* and *SbfI* (Fig. 3.5) to generate complementary sticky ends. The ligation mixture was used to transform *E. coli* Omnimax cells which were grown on LB agar with 100 µg/ml carbenicillin at 37°C overnight.

3.3.3 Confirming target plasmids using colony PCR and DNA sequencing

Colonies grown on LB agar with carbenicillin were picked with a sterile toothpick and quickly dipped in a 10 µl PCR mixture containing a *mexABoprM* forward primer and pBAD backbone reverse primer to select for the full insert in the correct orientation. Positive clones were identified by the presence of a band at ~5981 bp (predicted size

of PCR product) (Fig. 3.8). A random selection of three positive colonies were sent for DNA sequencing by Eurofins Genomics and an error-free clone was identified.

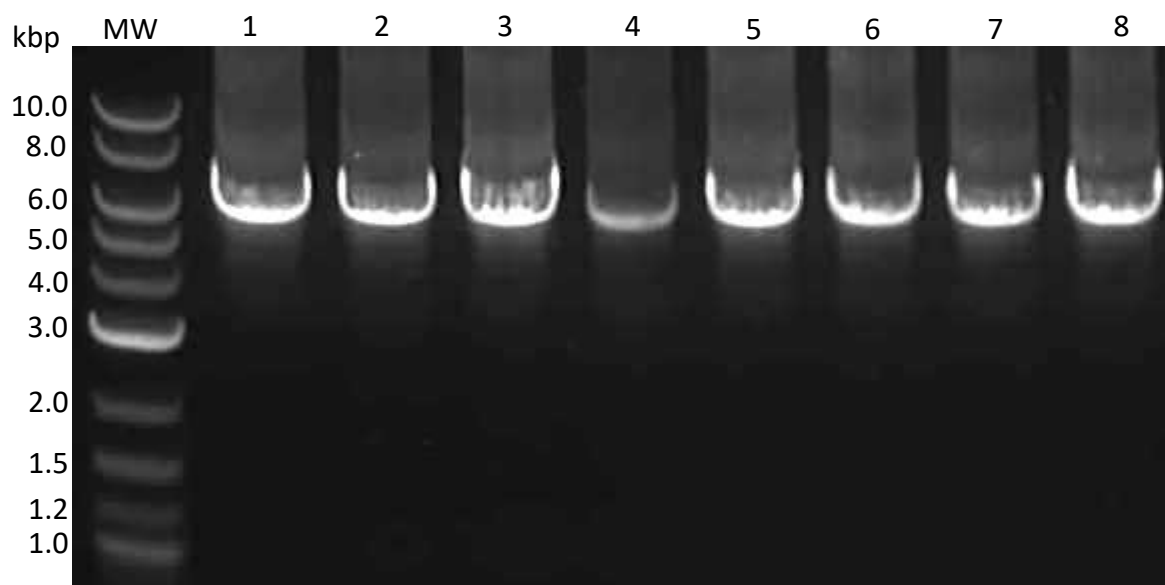


Figure 3.8. Colony PCR to identify pBAD plasmids with a *mexABoprM* insert. PCR was performed using *mexABoprM* forward and pBAD reverse primers and analysed on a 1% agarose gel. Positive clones were identified by the presence of a band at ~6 kb. Lanes designated ‘MW’ were loaded with a molecular weight marker.

3.4 Cloning the *mexCDoprJ* genes under control of the p_{BAD} promoter

3.4.1 Amplification of genes using PCR

The *mexCDoprJ* genes also exist as a gene cluster in the *Pseudomonas aeruginosa* genome, allowing all three genes to be amplified as a single fragment (5762 bp) from *P. aeruginosa* PAO1 genomic DNA (Fig. 3.9). GC enhancer was added to the PCR mixture to compensate for the high GC content of these genes (67.7%).

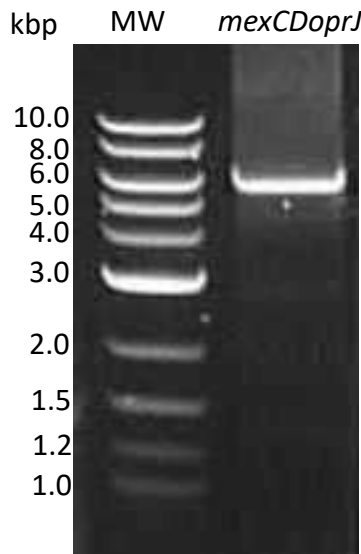


Figure 3.9. Amplification of the *mexCDoprJ* genes using PCR. PCR products were analysed on a 1% agarose gel. Lanes designated 'MW' were loaded with a molecular weight marker.

3.4.2 Digestion, ligation and transformation

The *mexCDoprJ* fragment was digested with *NheI* and *NsiI*, while the empty pBAD vector was digested using *AvrII* and *SbfI* to generate complementary sticky ends (Fig. 3.5). The two fragments were ligated together and the ligation mixture used to transform *E. coli* Omnimax cells which were then incubated at 37°C overnight on LB agar with 100 µg/ml carbenicillin.

3.4.3 Confirming target plasmids using colony PCR and DNA sequencing

Colonies grown on LB agar carbenicillin were picked with a sterile toothpick and quickly dipped in a 10 µl PCR mixture containing a *mexCDoprJ* forward primer and pBAD backbone reverse primer to select for the full insert in the correct orientation. Positive clones were identified by the presence of a ~5890 bp (predicted PCR product size) band on a 1% agarose gel (Fig. 3.10). A random selection of three positive

colonies were sent for DNA sequencing by Eurofins Genomics and an error-free clone was identified.

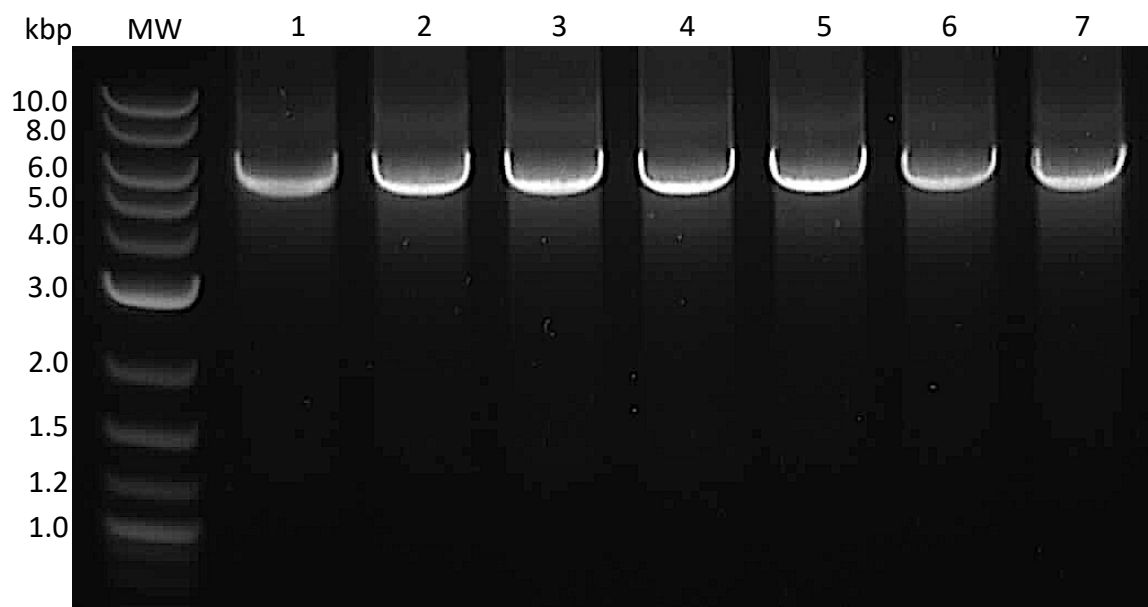


Figure 3.10. Colony PCR to identify pBAD plasmids with a *mexCDoprJ* insert. PCR was performed using *mexCDoprJ* forward and pBAD reverse primers and analysed on a 1% agarose gel. Positive clones were identified by the presence of a band at ~6 kb. Lanes designated 'MW' were loaded with a molecular weight marker.

3.5 Cloning the *emrABtolC* genes under control of the *p_{BAD}* promoter

3.5.1 Amplification of genes using PCR

Unlike the Mex systems, the genes encoding EmrAB-TolC are not co-localised in the *E. coli* chromosome. The *emrAB* genes (2542 bp) were amplified by PCR as a single fragment (Fig. 3.11A) from *E. coli* MG1655 (K12) genomic DNA and the *tolC* gene (1527 bp) amplified separately (Fig. 3.11B) using the same gDNA template.

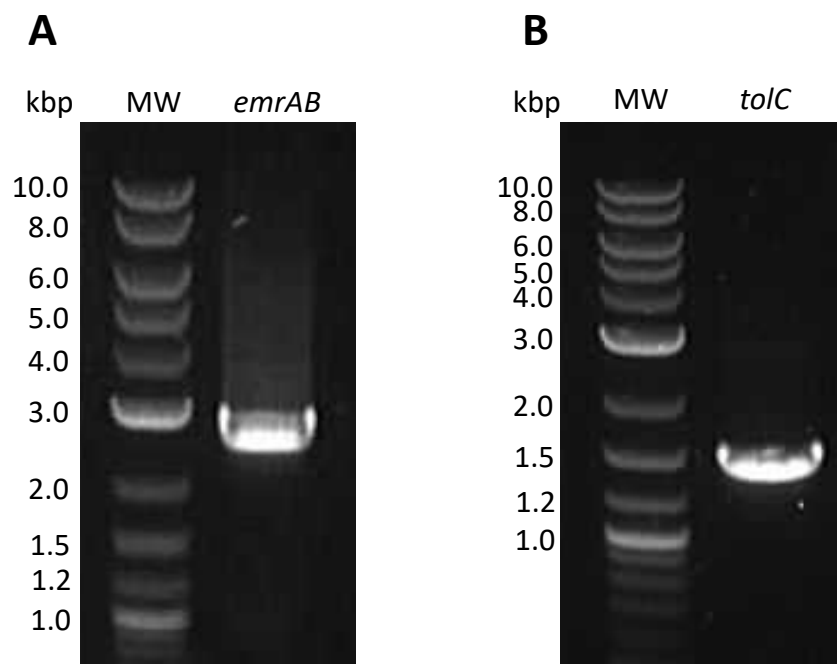


Figure 3.11. Amplification of the *emrAB* (A) and *tolC* (B) genes using PCR. PCR products were analysed on a 1% agarose gel. Lanes designated 'MW' were loaded with a molecular weight marker.

3.5.2 Digestion, ligation and transformation

The two fragments were digested using *SacI* and ligated together to yield a 4075 bp fragment, which was identified by size and extracted from a 1% agarose gel. The assembled fragment was then digested using *AvrII* and *SbfI* and ligated to the pBAD backbone that had been digested with the same enzymes (Fig. 3.5). The ligation mixture was used to transform *E. coli* C43 Δ *acrAB* cells which were grown on LB agar with 100 μ g/ml carbenicillin at 37°C overnight.

3.5.3 Confirming target plasmids using colony PCR and DNA sequencing

Colonies grown overnight in the presence of carbenicillin were picked and quickly dipped in PCR mixture containing an *emrAB* forward primer and pBAD backbone reverse primer to select for the correct insert in the correct orientation. Positive colonies were identified by the presence of a fragment ~4239 bp (predicted PCR

product size) (Fig. 3.12). Three positive colonies were randomly selected and plasmids isolated using a MiniPrep kit (Section 2.3.1) then sent for sequencing by Eurofins genomics to identify an error-free clone.

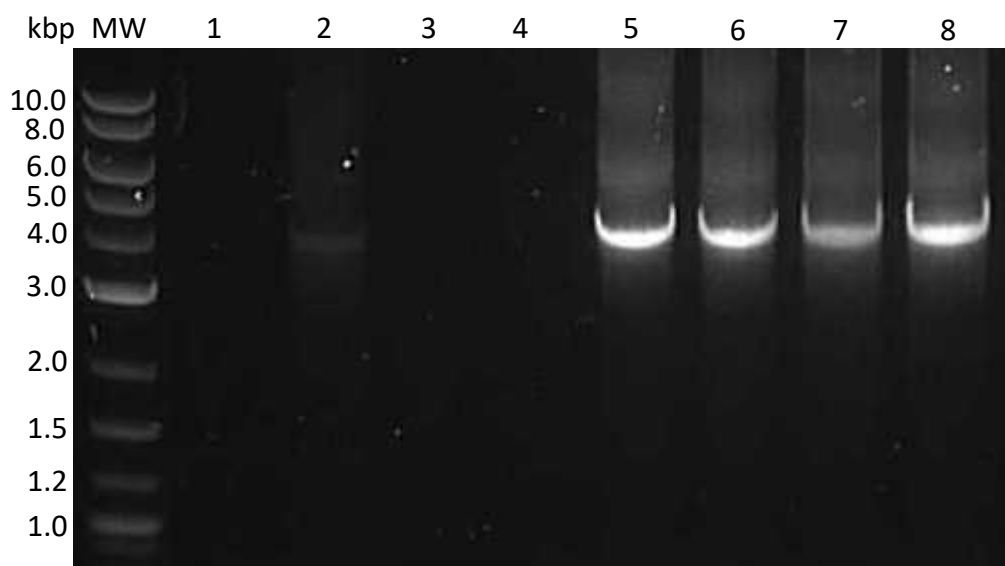


Figure 3.12. Colony PCR to identify pBAD plasmids with an *emrABtoIC* insert. PCR was performed using *emrAB* forward and pBAD reverse primers and analysed on a 1% agarose gel. Positive clones were identified by the presence of a band at ~4.2 kb. Lanes designated 'MW' were loaded with a molecular weight marker.

3.6 Cloning inner membrane transporters under control of the p_{BAD} promoter

3.6.1 Amplification of genes using PCR

All five target genes, *emrD*, *mdlB*, *mdtL*, *mdtM* and *msbA* were amplified by PCR from *E. coli* MG1655 genomic DNA (Fig. 3.13). PCR products were identified by their predicted sizes, which were 1236 bp, 1833 bp, 1227 bp, 1284 bp and 1803 bp, respectively (Fig. 3.13).

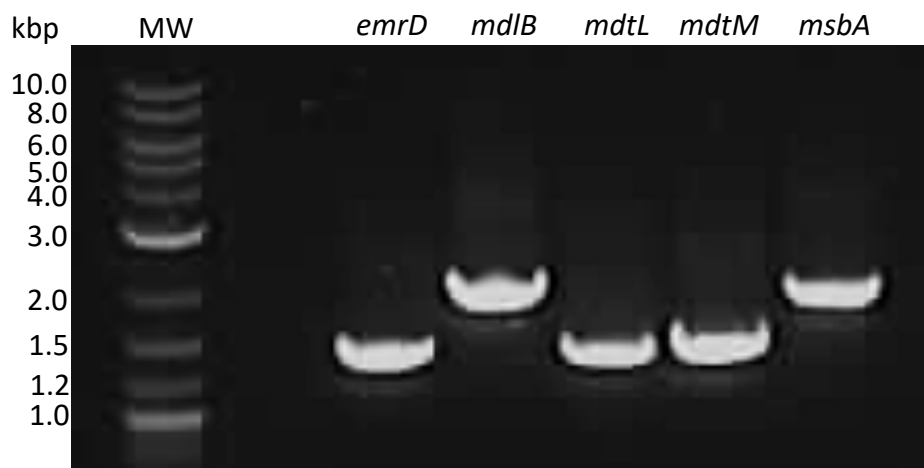


Figure 3.13. Amplification of inner membrane transporters using PCR. PCR products were analysed on a 1% agarose gel. Lanes designated ‘MW’ were loaded with a molecular weight marker and lane 2 was left empty.

3.6.2 Digestion, ligation and transformation

Following purification of the PCR products using a spin-column (Section 2.3.7), each gene was digested with *Nhe*I and *Nsi*I and ligated to the pBAD vector, which had been digested with *Avr*II and *Sbf*I to generate complementary sticky ends (Fig. 3.5). The ligation mixture was used to transform *E. coli* C43 Δ *acrAB* cells, which were grown overnight on LB agar with 100 μ g/ml carbenicillin at 37°C.

3.6.3 Confirming target plasmids using colony PCR and DNA sequencing

Colonies that grew in the presence of carbenicillin were chosen at random for colony PCR using one gene specific forward primer and a pBAD backbone reverse primer (Fig.3.13). Positive colonies were identified by the presence of a band at the expected molecular weight (see Section 3.6.1). Three positive colonies were chosen at random for each gene and their plasmids isolated using a MiniPrep kit (Section 2.3.1) and sent to Eurofins Genomics for insert sequencing.

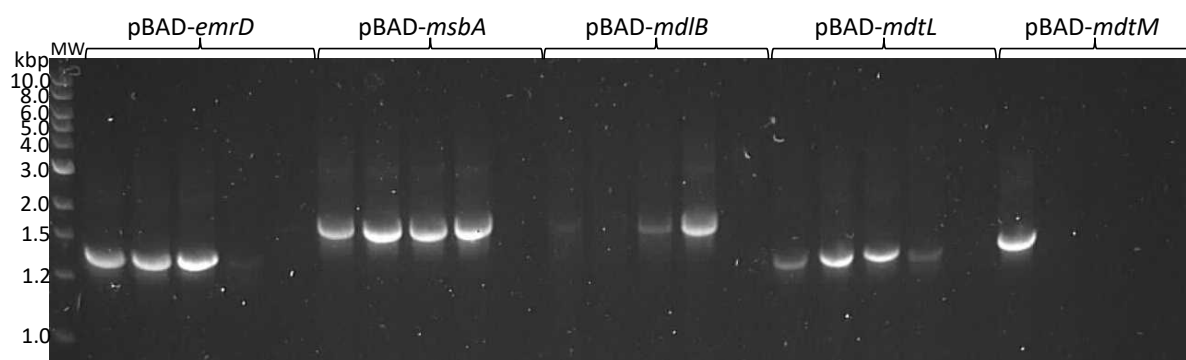


Figure 3.14. Colony PCR to identify pBAD plasmids with an inner membrane efflux pump gene insert. PCR was performed using gene specific forward and pBAD reverse primers and analysed on a 1% agarose gel. Positive clones were identified by the presence of a band at ~1.2 kb for *emrD*, *mdtL* and *mdtM* and ~2 kb for *mdlB* and *msbA*. Lanes designated 'MW' were loaded with a molecular weight marker.

3.7 Cloning the *acrA* under control of the *p_{BAD}* promoter

3.7.1 Amplification of the *acrA* gene using PCR

The *acrA* gene was amplified by PCR from *E. coli* MG1655 (K12) genomic DNA to generate a 1210 bp fragment (Fig. 3.15).

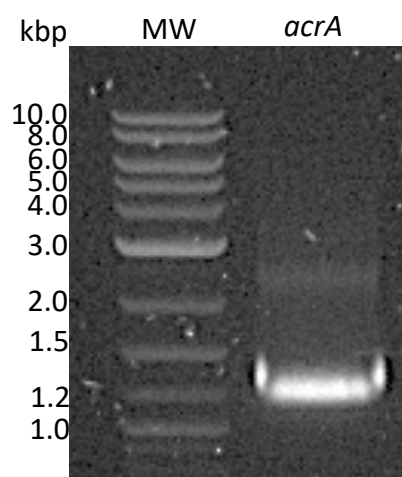


Figure 3.15. Amplification of the *acrA* gene using PCR. PCR products were analysed on a 1% agarose gel. Lanes designated 'MW' were loaded with a molecular weight marker.

3.7.2 Digestion, ligation and transformation

Following purification using a spin-column (Section 2.3.7), the PCR product was digested with *AvrII* and *SbfI* and ligated to the pBAD vector digested with the same enzymes (Fig. 3.5). The ligation mixture was used to transform *E. coli* C43 Δ *acrAB* cells which were then grown overnight on LB agar with 100 μ g/ml carbenicillin at 37°C overnight.

3.7.3 Confirming target plasmids using colony PCR and DNA sequencing

Colonies were chosen at random and screened by colony PCR using the *acrA* forward primer and pBAD backbone reverse primer (Fig. 3.16). Positive colonies were identified by the presence of a ~1409 bp fragment (predicted size of PCR product) on a 1% agarose gel (Fig. 3.16), then three selected at random to be subcultured for plasmid isolation using a MiniPrep kit (Section 2.3.1). Isolated plasmids were sent to Eurofins Genomics for insert sequencing and an error-free clone identified.

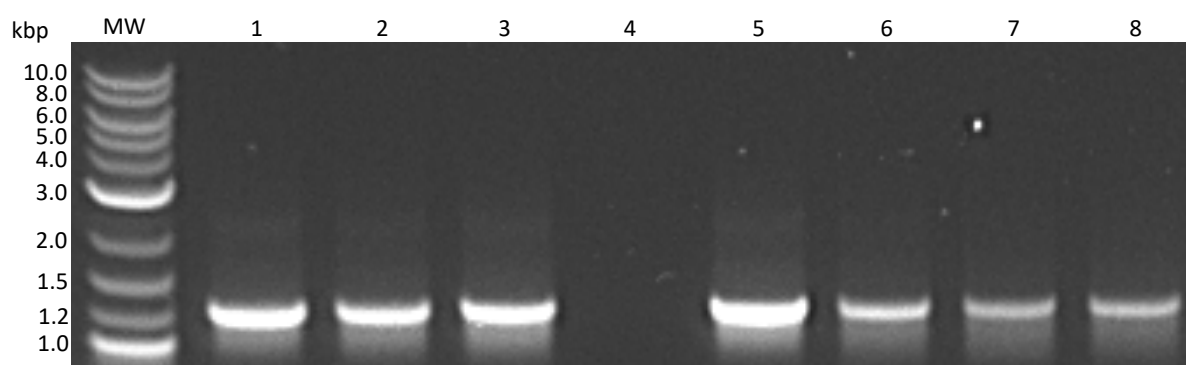


Figure 3.16. Colony PCR to identify pBAD plasmids with an *acrA* insert. PCR was performed using *acrA* forward and pBAD reverse primers and analysed on a 1% agarose gel. Positive clones were identified by the presence of a band at ~1.4 kb. Lanes designated 'MW' were loaded with a molecular weight marker.

3.8 Cloning tripartite efflux pumps under the p_{gntK} promoter

3.8.1 Amplification of genes using PCR

In order to simplify the cloning procedure, genes were amplified by PCR from the relevant pBAD plasmids and visualised on a 1% agarose gel (Fig. 3.17). The PCR was performed using one gene-specific forward primer and one pBAD backbone reverse primer in order to maintain the His₈-tag introduced by the pBAD vector. The *acrABtolC*-His₈, *emrABtolC*-His₈, *mexABoprM*-His₈ and *mexCDoprJ*-His₈ PCR products had predicted sizes of 6069 bp, 4239 bp, 5981 bp and 5890 bp respectively, which were used to identify them on a 1% agarose gel (Fig. 3.17).

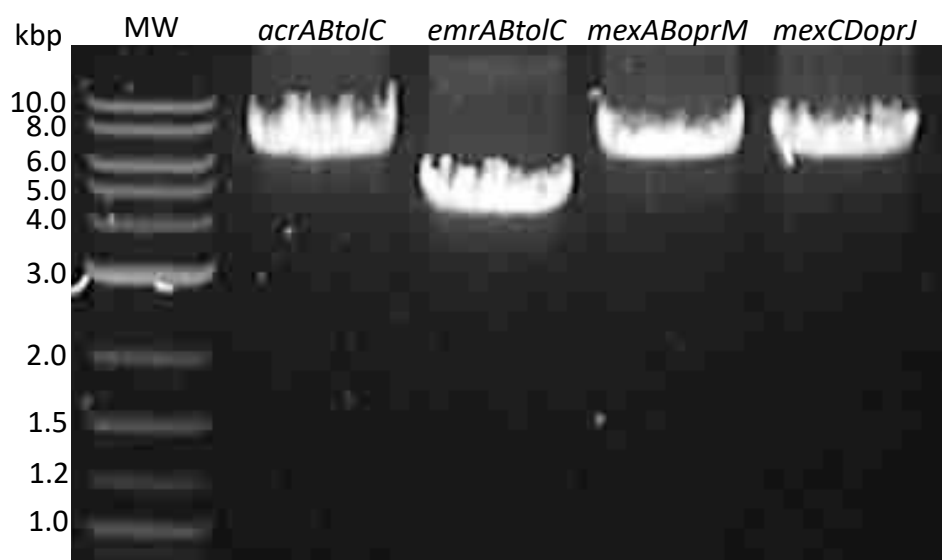


Figure 3.17. Amplification of tripartite efflux pump genes from pBAD constructs using PCR. PCR products were visualised on a 1% agarose gel. Lanes designated 'MW' were loaded with a molecular weight marker.

3.8.2 Digestion, ligation and transformation

The pBAD backbone contains a HindIII restriction site immediately downstream of the His₈-tag site, allowing cleavage and ligation into an appropriate vector without loss of the His₈-tag. The pGNTK backbone was therefore linearised by PCR to introduce both

an AvrII and HindIII restriction site downstream of the p_{gntK} promoter, allowing insertion of the His₈-tagged genes following amplification by PCR. The linearised vector was then digested with AvrII and HindIII (Fig. 3.18) and stored at -20°C to be used for all inserts.

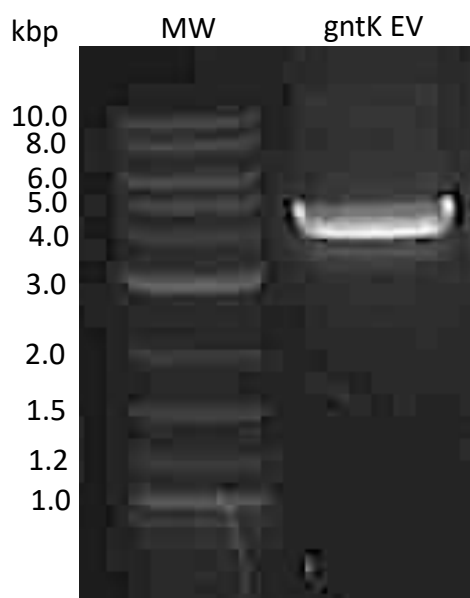


Figure 3.18. Linearisation and digestion of the pGNTK empty vector. The empty pGNTK vector was linearised by PCR then digested with AvrII and HindIII resulting in a ~4.1 kb product. The cleaved product was purified using a spin-column (Section 2.3.7) and analysed on a 1% agarose gel. Lanes designated 'MW' were loaded with a molecular weight marker.

The *acrABtolC* and *emrABtolC* genes were digested with AvrII and HindIII, while *mexABoprM* and *mexCDoprJ* were digested using NheI and HindIII before being ligated to the pGNTK backbone. The ligation mixtures were used to transform *E. coli* C43 Δ *acrAB* cells which were then grown overnight on LB agar with 50 µg/ml kanamycin at 37°C.

3.8.3 Confirming target plasmids using colony PCR and DNA sequencing

Colonies that grew in the presence of kanamycin were selected for colony PCR. Each construct was screened using one gene specific forward primer and a pGNTK specific

reverse primer to select for correct inserts in the correct orientation. The predicted PCR product sizes were 5986 bp, 4156 bp, 5898 bp and 5899 bp for *acrABtolC*, *emrABtolC*, *mexABoprM* (Fig. 3.19A) and *mexCDoprJ* (Fig. 3.19B) respectively, and were used to identify colony PCR positive clones. Three positive colonies were selected at random and subcultured for plasmid isolation, then sent to Eurofins Genomics for sequencing of each insert.

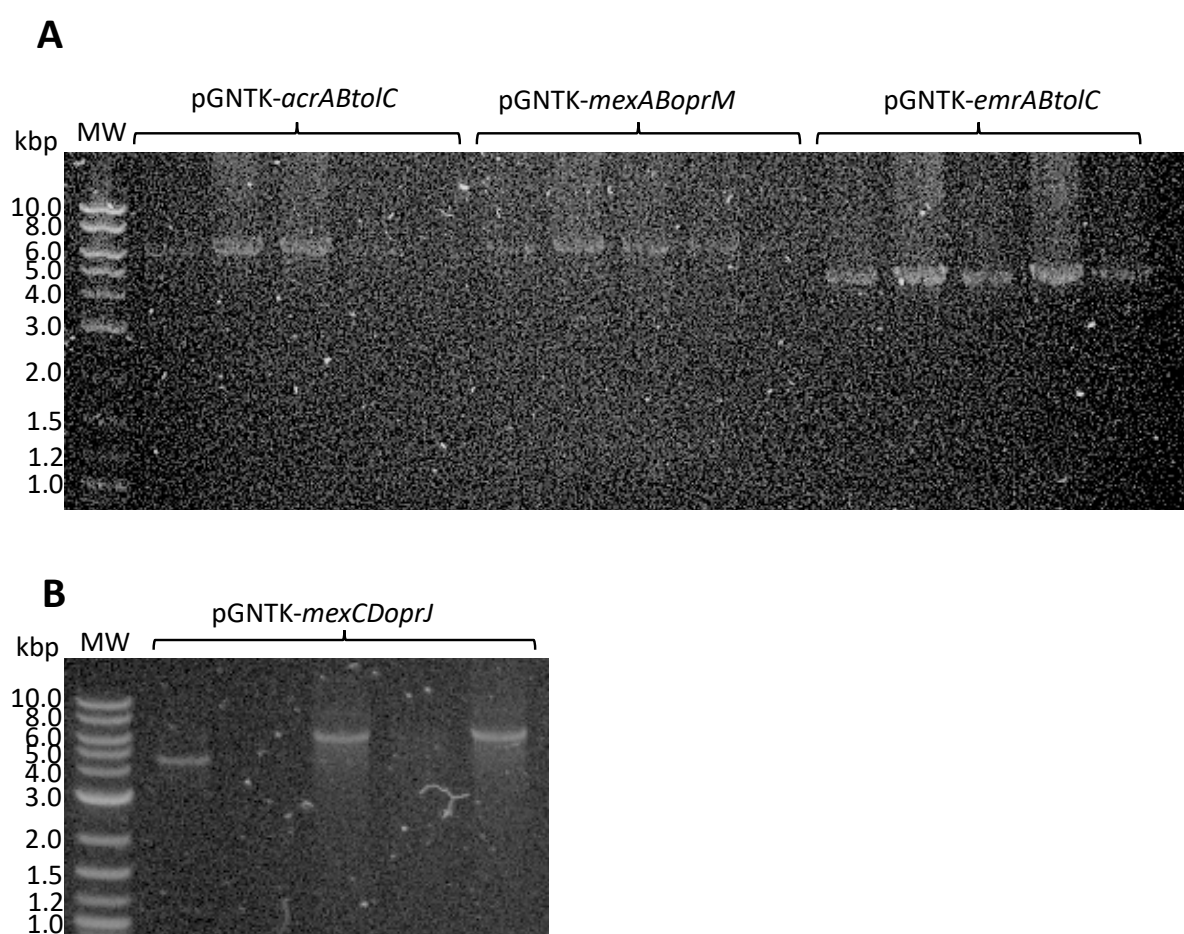


Figure 3.19. Colony PCR to identify pGNTK plasmids with tripartite efflux pump gene inserts. PCR was performed using *one* gene-specific forward and pGNTK reverse primers and analysed on a 1% agarose gel. Positive clones were identified by the presence of a band at ~4.2 kb for *emrABtolC* and ~6 kb for all other genes. Lanes designated 'MW' were loaded with a molecular weight marker.

3.9 Cloning inner membrane efflux pumps under the p_{gntK} promoter

3.9.1 Amplification of genes using PCR

In order to maintain the His₈-tag, inner membrane efflux pump genes were also amplified from their respective pBAD constructs by PCR using one gene-specific forward primer and one pBAD backbone reverse primer then identified based on their size on a 1% agarose gel (Fig. 3.20). Predicted PCR product sizes were 1399 bp, 1447 bp, 1390, 1966 bp and 1996 bp for *emrD*-His₈, *mdtM*-His₈, *mdtL*-His₈ (Fig. 3.20A), *msbA*-His₈ (Fig. 3.20B) and *mdlB*-His₈ (Fig. 3.20C), respectively.

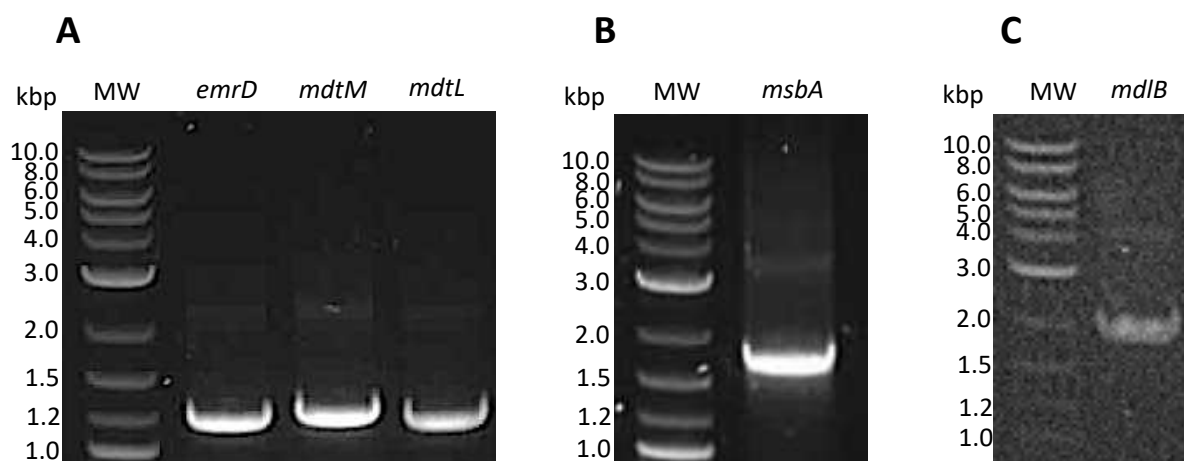


Figure 3.20. Amplification of inner membrane efflux pumps genes using PCR. PCR products were analysed on a 1% agarose gel. Lanes designated 'MW' were loaded with a molecular weight marker.

3.9.2 Digestion, ligation and transformation

Each of the five inserts was digested with NheI and HindIII and ligated to the pGNTK backbone, which has been digested with AvrII and HindIII (Fig. 3.18). The ligation mixture was used to transform *E. coli* C43 Δ *acrAB* cells that were grown at 37°C overnight on LB agar with 50 µg/ml kanamycin.

3.9.3 Confirming target plasmids using colony PCR and DNA sequencing

Colonies able to grow in the presence of kanamycin were picked and quickly dipped in 10 µl PCR reactions containing one gene-specific forward primer and a pGNTK-specific reverse primer. Positive colonies were identified based on the PCR product size on a 1% agarose gel (Fig. 3.21). Predicted product sizes were 1913 bp, 1316 bp, 1364 bp, 1307 bp and 1883 bp for *mdlB*, *emrD*, *mdtM*, *mdtL* and *msbA* respectively.

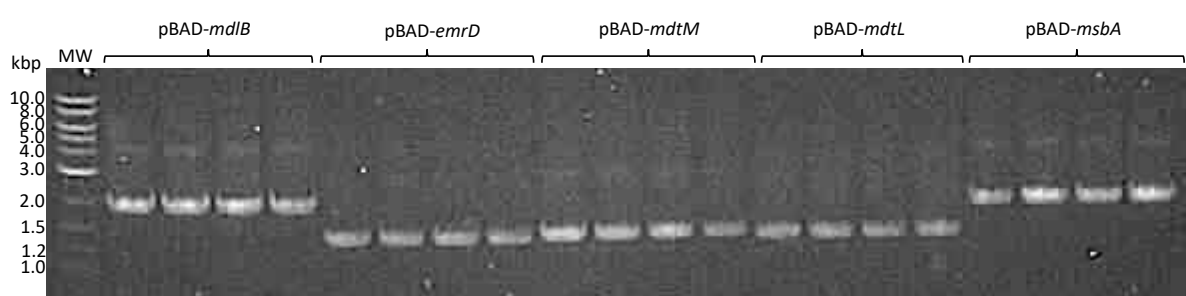


Figure 3.21. Colony PCR to identify pGNTK plasmids with an inner membrane efflux pump gene insert. PCR was performed using gene specific forward and pGNTK reverse primers and analysed on a 1% agarose gel. Positive clones were identified by the presence of a band at ~1.2 kb for *emrD*, *mdtL* and *mdtM* and ~2 kb for *mdlB* and *msbA*. Lanes designated 'MW' were loaded with a molecular weight marker.

3.10 Discussion

In order to study the efflux of butyl methacrylate and identify an efflux pump that would effectively transport this substrate out of the cell, target efflux pumps were identified from literature searches and preliminary *in vivo* studies performed at Ingenza Ltd., the University of Nottingham and the University of Strathclyde (Table 3.1). Nine different efflux pumps of three different families (Table 3.1) were cloned into two different *E. coli* expression vectors using a restriction enzyme-based approach (Section 3.1.4). The *acrA* gene was also cloned into the pBAD expression vector for future purification in order to study the purified AcrAB assembly (Fig. 3.16).

The MFS transporters EmrD, MdtM and MdtL, as well as the ABC transporters MdlB and MsbA were readily amplified by PCR (Fig. 3.13), digested and ligated into the pBAD expression vector (Fig. 3.14). Although the RND transporter AcrAB-TolC and MFS transporter EmrAB-TolC required assembly into a single DNA fragment, these too were easily cloned into the pBAD expression vector (Fig. 3.6; Fig. 3. 11). Amplification of the genes encoding the RND efflux pumps MexAB-OprM and MexCD-OprJ from *P. aeruginosa* gDNA (Fig. 3.7; Fig. 3.9) required the addition of GC enhancer due to their high GC content and large size (~6 kbp). Error-free DNA sequenvces of clones, each in the correct orientation, with an in-frame His₈-tag enabling both protein detection and purification, were identified for every gene cloned into the pBAD expression vector (see appendix A for full sequences). These tagged genes were successfully expressed in appropriate *E. coli* host strains (see Table 2.1) upon addition of arabinose (see Chapter Five). Additionally, the His₈-tagged genes could also be used as a template to subclone all nine tagged efflux pumps into the pGNTK expression vector (Fig. 3.19; Fig. 3.21), significantly simplifying the cloning procedure, particularly for the tripartite pumps that required additional assembly steps and/or PCR optimisation. The genetic constructs generated here are valuable tools for studying efflux proteins both in *E. coli* whole cells and in isolation, thanks to the addition of an affinity tag, and serve as an experimental platform for this project.

Chapter 4

Characterisation of the PACE protein Acel and its homologues

4.1 Introduction

4.1.1 *Acinetobacter baumannii*

Acinetobacter baumannii is a Gram-negative pathogen of significant clinical relevance. As part of the ESKAPE group of pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), *A. baumannii* is classified as one of the six leading causes of nosocomial infections worldwide (Pendleton *et al.*, 2013; Thombre *et al.*, 2019). The ESKAPE pathogens are characterised by their high virulence and multidrug resistance phenotypes, making them a serious threat to global health and the economy (Thombre *et al.*, 2019; Zhen *et al.*, 2019). *A. baumannii* is responsible for a broad range of infections in clinical settings but is most commonly associated with urinary tract infections, pneumonia and septicaemia, with mortality rates as high as 35% (Antunes *et al.*, 2014). It has previously been demonstrated that *A. baumannii* is able to survive on surfaces for several months (Kramer *et al.*, 2006; Wendt *et al.*, 1997), meaning effective decontamination of clinical environments and medical devices is vital in preventing the spread of nosocomial infections.

4.1.2 Discovery of Acel as an active chlorhexidine efflux pump

Antiseptics are an essential tool in preventing the spread of nosocomial infections and as such are used in hospital hand soaps, in the cleaning and treatment of wounds and even impregnated into wound dressings and medical devices. Chlorhexidine is a clinically relevant synthetic bisbiguanide antiseptic first described in the 1950s (Davies *et al.*, 1954) that has been shown to kill *A. baumannii* through alterations in membrane composition and general membrane damage (Biswas *et al.*, 2019). Chlorhexidine is included in the World Health Organisation (WHO) list of essential medicines (WHO,

2019), although increased levels of tolerance in a number of pathogens, including *Acinetobacter baumannii*, have been widely reported (Fernandez-Cuenca *et al.*, 2015; Hayashi *et al.*, 2017; Kampf, 2016; Thomas *et al.*, 2000; Wand *et al.*, 2017). Transcriptomic experiments performed in *A. baumannii* identified ~10-fold up-regulation of a hypothetical gene, now designated *acel*, upon exposure to sub-inhibitory concentrations of chlorhexidine (Hassan *et al.*, 2013). When cloned and expressed in *E. coli*, the Acel protein was localised to the inner membrane and conferred an 8-fold increase in resistance to chlorhexidine (Hassan *et al.*, 2013). Transport assays using radiolabelled chlorhexidine demonstrated that Acel is an active chlorhexidine efflux pump (Hassan *et al.*, 2013) belonging to the novel proteobacterial antimicrobial compound efflux (PACE) family of efflux pumps (Hassan *et al.*, 2015; Hassan *et al.*, 2018), of which Acel is the prototypical member.

4.1.3 The proteobacterial antimicrobial compound efflux (PACE) family

The PACE family of efflux pumps is characterised by 4 transmembrane helices arranged into two tandem bacterial transmembrane pair (BTP) domains which possess strong sequence conservation, suggesting they have arisen from a gene duplication event (Hassan *et al.*, 2018). Due to their small size, it is likely that PACE family proteins function as oligomers, although there are currently no structural data to confirm this. All PACE proteins also contain four conserved amino acids, most interestingly a glutamic acid residue in transmembrane helix one (Hassan *et al.*, 2018). In Acel, substitution of this residue for a neutral glutamine residue results in a protein that is able to bind chlorhexidine with comparable affinity to the wild-type, but has no observable transport activity (Hassan *et al.*, 2013). This glutamic acid residue is

therefore likely essential for proton translocation in a proton-driven transport mechanism in all PACE family members.

While PACE family proteins confer resistance to a number of synthetic biocides developed in the last century, homologous genes have been identified in bacterial genera that diverged millions of years ago (Hassan *et al.*, 2018), meaning these transporters evolved to perform a different physiological function. Polyamines such as spermine, spermidine, putrescine and cadaverine have previously been explored as potential physiological substrates of the PACE family proteins. Polyamines were investigated due to their natural abundance in bacterial cells, where they play important physiological roles, and their structural similarities to chlorhexidine as they consist of aliphatic carbon chains with multiple amino groups (Hassan *et al.*, 2019). Of those polyamines tested, cadaverine was of particular interest as it strongly induced expression of *aceI*, while deletion of the *aceI* gene also significantly reduced the minimum inhibitory concentration of cadaverine in *A. baumannii* (Hassan *et al.*, 2019).

To investigate this further, *aceI* and eight of its homologues were expressed in *E. coli* and characterised using radiolabelled transport assays and purified protein reconstituted into proteoliposomes. We demonstrate that Acel transports cadaverine, a diamine that is naturally abundant in many bacterial lineages, using a proton antiport mechanism and that short chain diamines represent the physiological substrates of PACE family efflux pumps. This discovery also makes PACE family proteins relevant in a biotechnological context, as cadaverine is widely used as a monomer for polyamide production (Kind *et al.*, 2014; Kwak *et al.*, 2017; Qian *et al.*, 2011; Wang *et al.*, 2018). As such, Acel and PACE family proteins are potentially valuable model systems for the study of bacterial efflux pumps and their biotechnological applications.

4.2 Screening expression levels of the PACE genes

4.2.1 Expression of the *aceI* wild-type and *aceI* E15Q mutant genes

The constructs used for the expression of *aceI* wild-type and *aceI* E15Q mutant were created by Hassan *et al.* (2013), as were the constructs used for expression of PACE family Acel homologues (Hassan *et al.*, 2015). These constructs use the pTTQ18 expression vector (Stark *et al.*, 1987) (Fig. 4.1), which has proven to be successful in expressing a wide range of bacterial membrane proteins in a heterologous *E. coli* host (Saidijam *et al.*, 2003; Szakonyi *et al.*, 2007; Ma *et al.*, 2014; Hassan *et al.*, 2015). The pTTQ18 vector uses the strong *tac* promoter, a fusion of the *lac* and *trc* promoters (Stark *et al.*, 1987), as well an ampicillin resistance cassette (AmpR) and ColE1 origin of replication for maintenance of the plasmid in *E. coli* grown in the presence of ampicillin or carbenicillin (Fig. 4.1). The partial LacI^q repressor encoded on the vector backbone binds the *lacO* operator region to reduce 'leaky' expression in the uninduced state, minimising effects of toxic gene expression during the pre-induction cell growth stage (Stark *et al.*, 1987). The gene of interest is placed under the control of the *tac* promoter, resulting in lactose or IPTG-inducible gene expression, while introduction of a hexahistidine affinity tag to the C-terminus of the recombinant protein (Fig. 4.1) allows for immobilised metal affinity chromatography (IMAC) purification and protein detection by Western blotting. The pTTQ18-*aceI*/His₆ construct is shown as an example (Fig. 4.1). Both *aceI* wild-type and E15Q mutant genes were well-expressed in *E. coli* BL21 from the pTTQ18 constructs and the His₆-tagged proteins were detected by Western blotting in crude cell lysates at comparable levels (see Section 2.6.1) (Fig. 4.2).

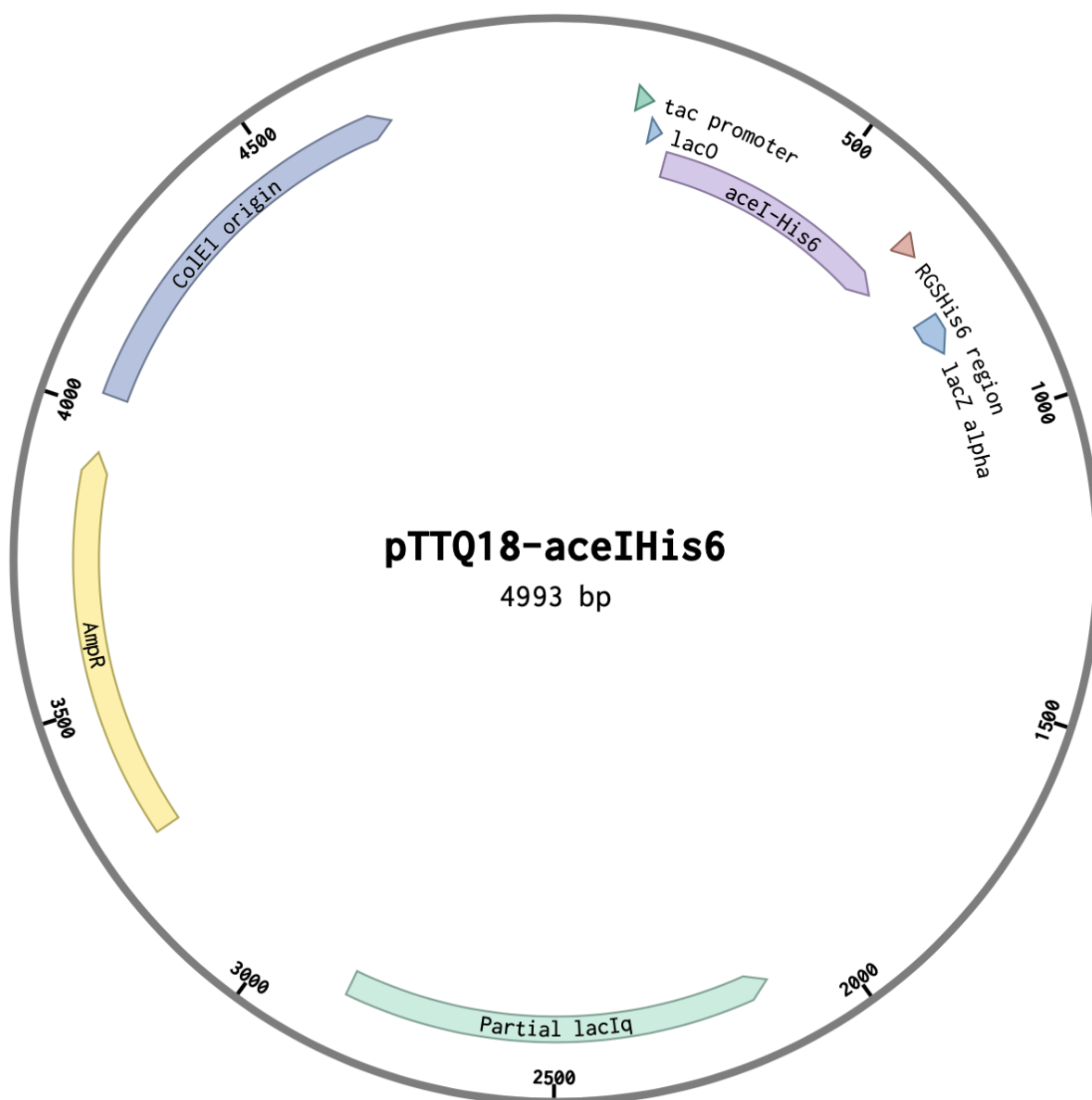


Figure 4.1. pTTQ18 PACE expression vector. The pTTQ18-aceIHis6 construct is shown as an example. In all other pTTQ18 constructs, the relevant gene of interest is substituted for the *aceI* gene with no other changes to the vector backbone.

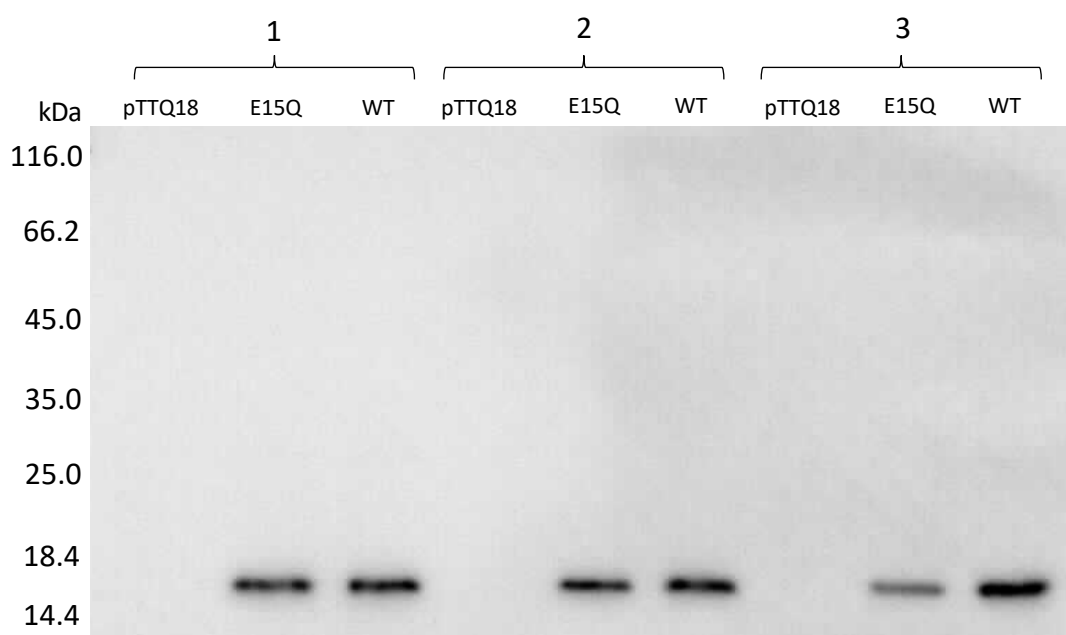


Figure 4.2. Screening production of Acel wild-type and Acel E15Q mutant proteins in *E. coli* BL21 cells used for [14 C]-cadaverine uptake assays. The empty pTTQ18 vector was used as a negative control. A molecular weight marker (MW) was loaded in the first lane (not shown). Whole cell samples were prepared as described in Section 2.2.5 in biological triplicate and analysed by Western blotting as described in Section 2.6.1. His-tagged proteins were detected using HisProbe-HRP conjugate.

4.2.2 Expression of Acel homologues

To investigate further the physiological role of PACE family proteins, eight PACE family homologues of Acel that were previously found to be well expressed in *E. coli* BL21 (Ahmad, 2017) were chosen for uptake experiments (Fig. 4.3). Cells were grown and induced in 10 ml volumes as described in Section 2.2.5, then disrupted and analysed by Western blotting (Section 2.6.1) (Fig. 4.4). Each gel was loaded with a sample of cells expressing the *acel* gene as a reference sample (Fig. 4.4). All genes were clearly expressed in *E. coli* and protein was detectable in all cases (Fig. 4.4), although to varying degrees.

TMARG	MEER-----NQAVESKQDGVALRTWRDRVRQVLAFAEAGGLVLIT	39
MLUT_15630	MSETHRAPGVDPASAPPSDVPRGPGGRDALVRRRVFRTPLLRVVYAVVFELLAILFTT	60
A1S_1503	-----MQGLKRRIVYVSSYEIIGMVISS	23
ACEI	-----MLISKRRLIHAISYEGILLVIA	23
VP1155	-----MTRNERIFHAVLFELMALAIIV	22
FBAL_3166	-----MSPRERVLSLLFEMIALAIVV	22
STY3166	-----MIKSKVFSFMQHDAIQRRSLPERIFHAVCFEGIATAILA	39
PFL_4558	-----MSLQKSLNERIFQAVGFELLAVLICT	26
PSPT0_3587	MNEQKD--SL-----NMTHREQPTQQPPVVHKTIRERALHATLFEVGGVILVA	47
	* :* :	
TMARG	PPFSWASGAPVLDLGLLALLALIAALWNALYNTVFDRLERFLHRRADLRPPWLVRVAHA	99
MLUT_15630	GILAAALGN---GGGPSLAVAVVSSTVALLWNIVFNSVFETLERRLGI---TGRPWVVRVAHA	116
A1S_1503	VGLALLAGDSVEHTGPLSMITTIATVWNFIYNIYKWEARQES---KSRTVKRRIAHA	80
ACEI	IALSFIFNMPMEVTGTLGVFMAVSVFWMNIFNHYFEKVEHKYN---WERTIPVRILHA	79
VP1155	PAAALITGKGSSDLALVGIGLSLYTVVWNYIYNLYFDKWFSGSNR---ADRSLAMRLGHT	78
FBAL_3166	PAGILLTGTDAGHMTATAIILSLMAMAWNYYVNLGFDVFGSNR---IDRSWGLRVGHG	78
STY3166	PTTAWLMQSVLEMGGLTILLATTAMIWNIIYNALFDRLLWPAHQ---VRRATAKVRALHA	95
PFL_4558	PLLSWIMDKPMADMGLVTIGIGLLALAWNIVFNGLFDRLLKRLQ---WVHNGWTRVLHA	82
PSPT0_3587	PLLAWIMNQLAMIGIMTVMISTVAMLWNMVYNALFDRLLRSRFG---FAMTAMTRILHA	103
	: ** ::* :: * *	
TMARG	LGFEGGLLFLTPVIVAWTGMGWLEALVADLGLALAYTVYAYVFNWCYDRLFPIGPTGEQ	159
MLUT_15630	VGFEGLIVFLVPAVALILGIGLGEAFLEAGLLVFFLVYAAVYAYAFDSVFGLPDSAAAR	176
A1S_1503	IGFQITLVMFLIPLIAWMMNISLVAAFWLDVAFIIIIPIYTFIFNWTFDKLFGLPASAQP	140
ACEI	IGFEGLLIATVPMIAYMMQMTVIDAFILDIGLTLCILVYTFIFQWCYDHIEDKFFPNAK	139
VP1155	VGFEGLLIFISIPVIAWFLEITFLALMLEAGFLVFFLFYATGFNWLVDKVPFGKMRKL	138
FBAL_3166	VGFEAGLVTVTIPVLMFSLNLGFVDALIMDIGFVFFLVYAIYINWAYDQFRARLERQGK	138
STY3166	LGFESGFIVIGVSIWAVLNVSLQAFTLEIGFFLFLPYTMLYNWAYDVLQRIVTRRQ	155
PFL_4558	LMFEGLVAVGVPMIAWNLISLLQAFILDIGVLLFFLPYTYVYHVVYDVLBDKWLLQQR	142
PSPT0_3587	IGFEAGLILAVVPLAAWWSISLMQAFWLDIGLLMFLPYTLFNWAYDNLRLRVQRRM	163
	: *: :: : : *: : .. : *: : : *: .	
TMARG	-----	159
MLUT_15630	AEA-----	179
A1S_1503	N--TAQQ--	145
ACEI	AASLH---	144
VP1155	LV-----	140
FBAL_3166	LAPLGQTS	146
STY3166	QRVSA---	160
PFL_4558	AH-----	144
PSPT0_3587	ARCEVL---	169

Figure 4.3. Sequence alignment of PACE family proteins. Sequence alignment was performed using the Clustal Omega alignment tool (Sievers *et al.*, 2011).

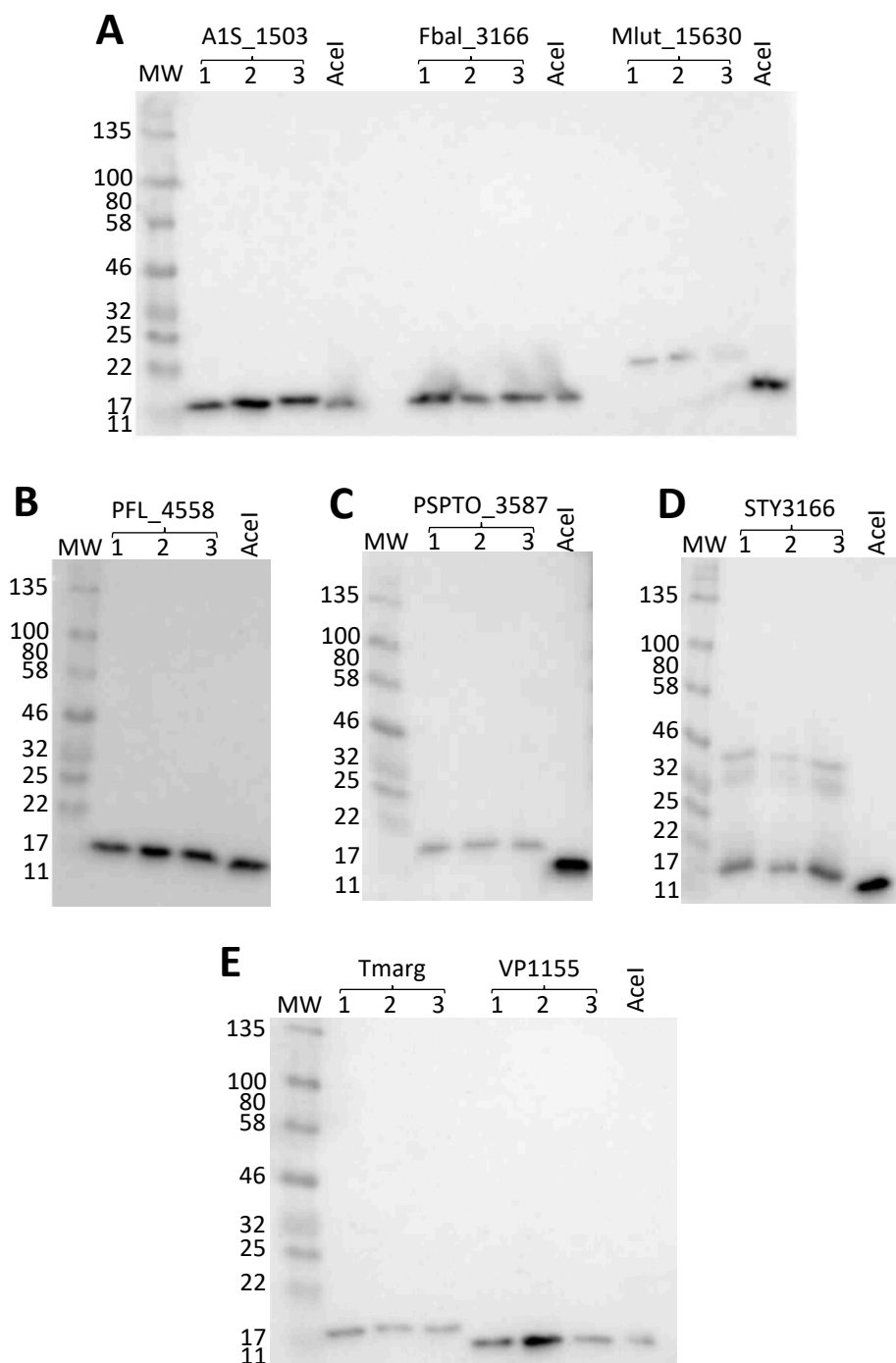


Figure 4.4. Screening production of PACE family proteins. Screening production of A1S_1503, Fbal_3166, Mlut_15630 (A), PFL_4558 (B), PSPTO_3587 (C), STY3166 (D), Tmarg and VP1155 (E) in *E. coli* BL21. Lanes designated 'MW' were loaded with a molecular weight marker and Acel was loaded as a reference standard. Whole cell samples were prepared as described in Section 2.2.5 and analysed as described in Section 2.6.1. His-tagged proteins were detected using HisProbe-HRP conjugate. Each image is automatically adjusted based on signal intensity, making Acel band intensity appear inconsistent.

4.3 Accumulation of [1,5-¹⁴C]-cadaverine by whole cells

4.3.1 Acel-mediated efflux of cadaverine

Cells expressing the *acel* gene maintained low intracellular concentrations of [¹⁴C]-cadaverine throughout the assay, with a mean accumulation of just 5.2 (±2.0 SD) pmol after 20 minutes (Fig. 4.5). Control cells carrying the pTTQ18 empty vector showed a mean accumulation of 56.8 (±2.9 SD) pmol after 20 minutes, 11-fold higher than cells expressing the *acel* gene ($p < 0.0001$, unpaired t-test) (Fig. 4.5).

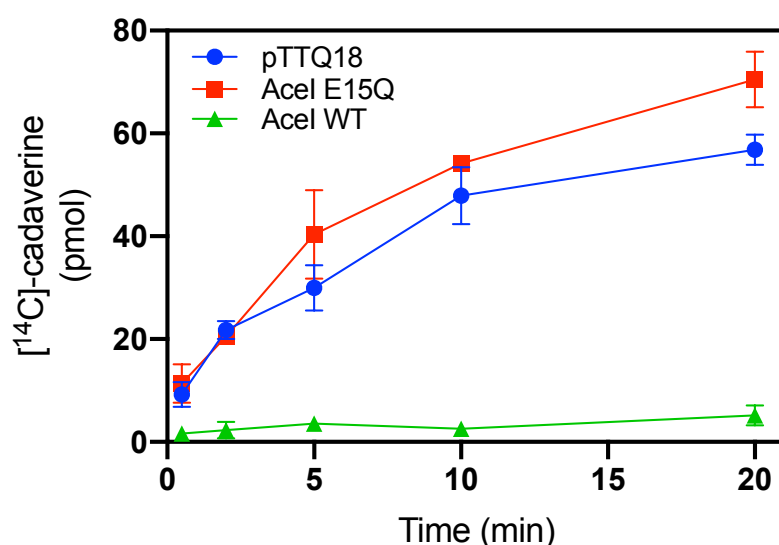


Figure 4.5. Accumulation of [¹⁴C]-cadaverine by energised *E. coli* whole cells. Measuring the effect of Acel wild-type (green) and Acel E15Q (red) proteins on accumulation of [¹⁴C]-cadaverine in *E. coli* BL21 energised whole cells. Gene expression was induced by addition of 0.2 mM IPTG and cells carrying the empty pTTQ18 expression vector (blue) were used as a negative control. Cells were prepared as described in Section 2.2.7 and assays performed as described in Section 2.4.4.2, using 45 mM D-glucose to energise cells before addition of 50 µM [1,5-¹⁴C]-cadaverine. Data shown are the mean of three biological replicates and error bars show standard deviation.

4.3.2 Inactivation of Acel transport

To determine if the glutamate residue at position 15, which is conserved in PACE family proteins, is essential for the transport mechanism of Acel, the E15Q mutant was also screened for [¹⁴C]-cadaverine transport. Cells expressing the *acel* E15Q mutant

gene accumulated 70.5 pmol (± 5.4 SD) of [^{14}C]-cadaverine, a 13.5-fold increase over the wild-type ($p < 0.0001$), despite the comparable expression level of these two genes (Fig. 4.2). This degree of [^{14}C]-cadaverine accumulation was comparable with pTTQ18 empty vector controls, demonstrating that neutralisation of the glutamate residue abolishes transport activity. Although cells expressing the *aceI* E15Q mutant gene accumulated slightly more [^{14}C]-cadaverine than the pTTQ18 control (Fig. 4.5); this was not statistically significant ($p = 0.66$) and could be explained by an increase in membrane permeability caused by overexpression of a membrane associated protein.

4.3.3 Efflux of cadaverine mediated by Acel homologues

Each of the eight Acel homologues was also screened for cadaverine transport using the [^{14}C]-cadaverine accumulation assay (Fig. 4.6). Of those tested, Fbal_3166, PFL_4558 and Tmarg were able to maintain intracellular [^{14}C]-cadaverine concentrations significantly lower than pTTQ18 controls (Fig. 4.6; Fig. 4.7). Cells producing A1S_1503, Mlut_15630, PSPTO_3587, STY3166 or VP1155 were unable to maintain an intracellular [^{14}C]-cadaverine concentration significantly lower ($p > 0.05$) than uninduced cells or cells carrying the pTTQ18 empty vector (Fig. 4.6; Fig. 4.7). The relative accumulation of [^{14}C]-cadaverine in cells producing each of the PACE family proteins after 20 minutes is summarised in Figure 4.7.

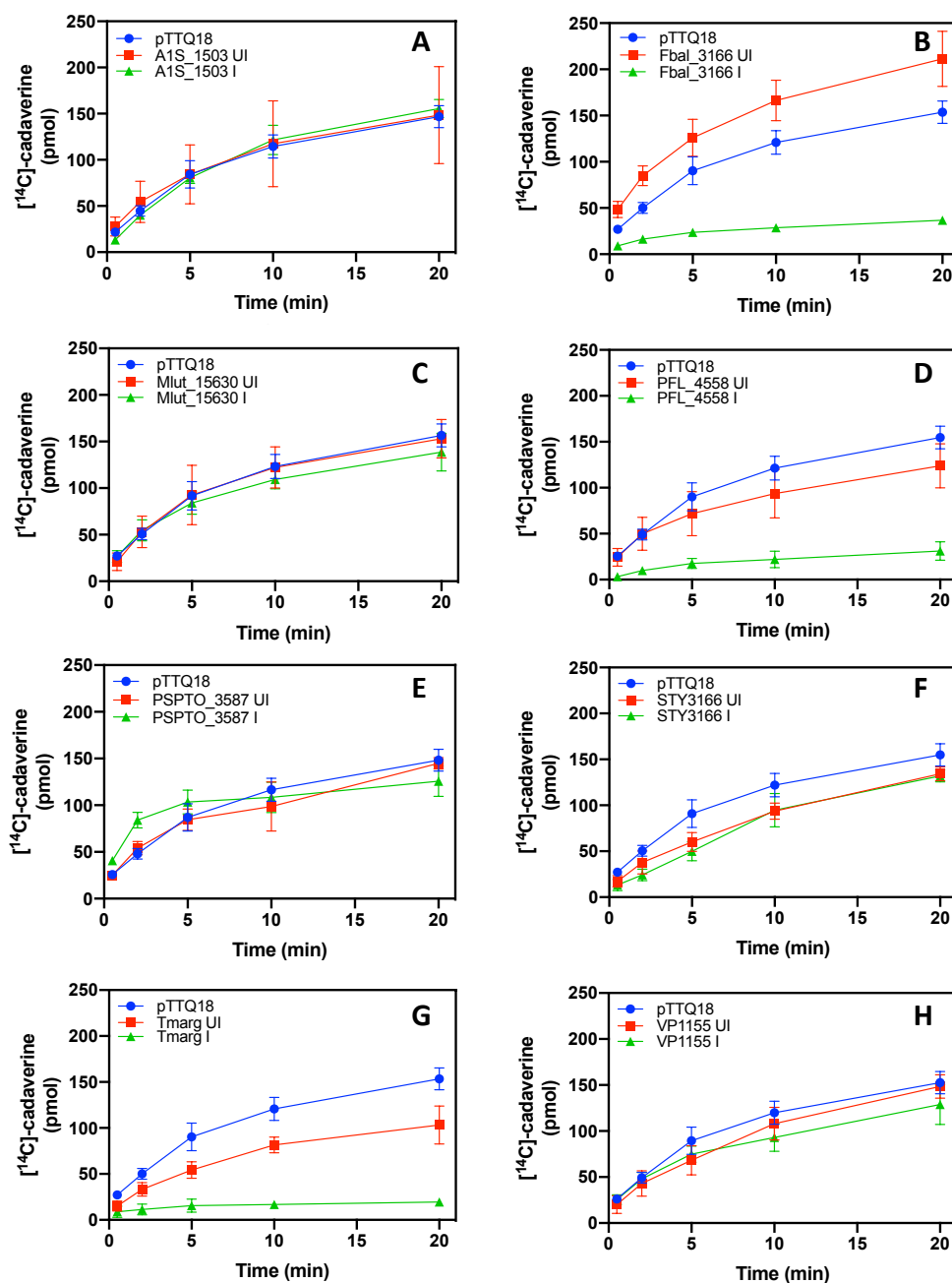


Figure 4.6. Accumulation of $[^{14}\text{C}]$ -cadaverine by energised *E. coli* whole cells. Measuring the effect of the PACE family proteins A1S_1503 (A), FbaI_3166 (B), Mlut_15630 (C), PFL_4558 (D), PSPTO_3587 (E), STY3166 (F), Tmarg (G) and VP1155 (H) (see Fig. 4.3) on the accumulation of $[^{14}\text{C}]$ -cadaverine in *E. coli* BL21 energised whole cells. Cells were prepared as described in Section 2.2.7 and assays performed as described in Section 2.4.4.2, using 45 mM D-glucose to energise cells before addition of 50 μM $[1,5\text{-}^{14}\text{C}]$ -cadaverine. Expression of PACE family genes was induced by addition of 0.2 mM IPTG (green), while uninduced cells (red) and cells carrying the empty pTTQ18 vector (blue) were used as negative controls. Data shown are the mean of three biological replicates and error bars show standard deviation.

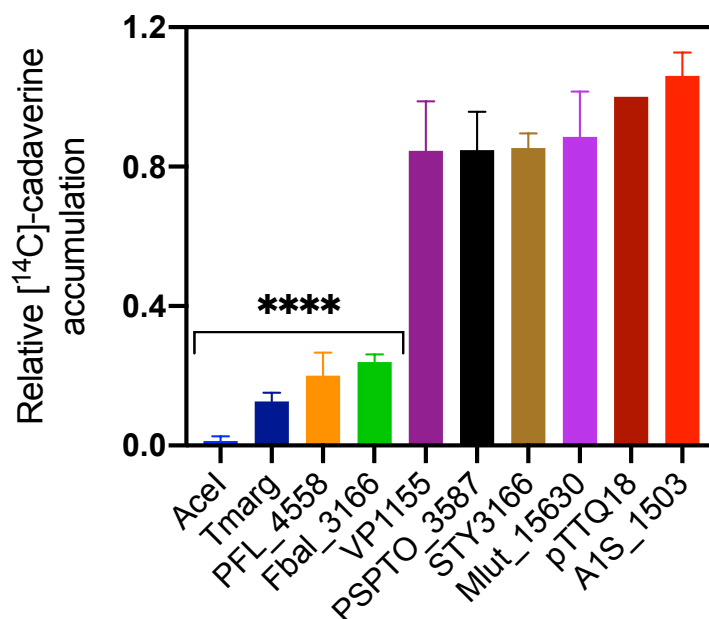


Figure 4.7. Relative accumulation of [^{14}C]-cadaverine by energised *E. coli* whole cells. Accumulation of [^{14}C]-cadaverine in *E. coli* BL21 cells expressing PACE family genes as a fraction of accumulation in pTTQ18 control samples. Cells were prepared as described in Section 2.2.7 and assays performed as described in Section 2.4.4.2, using 45 mM D-glucose to energise cells before addition of 50 μM [^{14}C]-cadaverine. Data are shown in order of the mean [^{14}C]-cadaverine accumulation after 20 minutes. Data shown are the mean of three biological replicates and error bars show standard deviation. Lower accumulation represents increased transport of [^{14}C]-cadaverine. Proteins that resulted in significantly lower intracellular [^{14}C]-cadaverine accumulation compared to pTTQ18 controls (one-way ANOVA with Dunnett's multiple comparisons test) are indicated as follows: **** $p \leq 0.0001$.

4.4 Measuring transport of cadaverine across proteoliposomes

4.4.1 Purification of Acel

IMAC purification of Acel was performed as described in Section 2.6.6.3 (Fig. 4.8) in the presence of n-Dodecyl β -D-maltoside (DDM), a mild detergent. Elution fraction four was typically used for proteoliposome reconstitution as it contained the greatest concentration of Acel protein (Fig. 4.8), which was present at a purity of approximately 39% as determined by densitometry (Section 2.6.2). Purified Acel always appears as three distinct bands during SDS-PAGE, as highlighted by Western Blotting (Fig.4.8),

presumably due to the presence of different oligomeric states of the protein once extracted from the membrane using a detergent. As such, all three of these bands are taken into account when performing densitometry measurements. Purification of PACE family proteins requires considerable optimisation and is discussed further in Section 4.5.

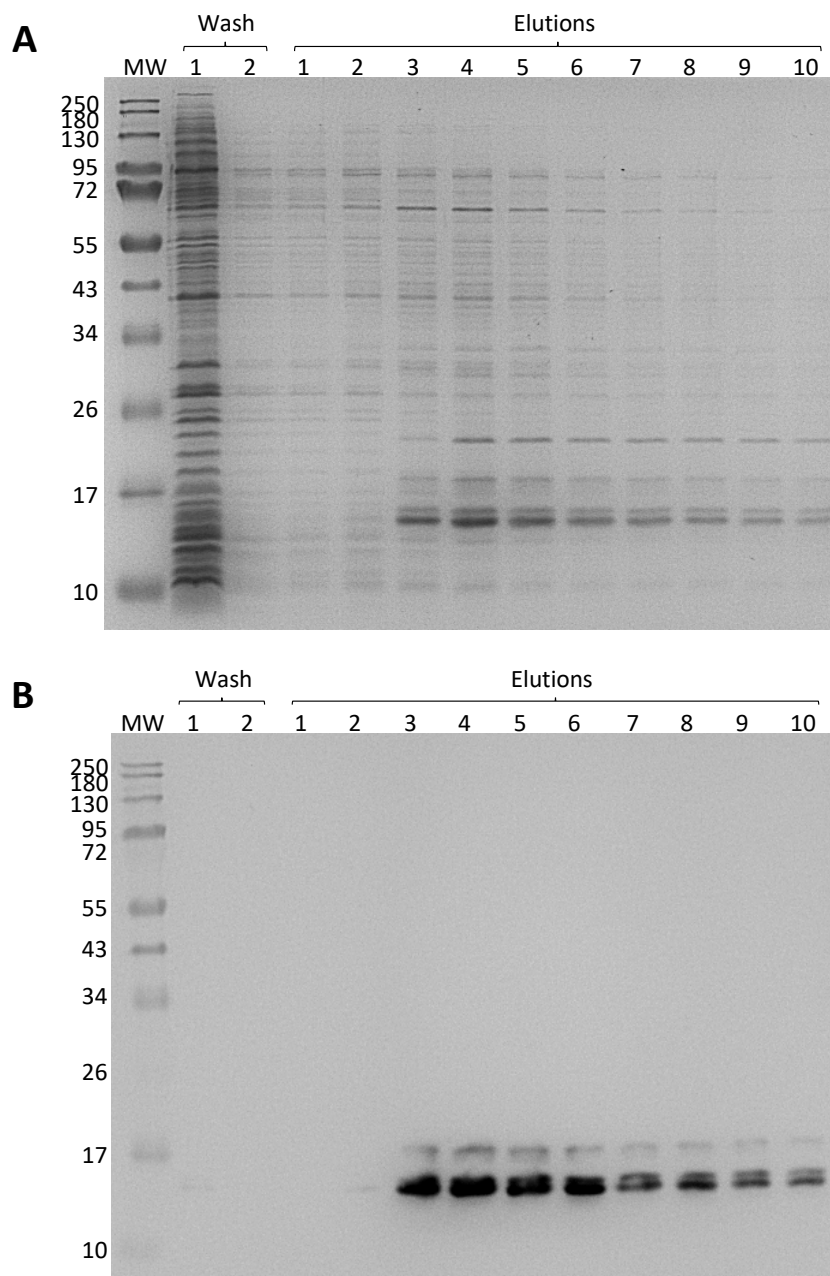


Figure 4.8. IMAC purification of Acel for reconstitution into proteoliposomes. Acel was purified following the protocol described in Section 2.6.6.3 and fractions analysed by SDS-PAGE (A) and Western blotting (B), as described in Section 2.6.1. Lanes designated ‘MW’ were loaded with a molecular weight marker.

4.4.2 Generation of Acel proteoliposomes

Lipids were rehydrated in reconstitution buffer (25 mM HEPES, 200 mM NaCl, 1 mM DTT, pH 7.0) to form large multilamellar vesicles (LMVs) and extruded to form small unilamellar vesicles (SUVs), as described in Section 2.8. Due to the instability of Acel in DDM detergent micelles, protein was purified and reconstituted into proteoliposomes as quickly as possible and always on the same day. Presumably due to the poor purity of Acel, determination of protein concentration in the IMAC column elutions was not possible using a spectrophotometer. However, the poor stability of Acel in DDM made lengthy protein quantification protocols unfavourable, so the maximum permitted volume (30 μ l of eluted protein) was added to the reconstitution mixture to ensure a sufficient Acel concentration while maintaining acceptable detergent and imidazole concentrations (see Section 2.8). The (proteo)liposomes were resuspended in assay buffer immediately before use (25 mM HEPES, 200 mM KCl, pH 8.0) to generate an electrochemical gradient across the membrane, with Na⁺ at pH 7.0 inside and isosmolar K⁺ at pH 8.0 outside. Addition of 5 nM valinomycin promotes downhill movement of the K⁺ into the liposome lumen, generating an electrical potential.

4.4.3 Using the fluorescent dye pyranine to measure the proton-coupled transport of cadaverine

The inclusion of pyranine, a pH sensitive hydrophilic dye, in the protein reconstitution step traps the dye in the (proteo)liposome lumen. Subsequent sedimentation and resuspension of the (proteo)liposomes removes any external pyranine, allowing real-time monitoring of lumen pH changes. The excitation maximum of pyranine shifts from 400 nm to 450 nm with increasing pH, which can be used to monitor movement of H⁺

across the (proteo)liposome membrane. Valinomycin (5 nM) was added after 50 seconds to generate an electrochemical gradient across the (proteo)liposome membrane and cadaverine (1 mM) added at 100 seconds to initiate the assay (Fig. 4.9). Empty liposomes show a gradual increase in the 450:400 nm fluorescence ratio over the course of the assay, likely due to slow equilibration of the liposome lumen (pH 7.0) with the external buffer (pH 8.0), with addition of cadaverine having no additional effect (Fig. 4.9). However, in Acel proteoliposomes, a cadaverine-dependent increase in the fluorescence ratio is observed indicating alkalinisation of the proteoliposome lumen ($p < 0.0001$, unpaired t-test). Movement of H^+ out of the lumen therefore appears to be stimulated by cadaverine, suggesting a cadaverine/ H^+ antiport mechanism (Fig. 4.9).

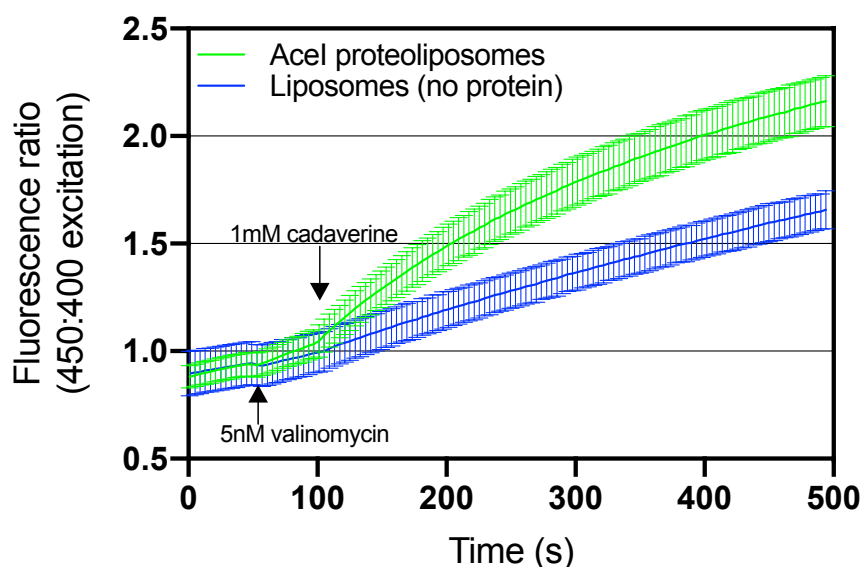


Figure 4.9. Cadaverine-induced pH changes inside Acel proteoliposomes. Monitoring pH changes in the lumen of (proteo)liposomes loaded with the pH sensitive dye pyranine. The excitation maximum of pyranine shifts from 400 nm to 450 nm with increasing pH, which was used to infer cadaverine/ H^+ antiport. (Proteo)liposomes were prepared with an internal pH of 7.0 with 200 mM NaCl and an external pH of 8.0 with isosmolar KCl, as described in Section 2.8. Valinomycin (5 nM) was added after 50 seconds to generate an electrochemical gradient across the (proteo)liposome membrane and cadaverine (1 mM) added at 100 seconds to initiate the assay. All data are the mean of three biological replicates, each with three technical replicates, and error bars show standard deviation.

4.5 Discussion

As the prototypical member of the PACE family of efflux pumps, the *aceI* gene has been heterologously expressed in *E. coli* since its initial discovery (Hassan *et al.*, 2013), as a means to simplify its characterisation in a well-characterised model organism that is more amenable to genetic manipulation and containment than the native *A. baumannii* host. *E. coli* is a particularly suitable expression host due to its lack of endogenous PACE family genes, which are found in <0.2% of sequenced *E. coli* genomes and have not been identified in any *E. coli* laboratory strains (Hassan *et al.*, 2013).

While both Acel wild-type and the E15Q mutant proteins were produced at comparable levels in *E. coli* BL21 (Fig. 4.2), some differences in production levels were observed for the eight Acel homologues (Fig. 4.4). It is therefore important to determine if observed differences in [¹⁴C]-cadaverine transport were simply artefacts of varying PACE expression levels. By calculating Pearson correlation coefficients, it was confirmed there was no significant correlation between gene expression level and [¹⁴C]-cadaverine accumulation ($r = -0.2877$, $p=0.45$). Regardless, further work to minimise the variation in expression levels of PACE genes may be useful, and could involve exploration of different expression vectors, *E. coli* expression strains, inducer concentrations, induction time periods and codon optimisation of genes for improved expression in *E. coli*.

As mentioned previously, Acel was initially identified as a chlorhexidine efflux pump (Hassan *et al.*, 2013) although chlorhexidine has only been synthesised and used during the last century (Davies *et al.*, 1954), meaning Acel must have evolved to perform a different physiological function. This led to the investigation of polyamines, which share a number of structural similarities to chlorhexidine including interspersed

and/or terminal amino groups, that are naturally abundant in bacterial cells (Shah and Swiatlo, 2008; Wortham *et al.*, 2007). Polyamines are involved in a number of important processes in bacteria including biofilm formation, cell signalling, cell growth, immune evasion and pathogenesis as well as oxidative and acid stress responses (Shah and Swiatlo, 2008; Wortham *et al.*, 2007). It stands to reason that some of these roles, for example cell signalling, may necessitate active export of polyamines. Alternatively, these efflux pumps may have evolved as a detoxification mechanism since accumulation of high intracellular concentrations of polyamines is known to inhibit cell growth (Hassan *et al.*, 2019). Previous studies have demonstrated that the polyamines cadaverine and putrescine induce expression of *acel* 20-fold in *A. baumannii* (Hassan *et al.*, 2019), while chlorhexidine induced expression just 10-fold (Hassan *et al.*, 2013). Similarly, while deletion of *acel* in *A. baumannii* results in an 8-fold reduction in tolerance to cadaverine and putrescine (Hassan *et al.*, 2019), the same deletion only reduced tolerance to chlorhexidine 2-fold (Tucker *et al.*, 2014). In this study, *E. coli* cells expressing the *acel* gene did not accumulate significant concentrations of [¹⁴C]-cadaverine over the course of the uptake assay unlike control cells (Fig. 4.5), meaning cadaverine is exported from the cell at a rate equal to or greater than the rate of uptake/diffusion. Similarly, deletion of *acel* in *A. baumannii* led to significantly increased [¹⁴C]-cadaverine accumulation (Hassan *et al.*, 2019). All of these data suggest that these polyamines are the physiological substrate of the Acel efflux pump, hence their investigation here.

Interestingly, the Acel E15Q mutant protein did not prevent accumulation of [¹⁴C]-cadaverine (Fig. 4.5). This is consistent with previous findings that Acel E15Q is unable to transport or confer resistance to chlorhexidine (Hassan *et al.*, 2013), suggesting that the glutamate residue at position 15 is essential for all Acel function.

This is unsurprising as this glutamate residue is one of the few that are conserved in all PACE genes identified to date (Hassan *et al.*, 2018). Conserved acidic residues are also found in small multidrug resistance (SMR) and major facilitator superfamily (MFS) transporters and are essential for proton translocation and therefore energisation of transport (see Section 1.4) (Bay and Turner, 2006; Vishwakarma *et al.*, 2018). If the Acel substrate/H⁺ antiport mechanism theory is correct, this acidic glutamate residue at position 15 may similarly be involved in translocation of protons across the membrane making it essential to protein function, which has been demonstrated here (Fig. 4.5).

Of the eight Acel homologues screened (Fig. 4.3), Fbal_3166, PFL_4558 and Tmarg were also able to prevent accumulation of [¹⁴C]-cadaverine in *E. coli* (Fig. 4.6; Fig. 4.7). These data suggest that transport of cadaverine and potentially other polyamines is conserved in the PACE family, supporting the theory that short chain diamines/polyamines represent the physiological substrates of PACE family efflux pumps.

Reconstitution of membrane proteins is a powerful technique for studying the function of isolated membrane proteins, a full description of which can be found in Section 1.2.8. Unfortunately, purification of Acel to high purity has remained challenging and is made more difficult by its propensity to precipitate once extracted from the membrane using detergents, meaning purification must be performed as quickly as possible at 4°C and all experiments performed on the day of purification. Work is currently being performed to improve purification of Acel and its homologues (Nikolova, unpublished), in particular those that are inherently more stable than Acel such as Tmarg from the thermophilic bacterium *Tepidiphilus margaritifer* (Manaia *et al.*, 2003), in order to gain both functional and structural insights into this family of

novel proteins. Regardless, in our reconstituted system we were able to observe a protein-dependent increase in the rate of proteoliposome lumen alkalinisation upon external addition of cadaverine (Fig. 4.9), which was measured using the pH sensitive dye pyranine. This is consistent with a cadaverine/H⁺ antiport mechanism, in which cadaverine is transported into the lumen of the proteoliposomes in exchange for H⁺. These data, along with the E15Q loss-of-function mutation which has also been shown to be non-functional in the reconstituted system (Hassan *et al.*, 2019), support a cadaverine/H⁺ antiport mechanism. However, it is possible that transport of unprotonated cadaverine into the proteoliposome lumen occurs, perhaps driven by an alternative cation such as Na⁺, where it can then accept protons and cause alkalinisation of the lumen. Further experiments should be carried out without addition of Na⁺ and in the presence of a Na⁺ chelating agent to eliminate this possibility.

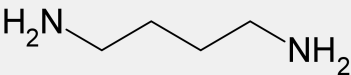
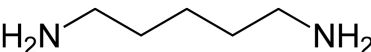
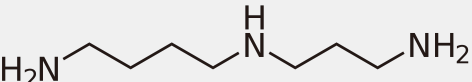
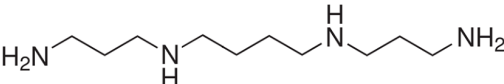
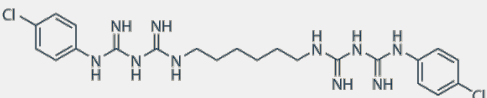
It is important to mention that purification and reconstitution of Fbal_3166 was also attempted in the hope of performing similar measurements of cadaverine transport. While purified protein was obtained at a purity comparable to Acel and successfully reconstituted, Fbal_3166 proteoliposomes invariably demonstrated a rapid cadaverine-independent alkalinisation of the proteoliposome lumen upon addition of valinomycin (data not shown). The reason for this apparent efflux of protons in the absence of substrate is currently unknown but further work is being performed to better understand this anomaly and the function of Fbal_3166 and other Acel homologues in the reconstituted system.

Although cadaverine was the primary focus in this study, addition of other polyamines (Table 4.1) in the reconstituted system has been shown to inhibit [¹⁴C]-cadaverine efflux, particularly the diamine putrescine (Hassan *et al.*, 2019), suggesting competition for the Acel efflux pump. Interestingly spermine, a tetraamine, and

spermidine, a triamine (Table 4.1), induce *aceI* expression and inhibit [^{14}C]-cadaverine transport in proteoliposomes both to a much lesser extent than putrescine (Hassan *et al.*, 2019). These observations suggest that Acel shows specificity towards diamines and it would therefore be worthwhile screening the activity of those Acel homologues that did not appear to transport cadaverine (Fig. 4.6; Fig. 4.7) with other radiolabelled polyamines, such as those shown in Table 4.2, in order to determine their respective specificities.

The diverse roles that polyamines play in bacteria make the discovery of polyamine transport proteins of particular importance. As a member of the ESKAPE group of pathogens and therefore one of the six most common nosocomial infections worldwide, it is essential to develop new treatments against *A. baumannii* infections.

Table 4.1. Potential polyamine substrates of PACE family efflux pumps

Name	Structure
Putrescine	
Cadaverine	
Spermidine	
Spermine	
Chlorhexidine	

Since polyamines play vital roles in biofilm formation, cell signalling, virulence and immune evasion (Shah and Swiatlo, 2008; Wortham *et al.*, 2007), transporters of these substrates could represent promising therapeutic targets. For example, deletion of polyamine transporters has previously been shown to significantly attenuate virulence of *Streptococcus pneumoniae* in *in vivo* models (Rai *et al.*, 2016; Ware *et al.*, 2006). The high level of conservation seen in PACE genes across a number of Gram-negative pathogens (Hassan *et al.*, 2018) means development of targeted therapeutics may have broader implications in the treatment of infections caused by other members of the ESKAPE group of pathogens.

Cadaverine is also an important molecule in the field of biotechnology, with extensive research being performed to explore the renewable production of cadaverine in bacteria as a monomer for polyamides such as Nylon (Kind *et al.*, 2014; Kwak *et al.*, 2017; Qian *et al.*, 2011; Wang *et al.*, 2018). Much like butyl methacrylate, bio-production of cadaverine can be limited by its toxicity (Wang *et al.*, 2018), in which case introduction of a cadaverine efflux pump, such as Acel, may help maintain subtoxic cellular concentrations and ultimately improve yield. As such, Acel serves as a useful model system for the characterisation of bacterial efflux pumps in this biotechnological context.

Chapter 5

Expression and characterisation of bacterial efflux
pumps using the fluorescent dye Nile Red

5.1 Introduction

5.1.1 Characterising efflux using fluorescent dyes

Fluorescent efflux substrates have become a valuable tool in quantifying efflux thanks to their availability, affordability and ease of measurement. Various fluorescent dyes have been widely utilised in the study of not only bacterial efflux pumps, but also in fungi to study antifungal resistance (Nakamura *et al.*, 2001; Ivnitski-Steele *et al.*, 2009) and in human cancer cell lines to understand the role of efflux in resistance to chemotherapeutic agents (Ansbro *et al.*, 2013; Patwardhan *et al.*, 2010). Fluorescent dyes are valuable surrogate molecules for characterising efflux as they can be easily quantified *in situ* in real-time, circumventing the need for lengthy analysis of supernatant and cell lysate samples and avoiding the use of expensive and hazardous radioisotope labelled substrates. The promiscuity of bacterial efflux pumps, particularly those belonging to the RND family, has led to the utilisation of a number of different fluorescent dyes in the study of efflux, each with different properties and therefore use cases.

Most dyes used to quantify bacterial efflux exhibit environment-sensitive fluorescence, allowing easy monitoring of the association and dissociation of these dyes with biological molecules in real-time. For example, dyes such as ethidium bromide and Hoechst 33342 are highly fluorescent when bound to nucleic acids in the cytoplasm, but have relatively low fluorescence outside of the cell (Adhikary *et al.*, 2003; Blair and Piddock, 2016; Vadevanyan *et al.*, 2003). Conversely, binding of acriflavine to nucleic acids reduces its fluorescence compared to in the aqueous buffer (Hassan *et al.*, 2018). To measure efflux directly, cells can be preloaded with these dyes in the presence of an uncoupling agent such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP) to dissipate the proton motive force and thus halt any

proton-driven membrane transport. Excess dye and uncoupling agent can then be washed away and cells re-energised with an appropriate carbon source, such as glucose or glycerol, after which membrane transport resumes and efflux of the dye can be observed. Alternatively, accumulation of a fluorescent dye can be measured and used to infer efflux activity indirectly; cells that are able to export a substrate actively will maintain very low intracellular concentrations, while cells unable to do so will show gradual accumulation of the dye and thus a change in fluorescence.

5.1.2 Lipophilic fluorescent efflux substrates and their applications

While cytoplasmic dyes are useful in understanding transport across the inner membrane of Gram-negative bacteria, RND efflux pumps have long been thought to recruit their substrates from the periplasm (Elkins and Nikaido, 2002; Lomovskaya and Totrov, 2005; Nikaido and Takatsuka, 2009). Efflux of cytoplasmic dyes is typically slow, most likely due to a multi-step mechanism of efflux including dissociation from an intracellular target, transport across the inner membrane and finally an RND-mediated efflux step (Blair and Piddock, 2016). For these reasons, lipophilic dyes that accumulate in the membranes and/or periplasm, such as Nile Red and 1,2-dinaphthylamine can be powerful tools in the study of RND efflux. Both of these dyes exhibit high fluorescence in non-polar environments (e.g *E. coli* membranes) but low fluorescence in aqueous solution (Bohnert *et al.*, 2010; Bohnert *et al.*, 2011), allowing movement from the membranes and periplasm to the extracellular buffer, or vice versa, to be easily monitored (Fig. 5.1). Crucially, Nile Red has successfully been used to investigate transport of non-fluorescent substrates through the development of a competition assay (Bohnert *et al.*, 2011). In these assays, cells can be treated with substrates suspected to compete with Nile Red for the same efflux pump, in which

case a dose-dependent decrease in Nile Red efflux would be observed due to competition for the same binding site(s) (Bohnert *et al.*, 2011). The proven utility of Nile Red as well as its affordability and availability led to its application in this study.

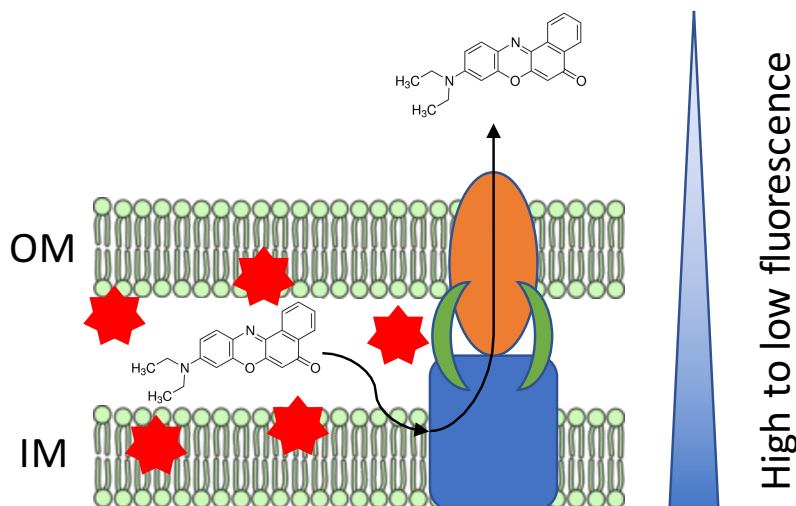


Figure 5.1. Principle of the Nile Red efflux assay Nile Red is lipophilic and accumulates in the membranes and periplasm, where it is highly fluorescent. Nile Red is recruited by RND efflux pumps and transported across the outer membrane to the aqueous buffer, where it has low fluorescence. OM – outer membrane, IM – inner membrane.

It is demonstrated here that RND efflux pumps originating from *E. coli* and *P. aeruginosa* are well-expressed in laboratory strains of *E. coli* and that these pumps are localised to the membrane(s) where they remain functional, as shown by measuring transport of Nile Red. Interestingly, Nile Red was useful in determining the solubility limit of BMA due to its hydrophobicity and its propensity to partition into an organic phase. Although efflux pumps not belonging to the RND family were also well expressed, none showed any measurable activity with Nile Red, perhaps due to their preference for cytoplasmic substrates. While initial Nile Red competition assays appeared promising, toxic levels of membrane protein expression were shown to be responsible for a decline in cell viability and therefore efflux activity, an effect that was

abolished by reducing gene expression levels. While this made Nile Red unsuitable as a means of measuring BMA transport, it is shown here to be a very useful tool when cloning RND efflux pumps into a heterologous host to ensure retention of efflux activity.

5.2 Assessing the compatibility of Nile Red and butyl methacrylate

Due to its hydrophobicity and therefore poor solubility, it was important to ensure that BMA both remained in solution at the concentrations used during efflux assays and had no undesirable interaction with Nile Red. For this reason, a 5 μ M solution of Nile Red was prepared in water, to which 5 mM BMA was added. At this concentration, BMA was insoluble and formed a separate colourless phase at the surface of the water. After five minutes at room temperature, partitioning of Nile Red into this organic phase was clearly visible, resulting in a bright pink colouration (Fig. 5.2).

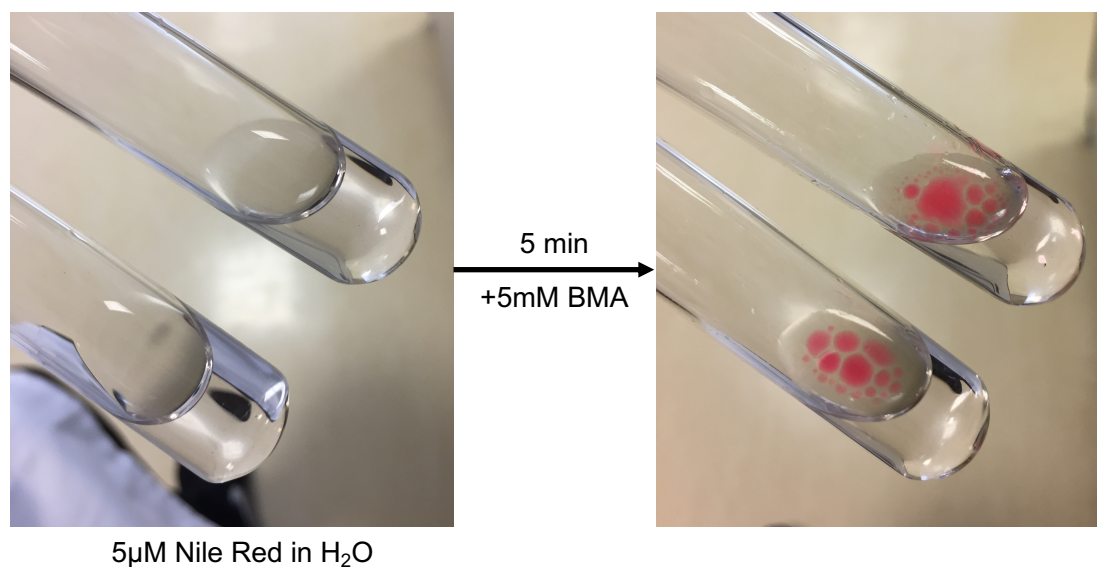


Figure 5.2. Observing the partitioning of Nile Red into an insoluble butyl methacrylate phase. A 5 μ M solution of Nile Red was prepared by diluting a 3 mM Nile Red stock solution prepared in DMSO into MilliQ water. Butyl methacrylate was added to a final concentration of 5 mM and mixed vigorously, then incubated at room temperature for 5 minutes.

This property of Nile Red provided a simple method to determine the solubility limit of BMA in aqueous solutions. A range of ethanol solutions with 5 μ M Nile Red were prepared to which 80 mM BMA was added (Fig. 5.3). This concentration of BMA was chosen to minimise the final ethanol concentrations that *E. coli* cells were exposed to, while avoiding liquid handling of very small volumes which can be the source of experimental errors. As mentioned previously, Nile Red displays significantly increased fluorescence in non-polar environments compared to in aqueous solutions, meaning its accumulation in the BMA phase is accompanied by an increase in fluorescence, which can be visualised under UV light (Fig. 5.3). At 80 mM BMA, large droplets of BMA/Nile Red were clearly visible at 25% and 40% ethanol, with some small droplets remaining at 45% ethanol (Fig. 5.3). At 50% ethanol, BMA remained in solution and no BMA/Nile Red droplets of any size were visible (Fig. 5.3).

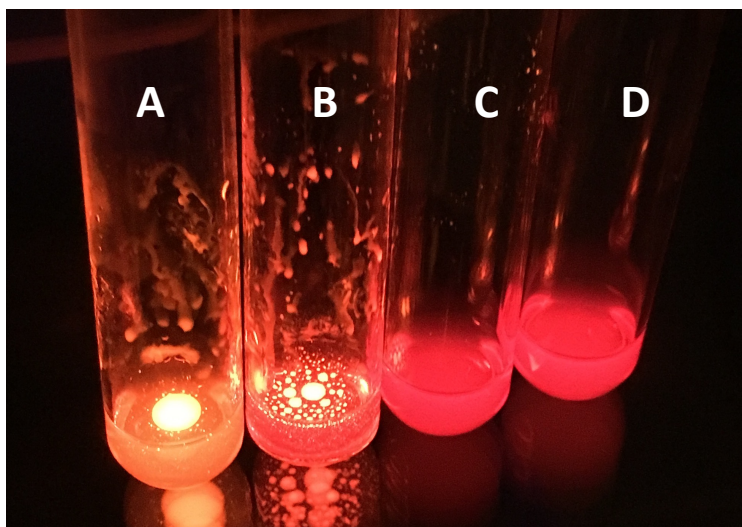


Figure 5.3. Using Nile Red to determine the minimum ethanol concentration required for butyl methacrylate stock solutions. Butyl methacrylate was added to a final concentration of 80 mM in solutions of 25% (A), 40% (B), 45% (C) and 50% (D) v/v ethanol with 5 μ M Nile Red. Samples were mixed vigorously and incubated at room temperature for 5 minutes before being observed on a Safelmager 2.0.

This stock solution was then diluted at a range of concentrations in PBS with 5 μ M Nile Red to mimic conditions in the efflux assay. All concentrations up to and including 2 mM BMA remained in solution and had no effect on Nile Red fluorescence, while a 5 mM BMA control exhibited phase separation and a dramatic increase in Nile Red fluorescence (Fig. 5.4). Concentrations up to and including 2 mM BMA were therefore deemed acceptable for Nile Red efflux assays.

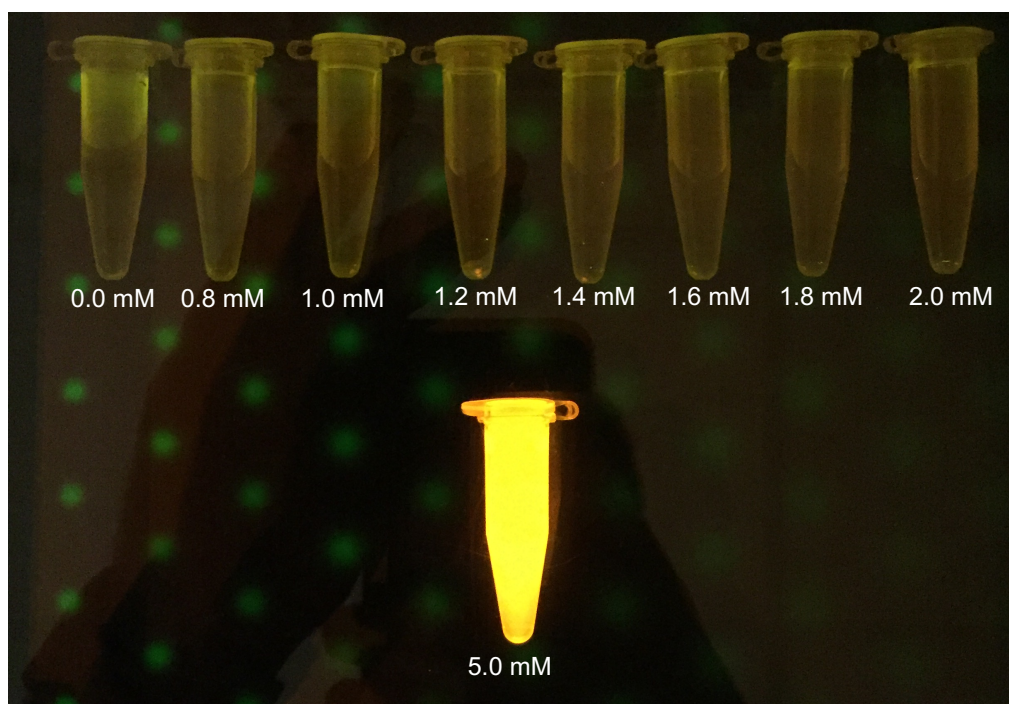


Figure 5.4. Monitoring the solubility of butyl methacrylate in efflux assay buffer and its interaction with Nile Red. Nile Red was added to PBS (pH 7.2) at a final concentration of 5 μ M to mimic conditions in the efflux assay. BMA was then added from an 80 mM stock solution in 50% ethanol to the concentrations shown. Samples were mixed and incubated at room temperature for 5 minutes before being visualised on a Safelmager 2.0.

5.3 Measuring RND-Mediated Efflux of Nile Red

5.3.1 Screening expression of RND efflux pumps

Before functional assays could be performed, each cloned RND efflux pump was tested for expression in *E. coli* BL21, a strain widely used for the production of

recombinant membrane proteins (Zhang *et al.*, 2015; Karyolaimos *et al.*, 2018). Expression testing is essential as cloned genes, particularly those originating from a heterologous host organism, will not necessarily be expressed in laboratory *E. coli* strains under standard conditions. When analysing expression of membrane proteins, it is important to consider their anomalous migration patterns observed in SDS-PAGE gels. It has long been reported that membrane proteins, due to variations in binding of the detergent SDS and retention of helical secondary structure under typical denaturing conditions, show significant deviations from their theoretical mobility on an SDS-PAGE gel (Miyake *et al.*, 1978; Therien *et al.*, 2001; Rath *et al.*, 2009; Wolkenhorst *et al.*, 2009), a phenomenon that was also observed here (Fig 5.5). Consequently, the appearance of a single band in only the induced sample of the relevant Western blot at approximately the predicted molecular weight was interpreted as successful expression of the gene(s) of interest. Functional assays were also vital in determining that genes were successfully expressed resulting in correctly folded, functional protein (Fig. 5.9). Full descriptions of all of the plasmids used here can be found in Chapter Two. Each of the 3 cloned RND pumps was well-expressed in *E. coli* BL21 and their abundance in the mixed membrane fraction was quantified using densitometry (Fig. 5.5). Interestingly, TolC of the AcrAB-TolC system constituted up to 38% of total membrane protein in *E. coli* BL21, while AcrB represented just 8% as measured by densitometry (Fig. 5.5). Both *mexAB-oprM* and *mexCD-oprJ* also appeared to be well-expressed, with MexB and OprM comprising 6% and 5% of total membrane protein, respectively, and MexD and OprJ representing 3% and 7% of total membrane protein, respectively (Fig. 5.5).

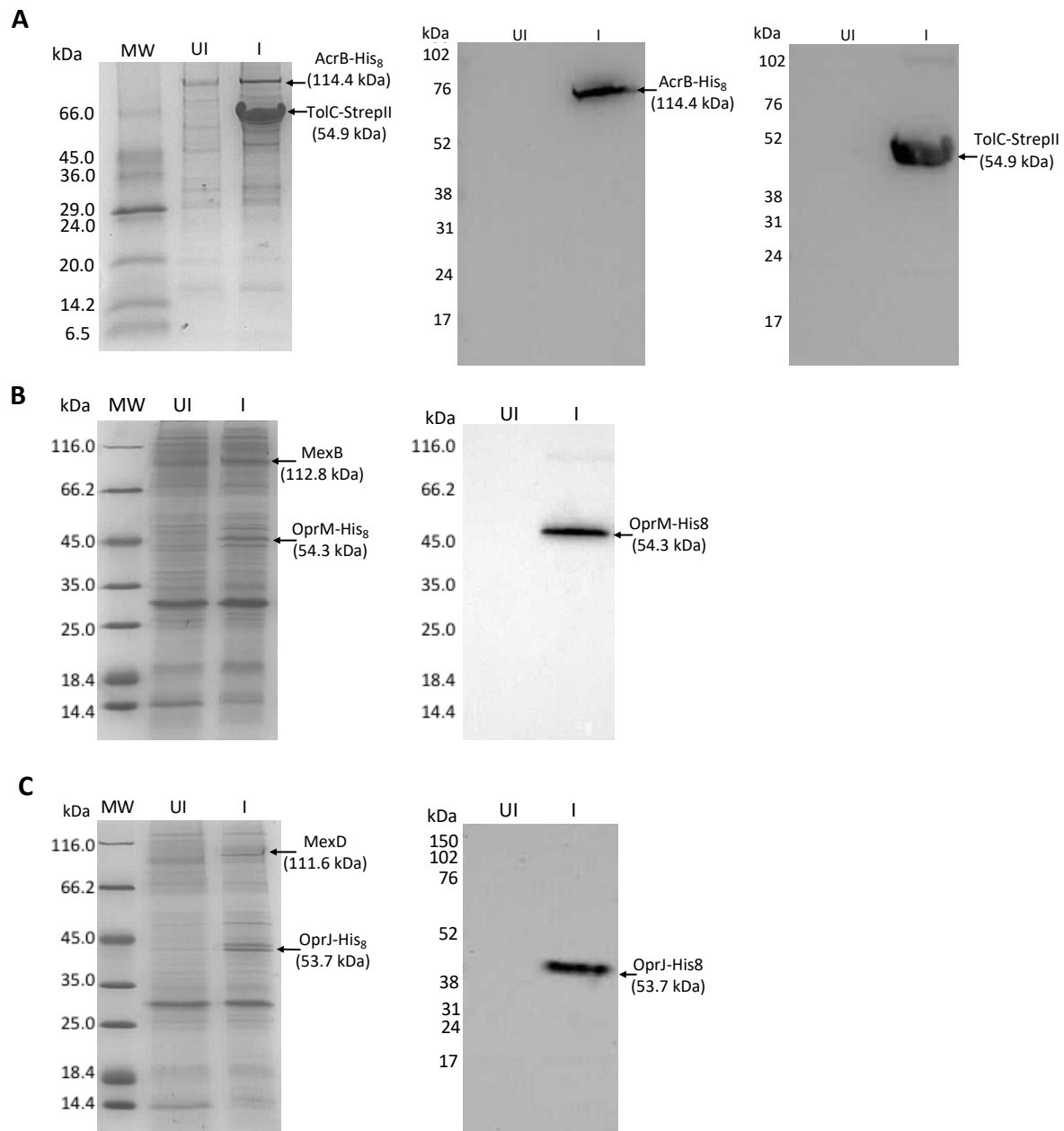


Figure 5.5. Screening RND efflux pump expression. Screening production of protein from pBAD-*arcABHis₈*-*tolC*Strep (A), pBAD-*mexAB-oprMHis₈* (B) and pBAD-*mexCD-oprJHis₈* (C) by SDS-PAGE (left) and Western blot (right). Membrane fractions of uninduced (UI) and induced (I) cells were prepared using the water lysis method as described in Section 2.6.2 from 50 ml cultures grown exactly as described in Section 2.2.5. His-tagged proteins were detected using HisProbe-HRP conjugate while Strep-tagged proteins were detected using StrepMAB-Classic HRP conjugate antibody. Lanes designated ‘MW’ were loaded with a molecular weight marker. Protein molecular weights shown were calculated from the relevant protein sequence using Benchling Biology Software.

5.3.2 Optimisation of Nile Red efflux assay

Initial attempts showed that the combination of 5 μ M Nile Red and 10 μ M CCCP was sufficient to preload the cells with dye during a 30 minute incubation period, consistent with the findings of Bohnert *et al.* (2010) (Fig. 5.6). Upon addition of 28 mM glucose, initiation of efflux was extremely rapid and initial data points were missed in the time taken to close the fluorimeter lid (Fig. 5.6).

5.3.2.1 Selection of appropriate *E. coli* expression strain. It was evident that the BL21 host strain initially used for this assay had very high background efflux activity, with even uninduced cells showing rapid efflux of Nile Red upon addition of glucose, while the C43 Δ *acrAB* strain showed little to no background efflux activity (Fig. 5.6). Consequently, all constructs were transformed into the C43 Δ *acrAB* strain for future assays.

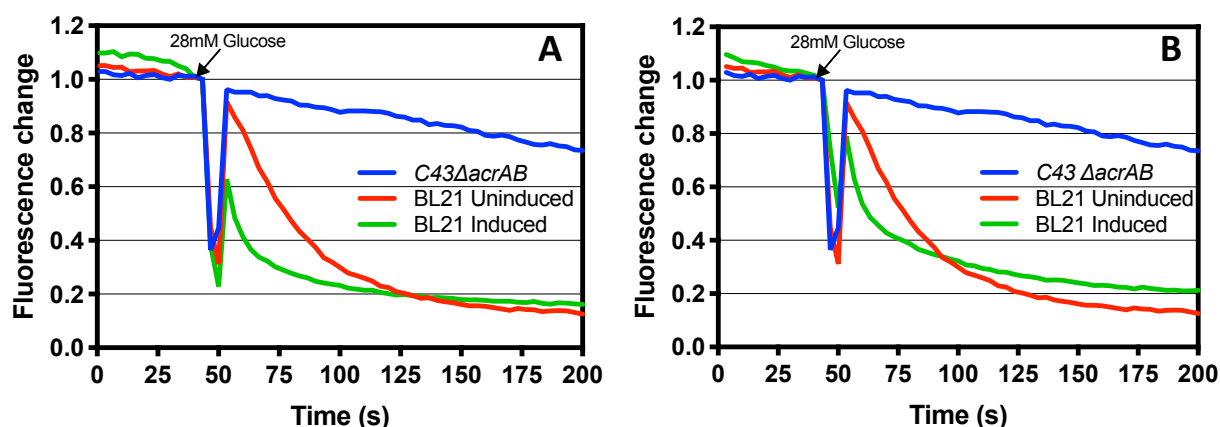


Figure 5.6. Measuring efflux of Nile Red from *E. coli* BL21 cells carrying pBAD-*acrABHis₈-toIcStrep* (A) or pBAD-*mexCDoprJHis₈* (B). Cells were preloaded with 5 μ M Nile Red in the presence of 10 μ M CCCP. After mild washing, efflux was initiated by addition of 28 mM D-glucose where indicated with an arrow. Data are shown as a fraction of the fluorescence intensity immediately before initiation of efflux.

5.3.2.2 Optimisation of glucose concentration used to initiate Nile Red efflux. A range of lower glucose concentrations were tested in an attempt to reduce the initial rate of efflux and facilitate early efflux measurements. These assays demonstrated that 1 μ M glucose was sufficient to initiate efflux of Nile Red at a reduced rate, but also highlighted the significant pre-energisation leakage of Nile Red from induced cells (Fig. 5.7). It was suspected that the multiple washes in PBS used to remove excess dye and CCCP were responsible for this pre-energisation leakage, perhaps due to physical damage to membranes or contamination with a carbon source.

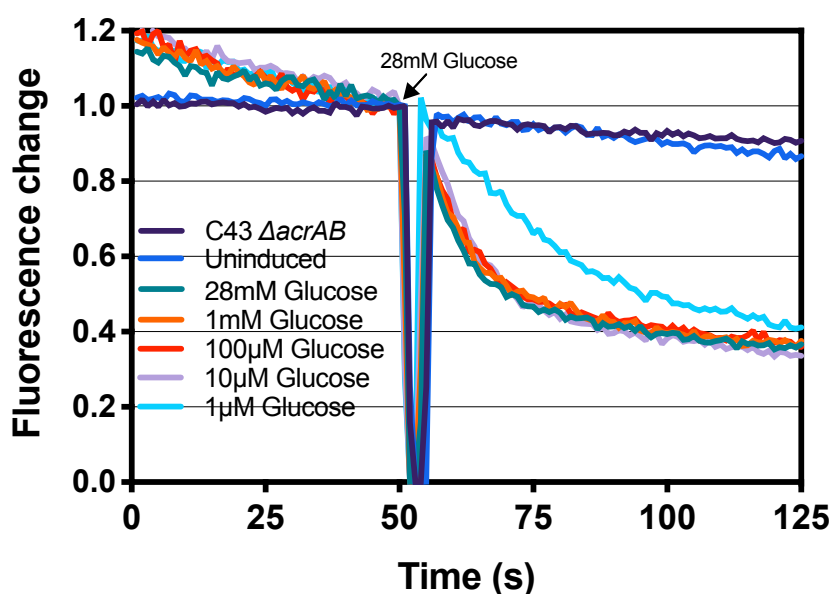


Figure 5.7. Reducing the rate of efflux from *E. coli* BL21 cells carrying pBAD-*acrABHis₈-to/CStrep* using reduced glucose concentrations. Cells were preloaded with 5 μ M Nile Red in the presence of 10 μ M CCCP. After mild washing, efflux was initiated by addition of D-glucose at a range of concentrations where indicated with an arrow. Data are shown as a fraction of the fluorescence intensity immediately before initiation of efflux.

5.3.2.3 Optimisation of wash steps used to remove excess CCCP and Nile Red

In order to overcome this pre-energisation leakage, the assay was repeated with fewer wash steps. This approach was successful in reducing pre-energisation dye leakage

but also increased the concentration of glucose required to initiate efflux from 1 μM to 10 μM (Fig. 5.8), suggesting that the effect was due to residual CCCP inhibiting membrane transport.

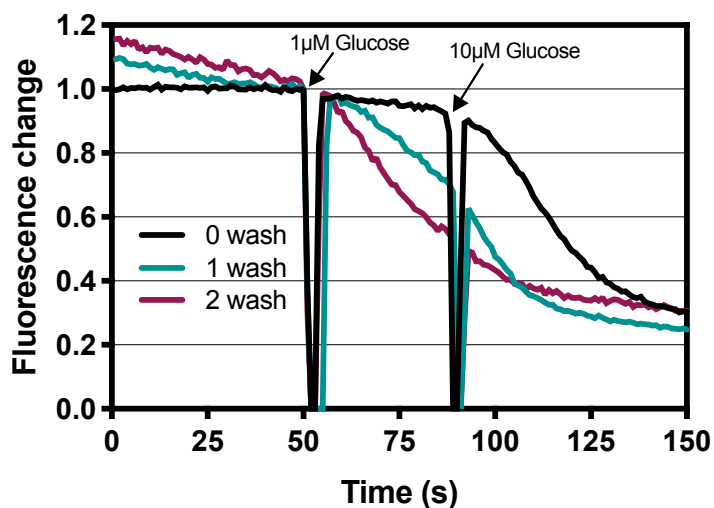


Figure 5.8. Assessing the effect of cell washes on Nile Red efflux in *E. coli* BL21 cells carrying pBAD-*acrABHis₈-tolCStrep*. Cells were preloaded with 5 μM Nile Red in the presence of 10 μM CCCP. After mild washing, efflux was initiated by addition of D-glucose at a range of concentrations where indicated with an arrow. Data are shown as a fraction of the fluorescence intensity immediately before initiation of efflux.

5.3.2.4 Measuring Nile Red efflux using the optimised efflux assay. Using these optimised conditions, rapid initiation of Nile Red efflux upon addition of glucose was observed for all three RND efflux pumps (Fig. 5.9). This proved Nile Red to be a useful tool in ensuring cloned RND pumps retain their activity when expressed in laboratory strains of *E. coli*. AcrAB-TolC and MexCD-OprJ showed high efflux activity resulting in a final mean Nile Red fluorescence reduction of 77.2% (± 0.4 SD) and 75.3% (± 0.4 SD), respectively, while MexAB-OprM displayed significantly lower levels of efflux with a final reduction of just 39.8% (± 1.2 SD) (Fig. 5.9). The final reduction in fluorescence

between uninduced and induced cells was statistically significant for all three constructs ($p < 0.0001$, unpaired t-test).

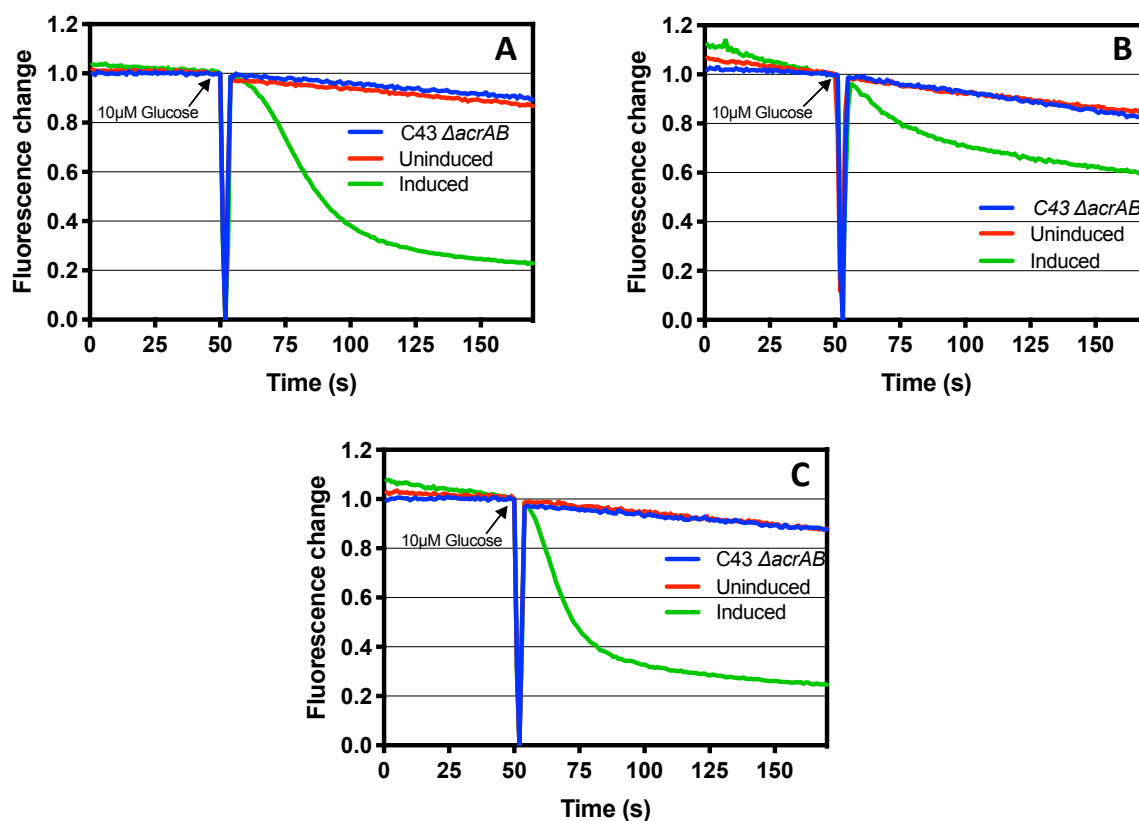


Figure 5.9. Optimised Nile Red efflux assay. Examples of efflux of Nile Red from *E. coli* C43 Δ acrAB carrying pBAD-acrABHis8-to/CStrep (A), pBAD-mexABoprMHis8 (B) or pBAD-mexCDoprJHis8 (C). Cells were prepared exactly as described in Section 2.4.2. D-Glucose was added after 50 s to a final concentration of 10 μ M to initiate efflux. Data are shown as a fraction of the fluorescence intensity immediately before initiation of efflux.

Although RND pumps are known for their substrate promiscuity, substrate specificity must still be considered as a factor in all membrane transport assays. It is likely that the reduced Nile Red efflux exhibited by MexAB-OprM is simply due to a lower affinity for Nile Red compared to the other 2 RND systems tested. Uninduced cells demonstrated little background efflux activity, with a final mean fluorescence signal reduction of just 7% to 15%, demonstrating that *E. coli* C43 Δ acrAB is an excellent strain for studying RND efflux pumps.

5.3.3 Measuring competition between efflux substrates

While the Nile Red efflux assay proved a useful measure of general efflux activity, it was important to investigate its utility in measuring BMA transport. In order to do this a competition assay, as described in Section 5.1.2, was developed. Due to its relatively poor Nile Red efflux activity, MexAB-OprM was omitted from competition assays. In the case of AcrAB-TolC, BMA concentrations up to and including 0.8 mM had no measurable effect on Nile Red efflux, while a clear dose-dependent decrease was observed between 1.0 and 2.0 mM ($r = -1.00$, $p=0.001$, Pearson correlation coefficient) (Fig. 5.10). However, no such competition was observed with MexCD-OprJ ($r = -0.60$, $p=0.12$) (Fig. 5.10). This suggested that the response observed with AcrAB-TolC was not simply a result of non-specific toxic effects of BMA, such as disruption of PMF, membrane damage and leakage, cell death or inhibition of glucose uptake or metabolism and thus served as a useful control.

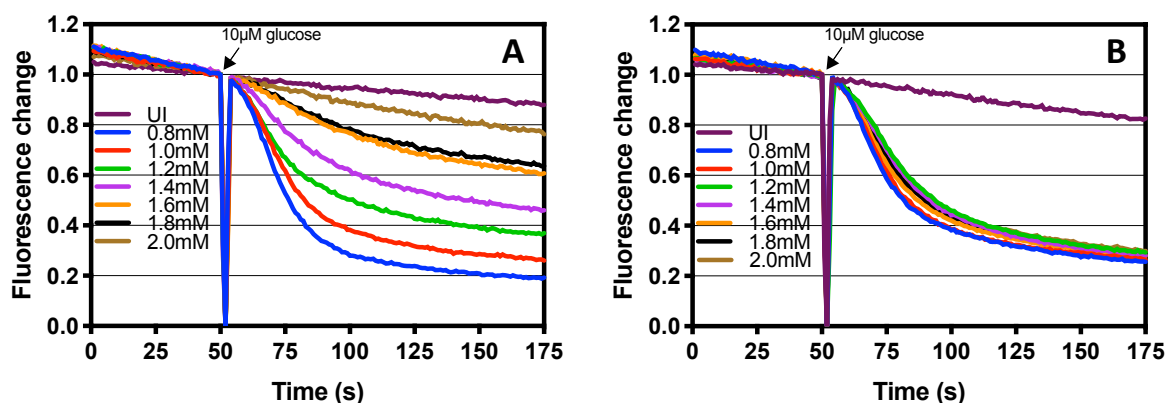


Figure 5.10. Effect of increasing concentrations of BMA on efflux of Nile Red. *E. coli* C43 Δ *acrAB* cells carrying pBAD-*acrABtolCHis*₈ (A) or pBAD-*mexCDoprJHis*₈ (B). Cells were prepared as described in Section 2.4.2 and preloaded with a range of BMA concentrations as indicated by colouring. D-Glucose was added to a final concentration of 10 μ M at 50s to initiate efflux. Data are shown as a fraction of the fluorescence intensity immediately before initiation of efflux.

However, cell viability assays revealed that expression of *acrABtolC* had a dramatic deleterious effect on cell viability following exposure to BMA (Fig. 5.11A). After just 10 minutes of exposure to 2 mM BMA, cells expressing *acrAB-tolC* from pBAD-*acrABHis₈-tolC*Strep showed a 119 (± 20 SD) fold reduction in the number of viable cells compared to uninduced control cells ($p \leq 0.0032$, Welch's unpaired t-test) (Fig. 5.11A). After 60 minutes, this difference between induced and uninduced cells was diminished but induced cells still showed a significant 29 (± 3 SD) fold reduction in viability compared to uninduced control cells ($p = 0.0161$) (Fig. 5.11A). This led to the theory that toxic levels of gene expression, particularly of *tolC*, observed from the pBAD-*acrABHis₈-tolC*Strep construct were responsible for both this hypersusceptible phenotype and the BMA dose-dependent decrease in efflux of Nile Red observed in competition assays (Fig. 5.10). The *tolC* gene present in the pBAD-*acrABHis₈-tolC*Strep construct is located independently from the *acrAB* genes under the control of its own pBAD promoter (Fig. 5.12A). Due to its relatively small size compared to *acrAB*, this means that *tolC* was likely transcribed and translated at a much faster rate than the *acrAB* genes, resulting in higher protein levels. The new expression plasmid pBAD-*acrABtolCHis₈* was constructed to reduce the level of *tolC* expression by bringing all three genes encoding AcrAB-TolC under a single promoter (Fig. 5.12B). This new construct, pBAD-*acrABtolCHis₈*, was successful in reducing production of TolC to just 7% of total membrane protein, as measured by densitometry, compared to 38% of total membrane protein when produced from pBAD-*acrABHis₈-tolC*Strep (Fig. 5.12). Production of AcrB remained unchanged, constituting 8% of total membrane protein when expressed from either construct (Fig. 5.12).

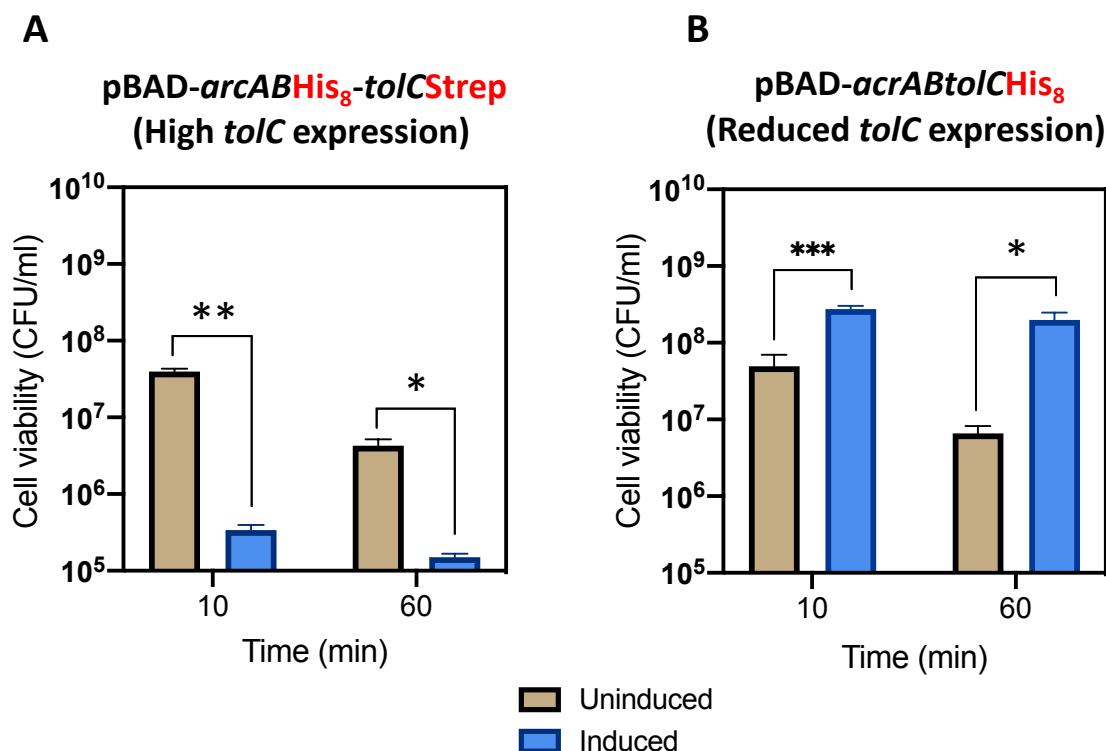


Figure 5.11. Assessing the effect of different *acrAB-tolC* expression levels on tolerance to butyl methacrylate in *E. coli* C43 Δ *acrAB*. For viability assays, cells were prepared and adjusted to the same starting count as described in Section 2.5.1 then energised by addition of 22 mM D-glucose. Butyl methacrylate was added to a final concentration of 2 mM to start the assay. After 60 minutes, viability counts were performed as described in Section 2.5.1. Error bars show standard deviation of three biological replicates and P values (Welch's unpaired t-test) are indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

In both cases, AcrA was indistinguishable from background on a Coomassie stained SDS-PAGE gel. The reduced expression of *tolC* from pBAD-*acrABtolCHis₈* was successful in reversing the hypersusceptible phenotype, with induced cells displaying a 6 (± 1 SD) fold greater cell viability compared to uninduced cells after ten minutes of exposure to 2 mM BMA ($p = 0.0004$) (Fig. 5.11B). After 60 minutes, a 30 (± 7 SD) fold increase in cell viability was observed ($p = 0.0207$) (Fig. 5.11B). Additionally, this construct resulted in significantly enhanced Nile Red efflux with a final mean

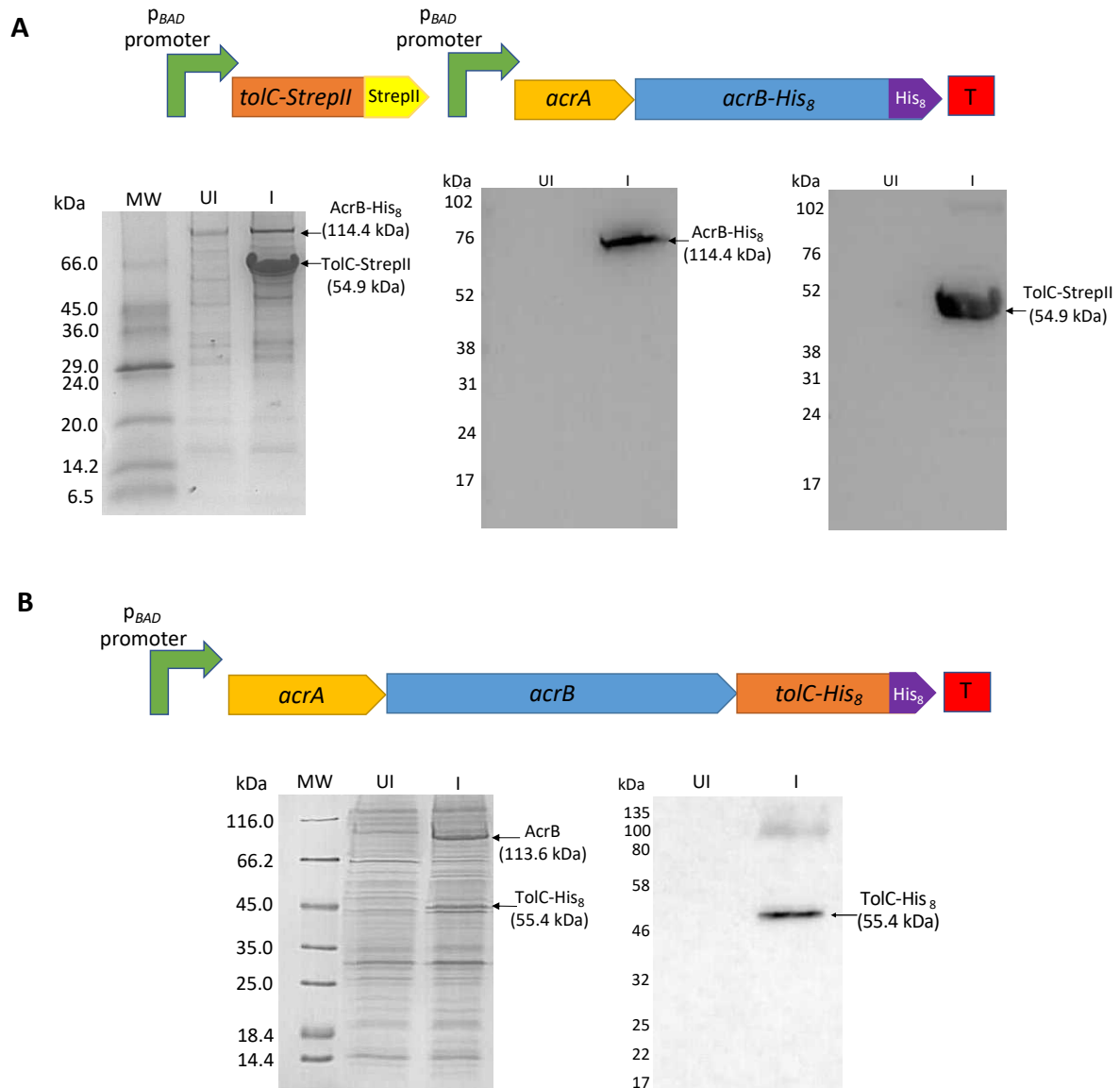


Figure 5.12. Comparing expression of the *acrABtolC* genes from pBAD-*arcABHis₈-tolCStrep* and pBAD-*acrABtolCHis₈* in *E. coli* C43 Δ *acrAB*. Screening production of protein from pBAD-*arcABHis₈-tolCStrep* (A) and pBAD-*acrABtolCHis₈* (B) by SDS-PAGE and Western blot. Membrane fractions of uninduced (UI) and induced (I) cells were prepared using the water lysis method as described in Section 2.6.2 from 50 ml cultures grown exactly as described in Section 2.2.5. His-tagged proteins were detected using HisProbe-HRP conjugate or StrepMAB-Classic HRP conjugate antibody. Lanes designated 'MW' were loaded with a molecular weight marker. Protein molecular weights shown were calculated from the relevant protein sequence using Benchling Biology Software. Schematics showing the layout of each construct are also shown.

fluorescence reduction of 82.4% (± 1.2 SD) (Fig. 5.13A), compared to 77.8% (± 1.1 SD) for the original expression plasmid (Fig. 5.9A) ($p=0.023$, unpaired t-test). However, expression of *acrABtolC* from this reduced expression construct abolished the BMA dose-dependent decrease in Nile Red efflux ($r = -0.49$, $p=0.18$) that was observed previously (Fig. 5.13B; Fig. 5.14). These data suggest that the apparent competition between BMA and Nile Red observed previously was in fact the additive effect of increasing BMA concentrations on cells that are already under stress from extremely high levels of *tolC* expression. The new pBAD-*acrABtolC*His₈ construct was therefore used for all future experiments.

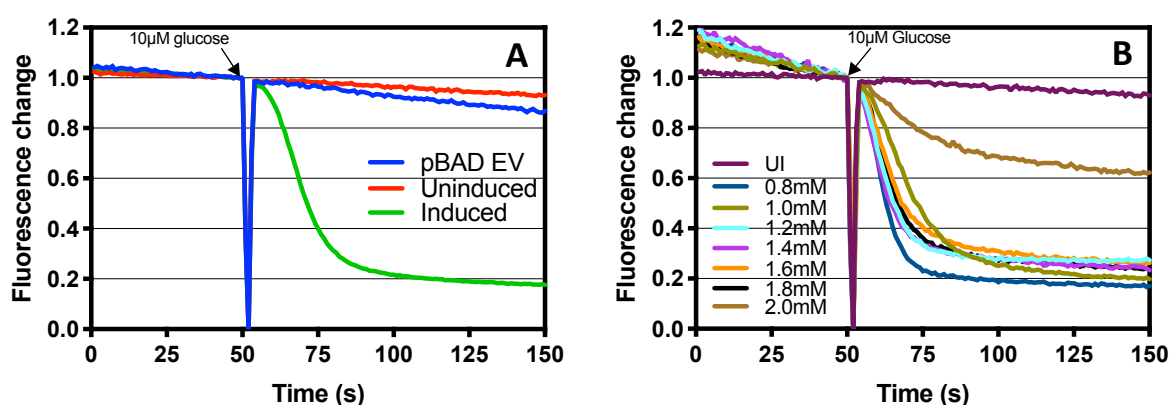


Figure 5.13. Activity of AcrAB-TolC expressed from pBAD-*acrABtolC*His₈. *E. coli* C43 Δ *acrAB* cells carrying pBAD-*acrABtolC*His₈ were screened for Nile Red efflux (A) and using the Nile Red competition assay (B). Cells were prepared as described in Section 2.4.2 (A and B) with the addition of a range of BMA concentrations (B only). Glucose was added to a final concentration of 10 μ M at 50s to initiate efflux. Data are shown as a fraction of the fluorescence intensity immediately before initiation of efflux.

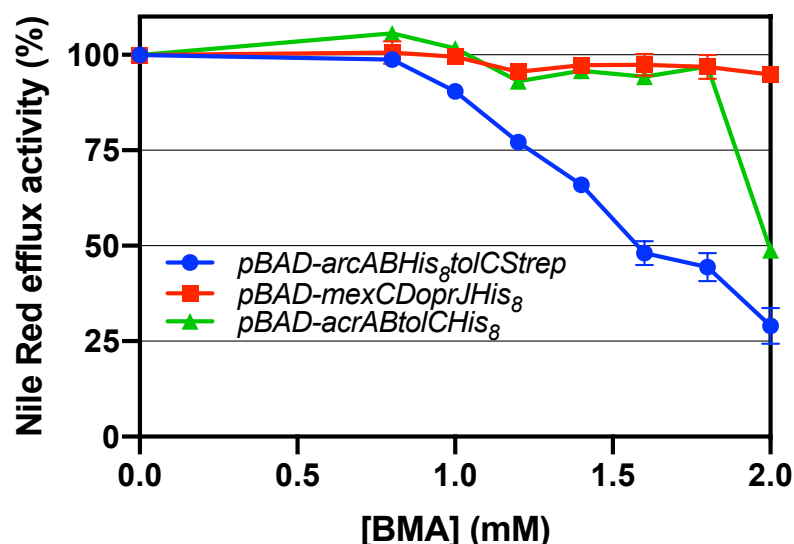


Figure 5.14. Effect of increasing BMA concentrations on Nile Red efflux. *E. coli* C43 Δ *acrAB* cells carrying various pBAD expression plasmids were prepared as described in Section 2.4.2 in the presence of increasing BMA concentrations. Glucose was added to a final concentration of 10 μ M at 50s to initiate efflux. The fluorescence endpoint was used to generate the dose-response curve.

5.3.4 Using Nile Red to test the *p_{gntK}* promoter system

The Nile Red and viability assays clearly highlighted the importance of maintaining sub-toxic production of membrane proteins, leading to the testing of the *gntK* expression system, a negative feedback system that automatically reduces gene expression upon detection of membrane stress (Boyarskiy *et al.*, 2016). Each of the three pumps tested was successfully expressed and detected by Western blot (Fig. 5.15), although due to the significantly reduced expression from these constructs, the relevant bands were not discernible from background on coomassie stained gels (data not shown). All three pumps showed statistically significant decreases in Nile Red fluorescence compared to empty vector controls ($p = <0.0001$), with a final mean reduction of 77.8% (± 1.1 SD), 33.4% (± 2.4 SD) and 56.1% (± 2.0 SD) for AcrAB-TolC, MexAB-OprM and MexCD-OprJ, respectively (Fig. 5.16).

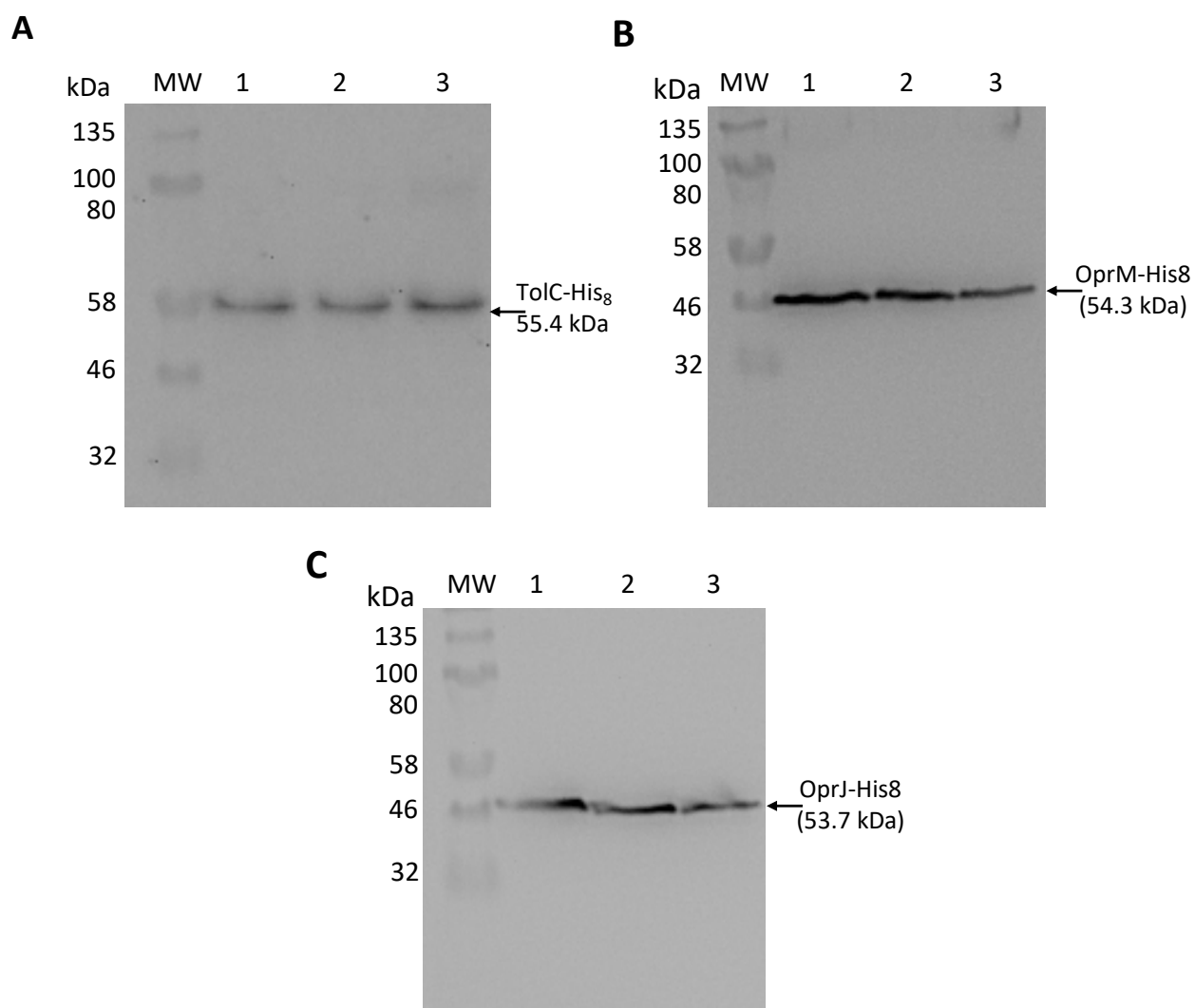


Figure 5.15. Screening RND pump expression from pGNTK constructs Screening production of protein from pGNTK-*acrABtolCHis₈* (A), pGNTK-*mexAB-oprMHis₈* (B) or pGNTK-*mexCD-oprJHis₈* (C). Membrane fractions were prepared in triplicate using the water lysis method as described in Section 2.6.2 from 50 ml cultures grown exactly as described in Section 2.2.5. His-tagged proteins were probed using HisProbe-HRP conjugate. Lanes designated 'MW' were loaded with a molecular weight marker. Protein molecular weights shown were calculated from the relevant protein sequence using Benchling Biology Software.

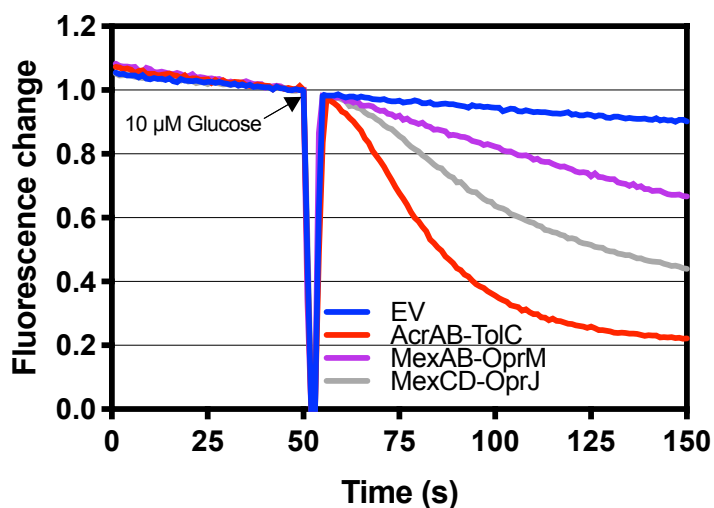


Figure 5.16. Nile Red efflux from cells carrying gntK expression constructs Efflux of Nile Red from *E. coli* C43 Δ *acrAB* carrying pGNTK-*acrABtolCHis*₈ (red), pGNTK-*mexAB-oprMHis*₈ (purple) or pGNTK-*mexCD-oprJHis*₈ (grey). Cells were prepared exactly as described in Section 2.4.2. Glucose was added after 50 s to a final concentration of 10 μ M to initiate efflux. Data are shown as a fraction of the fluorescence intensity immediately before initiation of efflux.

5.4 Nile Red as a potential substrate for non-RND pumps

Nile Red efflux assays were also performed on cells expressing a range non-RND efflux pumps (Fig. 5.17). Both MdlB and MsbA belong to the ATP-binding cassette (ABC) superfamily while EmrD, MdtL, MdtM and EmrB belong to the major facilitator superfamily (MFS), with EmrB forming a tripartite complex along with EmrA and TolC. Interestingly, none of these proteins showed any activity with Nile Red with each resulting in a final fluorescence reduction between 5% and 13%, which was not a statistically significant difference from uninduced control cells ($p > 0.05$) (Fig. 5.18). This is consistent with the hypothesis that RND pumps recruit hydrophobic substrates (eg. Nile Red) from the membranes/periplasm, while other pump families are responsible for transport of cytoplasmic substrates. This assay also suggests that despite forming a tripartite complex that spans both membranes, EmrAB-TolC may not export substrates from the periplasm.

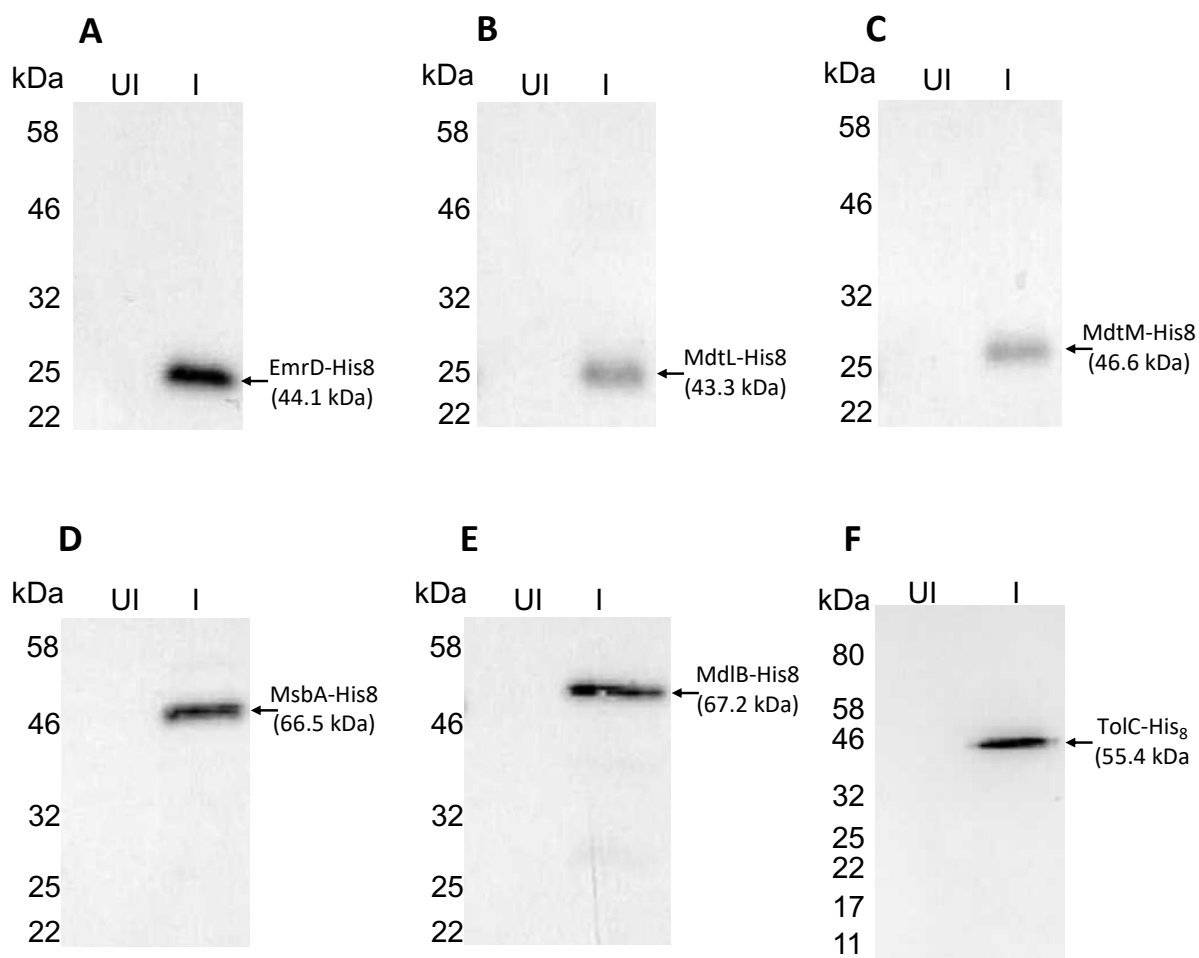


Figure 5.17. Screening non-RND efflux pump expression. Screening production of protein from pBAD-*emrD*His₈ (A), pBAD-*mdtL*His₈ (B), pBAD-*mdtM*His₈ (C), pBAD-*msbA*His₈ (D), pBAD-*mdlB*His₈ (E) and pBAD-*emrABtolC*His₈ (F). Membrane fractions of uninduced (UI) and induced (I) cells were prepared using the water lysis method as described in Section 2.6.2 from 50 ml cultures grown exactly as described in Section 2.2.5. The first lane of each gel was loaded with a molecular weight marker (not shown). His-tagged proteins were detected using HisProbe-HRP conjugate.

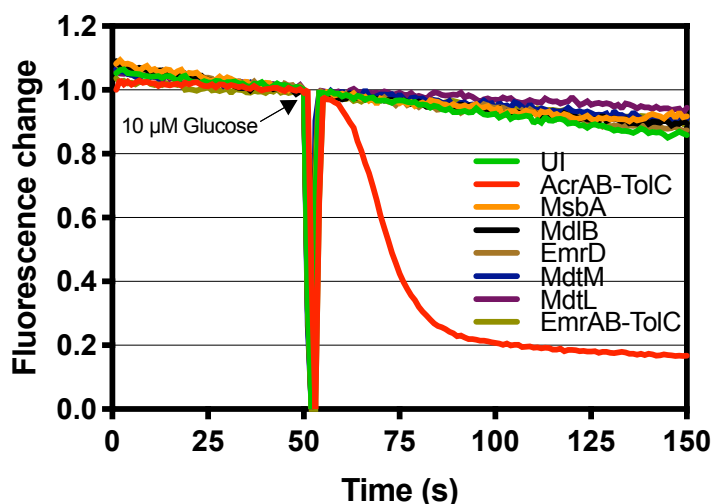


Figure 5.18. Effect of non-RND efflux pumps on Nile Red efflux. Cells expression a range of efflux pumps were screened using the Nile Red efflux assay. Cells were prepared as described in Section 2.4.2. Glucose was added after 50 s to a final concentration of 10 μ M to initiate efflux. Data are shown as a fraction of the fluorescence intensity immediately before initiation of efflux.

5.5 Discussion

Nile Red proved to be a valuable tool in determining the solubility limit of non-polar molecules such as butyl methacrylate, thanks to its propensity to partition into the organic phase where it displays dramatically increased fluorescence (Fig. 5.2; Fig. 5.3). This phenomenon has been observed previously and has led to the application of Nile Red in the detection and quantification of hydrophobic cellular components such as lipids (Krishna *et al.*, 1998; Chen *et al.*, 2009; Greenspan *et al.*, 1985) and has even been utilised to locate hydrophobic products in *E. coli* producer strains (Pinzon *et al.*, 2011). This approach could potentially be applied to a butyl methacrylate producer strain to identify where in the cell BMA accumulates, likely in the membranes, in order to further our understanding of toxicity of BMA in the cell.

In *E. coli*, basal expression of the *acrAB-tolC* genes is the primary mechanism for resistance to a range of toxic hydrophobic agents (Elkins and Nikaido, 2002). It is

therefore unsurprising that high background activity was observed with Nile Red, a hydrophobic dye, and abolished with the use of a $\Delta acrAB$ strain (Fig. 5.6). This high background activity would likely be observed with other lipophilic fluorescent dyes due to the promiscuity of AcrB, making a knockout of the chromosomal *acrB* gene essential when studying inducible plasmid-encoded RND efflux pumps in *E. coli*. The C43 $\Delta acrAB$ strain, a derivative of *E. coli* BL21 originally selected for its superior production of membrane proteins (Miroux and Walker, 1996), displayed very low background efflux while remaining amenable to genetic modification and protein production, hence its adoption for all further experiments.

The Nile Red activity assay proved a useful tool for ensuring cloned efflux pumps were expressed and assembled correctly and remained functional in *E. coli*. Nile Red efflux was observed for all 3 cloned RND systems in the optimised Nile Red efflux assay, with no observable efflux in control and uninduced cells (Fig. 5.9). In all cases, addition of 10 μ M glucose was sufficient to initiate rapid efflux of Nile Red from the cell following CCCP treatment and pre-loading with fluorescent dye, without the need for multiple wash steps. MexAB-OprM displayed a significantly lower rate of Nile Red efflux than AcrAB-TolC or MexCD-OprJ (Fig. 5.9C), although protein and dye loading levels were comparable, suggesting Nile Red is a poor substrate for the MexAB-OprM system.

The BMA competition assay initially appeared promising for AcrAB-TolC, though later experiments proved the apparent dose-response to be a result of the additive effect of BMA toxicity on cells pre-stressed through hyperexpression of the *tolC* gene (Fig. 5.14). Additionally, no competition between Nile Red and BMA was observed for MexCD-OprJ, despite this pump conferring significantly enhanced tolerance to BMA in viability assays (see Chapter Six). The Nile Red efflux assay is

therefore useful as a general activity assay but unsuitable for measuring BMA transport, necessitating alternative methods of study.

To better understand the findings of the competition assays, more experiments were performed using a new AcrAB-TolC construct designed to reduce expression, particularly of *tolC*. In pBAD-*acrABHis₈tolC*StrepII, *tolC* was in isolation under the control of its own p_{BAD} promoter (Fig. 5.12). In pBAD-*acrABtolCHis₈* all 3 genes are under the control of a single promoter (Fig. 5.12), which was found to both reduce TolC protein levels in the membrane and dramatically increase tolerance to extracellular BMA (Fig. 5.11). The greatest difference in cell viability between induced and uninduced cells was observed in samples taken after ten minutes of exposure to BMA (Fig. 5.11A), suggesting that overexpression of the *tolC* gene significantly increases the rate of entry of BMA into the cell. This increased rate of BMA ingress may be due to general membrane damage and increased permeability caused by toxic levels of membrane protein production, or by facilitated diffusion of BMA into the periplasm through the TolC protein itself. TolC has previously been implicated as a route of entry for sugars and large antibiotics such as vancomycin into the periplasm (Bavro *et al.*, 2008; Zgurskaya *et al.*, 2016) and of course allows passage of hydrophobic AcrB substrates out of the cell, indicating that facilitated diffusion of BMA through TolC into the periplasm may be possible. Fortunately, research has shown that depletion of TolC does not attenuate AcrAB-TolC-mediated efflux, suggesting very small quantities of TolC are required for correct function of this pump (Krishnamoorthy *et al.*, 2013). Reducing expression of *tolC* is therefore unlikely to impede AcrAB-TolC-mediated BMA efflux, consistent with our findings that reduced expression actually improved efflux of Nile Red (Fig. 5.13A), while also minimising any changes in membrane permeability caused by TolC or general toxic effects of

membrane protein production. By reducing the level of TolC in the membrane fraction (Fig. 5.12), we have demonstrated the importance of optimising protein expression levels and the effects of their overexpression on membrane integrity and cellular stress. Wagner *et al.* (2007) previously demonstrated that overexpression of membrane proteins causes severe cellular stress including the formation of large cytoplasmic aggregates/inclusion bodies and an apparent saturation of the Sec translocon. As a result of this, in addition to changes in membrane fluidity and permeability, there was a marked decrease in the abundance of respiratory chain complexes, which will have profound effects on the cell (Wagner *et al.*, 2007).

To overcome this, strains have been developed to reduce the rate of membrane protein expression therefore alleviating toxicity and, counter-intuitively, increase yields of correctly folded, membrane-localised protein (Mathieu *et al.*, 2019; Nannenga and Baneyx., 2011; Schlegel *et al.*, 2015; Wagner *et al.*, 2007). Further investigations suggest that toxic overexpression selects for non-productive cells, thus reducing overall protein yields and in some cases finding that uninduced cells were ultimately more productive than induced cells when using a T7 based expression system (Zhang *et al.*, 2015). These observations are consistent with the findings presented here that reducing the expression level not only resulted in a strain with enhanced BMA tolerance but also increased Nile Red efflux capacity. We have demonstrated that overexpression of membrane proteins has profound deleterious effects in *E. coli* and, particularly for functional assays, it is vital to optimise expression levels to prevent protein toxicity and maximise the production of correctly folded, functional transporters.

These observations led to the utilisation of the p_{gntK} promoter, a negative feedback expression system that is able to regulate expression of membrane proteins

automatically in response to membrane stress (Boyarskiy *et al.*, 2016). This system resulted in optimal expression of an *n*-butanol transporter without the need for optimisation, resulting in a strain with increased *n*-butanol tolerance as well as a 40% increase in *n*-butanol yield when co-expressed with the production pathway (Boyarskiy *et al.*, 2016). These findings demonstrate the utility of such an expression system in an industrially relevant context. The *gntK* system would also be more appropriate for use in a large-scale industrial process, as no intervention is required to induce gene expression and the need for expensive inducing agents is circumvented, making the process more economically viable at scale. In our hands, all 3 systems remained active in the Nile Red assay following expression from the *p_{gntK}* promoter (Fig. 5.16).

None of the 6 MFS and ABC superfamily efflux pumps were able to transport the membrane-associated Nile Red (Fig. 5.18). While it is important to consider and investigate further the specificity of these transporters and the possibility that Nile Red is simply an unsuitable substrate, these observations could also strengthen the hypothesis that these families of transporters generally recruit their substrate from the cytoplasm rather than the membranes/periplasm. This is important to consider in the context of a BMA producing strain, as in order to effect efflux from the cytoplasm to the external medium it may be necessary to exploit both an inner membrane pump to transport BMA from the cytoplasm to the periplasm and an RND pump to transport BMA across the outer membrane. Studies have demonstrated that both an RND and an inner membrane efflux pump are required to confer high levels of resistance to a number of antibiotics and other cytoplasmic substrates such as ethidium (Lee *et al.*, 2000; Tal and Schuldiner, 2009). Expression of a single efflux pump or multiple pumps of the same family was ineffective in restoring resistance to the same levels (Lee *et*

et al., 2000), suggesting that the RND component is essential for full removal of substrate from the periplasm and in preventing re-entry of substrate into the cell.

EmrB, an MFS transporter, may also be of particular interest in this context. Just like RND pumps, EmrB forms a tripartite complex that spans both the inner and outer membranes through interactions with EmrA, a periplasmic linker protein, and the familiar outer membrane channel TolC (Piddock, 2006). Unlike RND pumps, it has been suggested that EmrB is able to capture substrates from the cytoplasm and transport them directly across both membranes through the tripartite complex (Tanabe *et al.*, 2009, Zgurskaya *et al.*, 2009), although there is very little experimental evidence to support this hypothesis at present and it seems to have been inferred from homology to other, more highly characterised transporters such as QacA (Zgurskaya *et al.*, 2009). EmrB lacks the large soluble periplasmic domain found in RND transporters (Tanabe *et al.*, 2009) and was also unable to transport Nile Red in our efflux assay (Fig. 5.18) supporting the suggestion that it does not capture substrates from the periplasm, although more work is needed to understand this.

Chapter 6

Assessing the effect of isobutyrate and
methacrylate esters on cell viability

6.1 Introduction

Owing to their hydrophobicity, organic solvents rapidly partition into biological membranes leading to accumulation of high local concentrations and exacerbation of toxic effects (Sikkema *et al.*, 1995). This accumulation in membranes would lead to alterations in membrane fluidity and integrity, resulting in increased membrane permeability and subsequent disruption of pH homeostasis and dissipation of electrochemical gradients (Sikkema *et al.*, 1995; Eberlein *et al.*, 2018). Additionally, solvents may interact directly with membrane-bound proteins, binding to hydrophobic sites to inhibit enzyme activity (Backes *et al.*, 1993; Slater *et al.*, 1993) or displacing native lipids, thereby disrupting key protein-lipid interactions that modulate enzyme activity and stabilise quaternary protein structures (Contreras *et al.*, 2011; Pyle *et al.*, 2018; Pyle *et al.*, 2019). These severe perturbations to essential membrane-localised biological processes could result in a diminution of ATP synthesis and ultimately loss of cell viability (Sikkema *et al.*, 1995; Oh *et al.*, 2012; Eberlein *et al.*, 2018). It is vital to characterise and overcome this loss of viability brought about by butyl methacrylate (BMA) and other esters in the context of an industrial production strain, where loss of viability means loss of productivity.

Consequently, viability assays were performed using twelve different esters in order to identify suitable alternative target products with reduced toxicity compared to BMA. Eight different efflux pumps were also screened for their ability to export and therefore enhance tolerance to these esters. Of the esters tested, five were found to be significantly less toxic to *E. coli* than butyl methacrylate, while expression of *acrAB-tolC*, *mexAB-oprM* or *mexCD-oprJ* provided significant protection against all seven toxic esters. Non-RND pumps were less effective in enhancing tolerance to these compounds, with only EmrD and MdtM providing significant protection against some

of the esters tested here. It is also demonstrated that overexpression of membrane proteins has additive deleterious effects on cells exposed to butyl methacrylate, highlighting the importance of optimising gene expression in the study of membrane proteins and in the final production strain. These findings demonstrate that bacterial efflux pumps, particularly those belonging to the RND family, are effective in significantly enhancing tolerance to a range of toxic esters and may have valuable applications in a methacrylate or isobutyrate production strain and in the broader field of biotechnology.

6.2 Determining the effect of efflux pump expression on tolerance to butyl methacrylate

6.2.1 Identifying efflux pumps that confer enhanced tolerance to BMA

The ability to transport a toxic substrate actively out of the cell is usually accompanied by an increase in tolerance to that substrate, due to the maintenance of lower intracellular concentrations and therefore reduced toxicity. In order to investigate this, viability assays were performed using extracellular addition of BMA in the presence and absence of all cloned efflux pumps. As discussed in Chapter 5, expression of *acrABtoIC* from pBAD-*acrABtoIC*His₈ resulted in a 30-fold (± 7) increase in cell viability following one hour of exposure to 2mM BMA compared to cells carrying the pBAD empty vector ($p = 0.0207$, Welch's unpaired t-test) (Fig. 6.1). Both MexAB-OprM and MexCD-OprJ were also effective in enhancing tolerance to BMA, with induced cells demonstrating a 22 (± 1 SD) fold increase ($p = 0.0006$) and a 10 (± 4 SD) fold increase ($p = 0.0485$) in cell viability, respectively, compared to control cells carrying the pBAD empty vector (Fig. 6.1). Of the non-RND pumps tested, only MdtM provided significant protection against BMA with a 6 (± 1 SD) fold increase in cell viability ($p = 0.0033$).

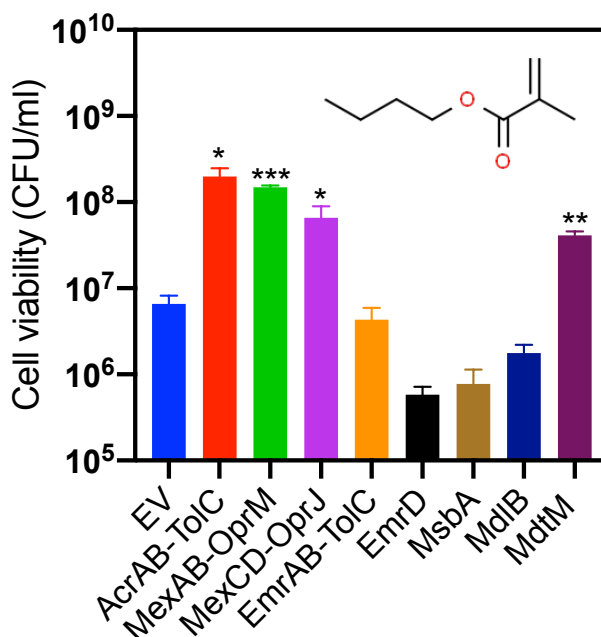


Figure 6.1. Assessing the effect of efflux pump expression on tolerance to butyl methacrylate in *E. coli* C43 Δ *acrAB*. Cells were prepared and adjusted to the same starting count as described in Section 2.5.1 then energised by addition of 22 mM D-glucose. Butyl methacrylate was added to a final concentration of 2 mM to start the assay. Cells treated with glucose only were used as a negative control and showed no significant change in viability over the course of the assay (data not shown). After 60 minutes, viability counts were performed as described in Section 2.5.1. Error bars show standard deviation of three biological replicates and P values (Unpaired Welch's t-test) are indicated as follows for efflux pumps that significantly enhanced tolerance to BMA compared to cells carrying the pBAD empty vector (EV): * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. The structure of BMA is shown for reference.

6.3 Screening the effect of efflux pump expression on tolerance to alternative methacrylate and isobutyrate esters

6.3.1 Identification of esters with reduced toxicity

Acrylic polymers can be derived from a number of different ester feedstocks, some of which may be significantly less toxic than BMA. It was therefore important to test a selection of other esters that may be suitable alternative target molecules to BMA with reduced toxicity, allowing higher yields to be achieved. Of the 11 esters tested, isopropyl isobutyrate (IPIB), isopropyl methacrylate (IPMA), butyl isobutyrate (BIB),

2-ethylhexyl isobutyrate (EHIB) and 2-ethylhexyl methacrylate (EHMA) all had no effect on cell viability at a final concentration of 2 mM (Fig. 6.2). Therefore, these esters may represent appropriate target products in the bio-production of acrylic monomers due to their significantly reduced toxicity compared to BMA.

6.3.2 Determining the effect of efflux pump expression on tolerance to alternative esters

Conversely, pentyl isobutyrate (PIB), pentyl methacrylate (PMA), hexyl isobutyrate (HIB), hexyl methacrylate (HMA), heptyl isobutyrate (HepIB) and heptyl methacrylate (HepMA) were all toxic to *E. coli* at a concentration of 2 mM (Fig. 6.3). Interestingly, all three RND efflux pumps were able to provide significant protection against every one of these toxic esters (Fig. 6.3; Table 6.1). MdtM and EmrD were the only non-RND pumps found to confer enhanced tolerance to any of the toxic esters, although protection was only observed for pentyl isobutyrate, pentyl methacrylate and hexyl methacrylate (Fig. 6.3; Table 6.1). All changes in cell viability are summarised in Table 6.1. No statistically significant correlation was observed between the octanol/water partition coefficient ($\log P_{ow}$) values and ester toxicity ($r = -0.38$, $p = 0.26$) (Fig. 6.4).

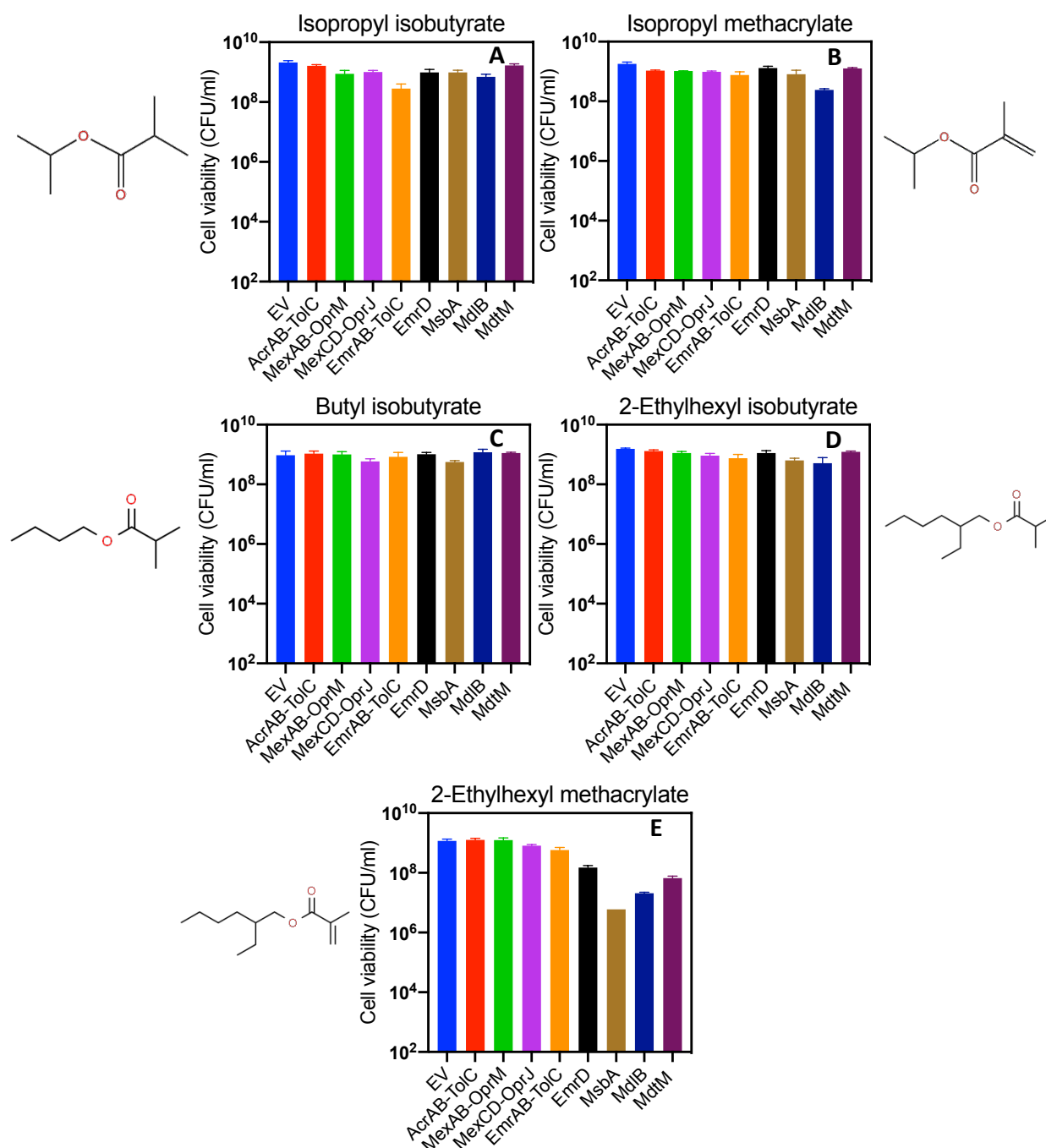


Figure 6.2. Assessing the effect of efflux pump expression on tolerance to a range of esters in *E. coli* C43 Δ *acrAB*. Cells were prepared and adjusted to the same starting count as described in Section 2.5.1 then energised by addition of 22 mM D-glucose. The appropriate ester was added to a final concentration of 2 mM to start the assay. Cells treated with glucose only were used as a negative control and showed no significant change in viability over the course of the assay (data not shown). After 60 minutes, viability counts were performed as described in Section 2.5.1. Error bars show standard deviation of three biological replicates. Ester structures are shown for reference.

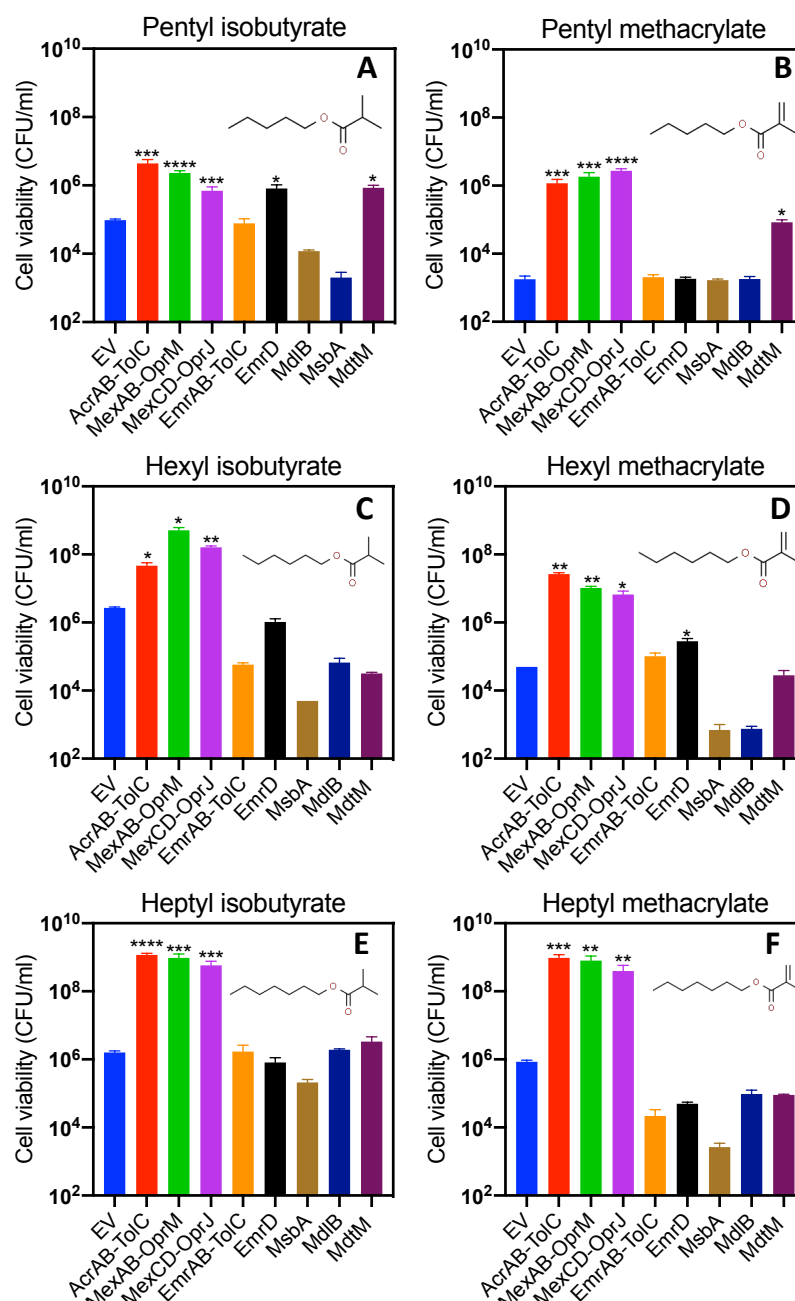


Figure 6.3. Assessing the effect of efflux pump expression on tolerance to a range of esters in *E. coli* C43 Δ *acrAB*. Cells were prepared and adjusted to the same starting count as described in Section 2.5.1 then energised by addition of 22 mM D-glucose. The appropriate ester was added to a final concentration of 2 mM to start the assay. Cells treated with glucose only were used as a negative control and showed no significant change in viability over the course of the assay (data not shown). After 60 minutes, viability counts were performed as described in Section 2.5.1. Error bars show standard deviation of three biological replicates and P values (Unpaired Welch's t-test) are indicated as follows for efflux pumps that significantly enhanced tolerance to BMA compared to cells carrying the pBAD empty vector (EV): * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Ester structures are shown for reference.

Table 6.1. Fold-increase in cell viability conferred by efflux pumps upon exposure to a range of esters. Values shown are means of triplicate data with standard deviation shown in parentheses.

Compound	AcrAB-TolC	MexAB-OprM	MexCD-OprJ	EmrD	MdtM
Butyl methacrylate	30 (± 7)	22 (± 1)	10 (± 4)	-	-
Pentyl methacrylate	645 (± 200)	1033 (± 329)	1533 (± 239)	-	47 (± 9)
Pentyl isobutyrate	46 (± 14)	24 (± 4)	7 (± 2)	8 (± 2)	9 (± 2)
Hexyl methacrylate	533 (± 50)	207 (± 25)	133 (± 35)	-	-
Hexyl isobutyrate	17 (± 4)	191 (± 39)	61 (± 5)	-	-
Heptyl methacrylate	1137 (± 273)	941 (± 337)	468 (± 216)	-	-
Heptyl isobutyrate	734 (± 78)	599 (± 176)	365 (± 116)	-	-

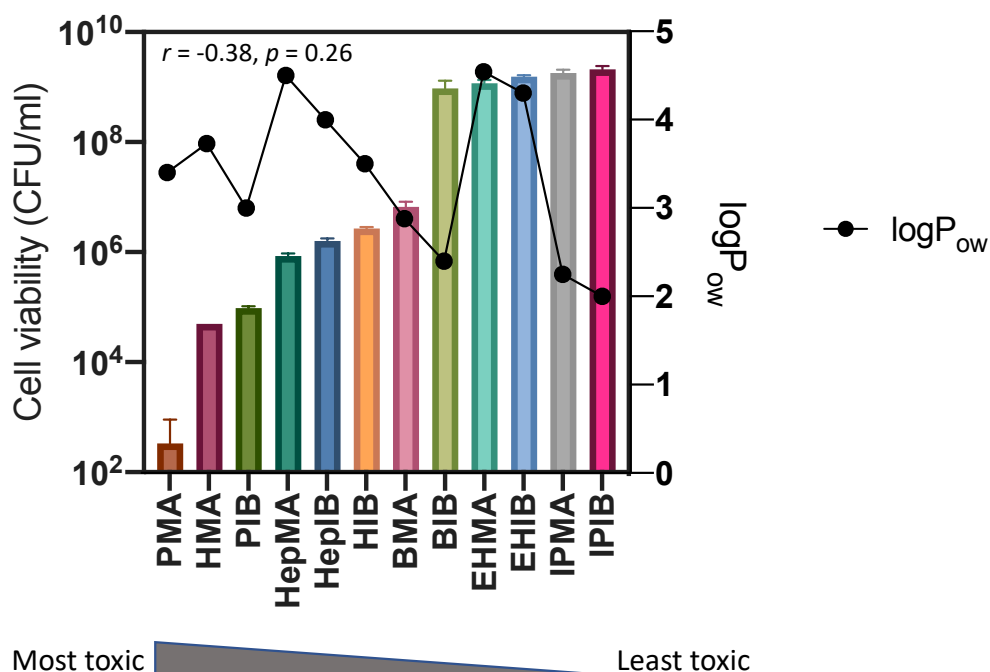


Figure 6.4. Comparing toxicity of different esters. *E. coli* C43 Δ acrAB cells carrying the pBAD empty vector were prepared and adjusted to the same starting count as described in Section 2.5.1 then energised by addition of 22 mM D-glucose. The appropriate ester was then added to a final concentration of 2 mM to start the assay. After 60 minutes, viability counts were performed as described in Section 2.5.1. Toxicity is ranked based on remaining viable cell count after 60 minutes of exposure to the relevant ester at a concentration of 2 mM. $\log P_{ow}$ values indicate the octanol/water partition coefficient. Error bars show standard deviation of three biological replicates and Pearson correlation coefficient (r) is also shown.

6.4 Discussion

The AcrAB-TolC efflux pump of *E. coli* is a model RND system known to transport and confer tolerance to a vast range of structurally dissimilar substrates, including organic solvents (Tsukagoshi and Aono, 2000; Wang *et al.*, 2013). Although overexpression of the *tolC* gene was found to have severe deleterious effects in *E. coli* upon exposure to BMA (see Chapter Five), AcrAB-TolC produced from the new pBAD-*acrABtolC*His₈ construct demonstrated significantly enhanced tolerance to external addition of BMA (Fig. 6.1). These data are consistent with previous findings that this efflux pump is involved in conferring tolerance to BMA in *E. coli*, where up-regulation and mutations in key regulators of gene expression have both been identified (Martins, unpublished; Menchavez, unpublished).

Previous work at the University of Strathclyde used *P. aeruginosa* transposon mutant libraries to identify genes involved in tolerance to BMA (El Bestawy, unpublished). Cells in which the *mexAB-oprM* gene cluster was disrupted showed the greatest attenuation of growth, followed closely by those with disruptions in the *mexCD-oprJ* cluster, suggesting that these RND efflux pumps are the major determinants of resistance to BMA in *P. aeruginosa*. MexAB-OprM is considered the major contributing factor to intrinsic multidrug resistance in *P. aeruginosa* and the MexB pump component shares 83.4% similarity and 70.0% identity with *E. coli* AcrB. As such, MexAB-OprM demonstrates significant substrate overlap with AcrAB-TolC and is known to transport organic solvents and other hydrophobic agents (Li *et al.*, 1998; Li and Poole, 1999; Nehme *et al.*, 2004). Similarly, expression of *mexCD-oprJ* is known to be induced by membrane damaging agents in *P. aeruginosa* (Li *et al.*, 1998; Fraud *et al.*, 2008; Morita *et al.*, 2003), with the MexD pump component sharing 69.1% similarity and 49.9% identity with *E. coli* AcrB. The data presented here are

consistent with previous findings that both of these pumps play a role in tolerance to organic solvents and now specifically BMA, with both conferring significantly enhanced tolerance to 2 mM BMA (Fig. 6.1; Table 6.1). MexAB-OprM appeared to provide a greater level of protection than MexCD-OprJ (Fig. 6.1, Table 6.1), consistent with findings at the University of Strathclyde that MexAB-OprM is the major determinant of tolerance to BMA in *P. aeruginosa* (El Bestawy, unpublished).

In addition to enhancing tolerance to BMA, all three of the RND pumps tested here were able to confer significantly enhanced tolerance to PIB, PMA, HIB, HMA, HepIB and HepMA, (Fig 6.4; Table 6.1). RND pumps possess a hydrophobic trap in the substrate binding pocket that is lined with hydrophobic phenylalanine residues and is conserved in Enterobacteriaceae, including in AcrB of *E. coli* as well as in MexB and MexD of *P. aeruginosa* (Nakashima *et al.*, 2013; Ramaswamy *et al.*, 2017; Aron and Opperman, 2018). Substrate recognition and binding in AcrB is known to occur primarily through hydrophobic interactions (Yu *et al.*, 2003a; Yu *et al.*, 2003b; Vargiu *et al.*, 2018) and pumps lacking the hydrophobic trap have significantly different substrate profiles, including more hydrophilic antibiotics (Ramaswamy *et al.*, 2017; Aron and Opperman, 2018), suggesting that this conserved feature may be responsible for the general preference of RND pumps for hydrophobic substrates. In their biological context this preference for hydrophobic substrates seems logical considering that RND pumps are generally thought to recruit their substrates from the periplasm (Elkins and Nikaido, 2002; Lomovskaya and Totrov, 2005; Nikaido and Takatsuka, 2009) where hydrophobic agents, such as the esters tested here, are likely to accumulate.

The p_{gntK} promoter system (see Section 5.3.4) was also tested using viability assays for its reported ability to automatically optimise expression of integral

membrane proteins, but anomalous growth and difficulties in obtaining consistent starting inocula were observed, consistent with findings at Ingenza Ltd. (S. Baxter, personal communication, June 2018). Obtaining consistent starting inocula is absolutely essential when performing viability assays in order to accurately and reproducibly quantify changes in the cell population, making these constructs unsuitable for this type of assay. Additionally, any detrimental effects on the growth rate of the host organism would be unacceptable in the final production strain, meaning considerable characterisation and optimisation is required before the *p_{gntK}* expression system would be suitable for use in this context.

The inner membrane efflux pumps had limited efficacy in viability assays. In fact, to different degrees, their expression significantly reduced viability (Fig. 6.2; Fig. 6.3). Although no obvious signs of stress were observed when expressing these non-RND genes in isolation, this decrease in viability is most likely due to the additive effects of membrane protein overexpression and ester toxicity, as observed with *tolC* (Fig. 6.1). As mentioned previously, the *p_{gntK}* promoter system was intended to solve this issue but resulted in anomalous growth and very poor reproducibility. Further work should therefore be performed to determine empirically appropriate levels of expression of these genes in order to minimise toxic effects of membrane protein production. It is also important to consider that external addition of esters may not be an appropriate assay of the activity of inner membrane transporters. As discussed previously, solvent toxicity appears to arise from partitioning into, and disruption of, membranes. There is currently no evidence to suggest that, when added externally, these esters are able to cross the inner membrane barrier and enter the cytoplasm, where they might then be captured and transported by an inner membrane pump. However, co-expression of both a non-RND inner membrane transporter and an RND

pump is known to be essential for resistance to a number of hydrophobic drugs (Holdsworth and Law, 2012; Tal and Schuldiner, 09), suggesting that some hydrophobic agents, and therefore possibly esters, freely enter the cytoplasm and are then exported in a two-step mechanism. This is particularly relevant in the production strain, where BMA is produced in the cytoplasm and may require transport from the cytoplasm to the periplasm by a non-RND inner membrane transporter, where it could then be recruited by an RND pump for expulsion across the outer membrane. It is therefore more appropriate and of high priority to screen the effect of these inner membrane pumps, both alone and in combination with an RND pump, in the production strain in order to better understand transport of these esters in the relevant biological context.

Interestingly, EmrD and MdtM did enhance tolerance but only to BMA, PIB, PMA and HMA (Fig. 6.3; Table 6.1). This may indicate specificity of these transporters for these esters, or alternatively could suggest that these esters are able to penetrate the inner membrane and enter the cytoplasm, making non-RND-mediated transport from the cytoplasm an effective tolerance mechanism. The crystal structure of EmrD reveals the presence of a hydrophobic core similar to RND pumps, consistent with its preference for hydrophobic substrates (Yin *et al.*, 2006; Sun *et al.*, 2014). Homology models have indicated that this hydrophobic core is conserved in MdtM (Alegre *et al.*, 2016) and is likely responsible for the transport of hydrophobic compounds observed in these transporters. These structural data combined with the viability assays presented here make EmrD and MdtM the most interesting of the non-RND proteins and warrants further characterisation of these pumps in the production strain.

Although IPIB, IPMA, BIB, EHIB and EHMA had no effect on cell viability at a concentration of 2 mM, it is important to consider that other native tolerance

mechanisms may be active in the *E. coli* C43 Δ *acrAB* strain that have not been accounted for. For example, other constitutively expressed efflux pumps present in this strain could be providing protection against these compounds and further studies to identify them, for example using gene knockout studies, may be valuable. Microscopic examination of cells may also be worthwhile to see if cells display any typical signs of stress, such as filamentation and other morphological changes, to better understand the toxicity limits of these esters. Although production of these substrates will necessitate further downstream processing, such as traditional chemical conversion to more useful acrylic polymer precursors, the higher yields made achievable with a reduced toxicity bottleneck may ultimately result in a more economically viable process. Further investigation into bio-production of these compounds is therefore of great value. Interestingly, despite it being widely reported that toxicity of hydrophobic agents correlates with the octanol/water partition coefficient ($\log P_{ow}$) (Sikkema *et al.*, 1995; Tsukagoshi and Aono, 2000; Ramos *et al.*, 2002; Eberlein *et al.*, 2018; Dyrda *et al.*, 2019), no correlation was observed here ($r = -0.38$, $p = 0.26$) (Fig. 6.4), perhaps because these $\log P_{ow}$ values do not reflect the complex and dynamic nature of biological membranes (Loureiro *et al.*, 2018). Model lipid systems and small-angle X-ray scattering (SAXS) have been used at the University of Nottingham to show that incorporation of BMA reduces lipid transition temperature without altering the thickness of the bilayer, suggesting that BMA uncouples lipids by becoming incorporated between hydrophobic lipid tail groups (Yeh, unpublished). Generally, branched esters were significantly less toxic than unbranched, with the exception of butyl isobutyrate (Fig. 6.2C), which could suggest that branched esters are less able to penetrate the membrane and/or pack between lipid tails due to their increased steric bulk. Of course, those compounds that

appeared to be non-toxic at 2 mM must be tested at higher concentrations to fully understand their respective toxicities. Molecular dynamics simulations have also been used to model diffusion of small molecules, including organic solvents, across biological membranes (Bemporad and Essex, 2004; Awoonor-Williams and Rowley, 2016; Chipot and Comer, 2016) and could be useful in the future to shed light on the movement of esters through model *E. coli* membranes.

Chapter 7

Measuring cell viability and the metabolic burden
of RND efflux pumps using a luciferase-based
ATP assay

7.1 Introduction

Luciferases are a diverse group of enzymes found in a range of both prokaryotic and eukaryotic organisms that catalyse oxidation of a luciferin substrate to produce light (Thorne *et al.*, 2010; Close *et al.*, 2012). Since the first luciferin gene was successfully cloned (de Wet *et al.*, 1987), the applications for these enzymes in the field of biological research have been manifold including in hygiene and infection monitoring (Kramer *et al.*, 2008; Papon *et al.*, 2014), *in vivo* imaging of tumours (Zinn *et al.*, 2009; Tiffen *et al.*, 2010), drug susceptibility testing (Tenhami *et al.*, 2001; Vocat *et al.*, 2015), DNA pyrosequencing (Ronaghi, 2001) and as a reporter of gene expression in both prokaryotes and eukaryotes (Koncz *et al.*, 1990; Lewenza *et al.*, 2005; Thorne *et al.*, 2010). The typical reaction catalysed by luciferase enzymes is as follows:



Luciferase enzymes of eukaryotic origin invariably carry out ATP-dependent reactions, generating a luminescence signal proportional to ATP concentration. ATP is present in all living cells at a relatively constant concentration and degrades rapidly following cell death (Karl, 1980; Webster *et al.*, 1985), meaning ATP concentration and thus luminescence signal is closely correlated to the number of viable cells in a given sample (Hammes *et al.*, 2010; Turner *et al.*, 2010; Finger *et al.*, 2012). Luciferase-based assays have therefore been developed as rapid, high-throughput means of quantifying cell viability. Additionally, since ATP synthesis is primarily driven by movement of protons down their concentration gradient through ATP synthase (Maloney *et al.*, 1974), changes in cellular ATP concentration may be used to infer

perturbation of the proton motive force (PMF) (Bergersen and Taylor, 1985; Farha *et al.*, 2013; Kurre *et al.*, 2013).

A luciferase-based assay was investigated as an alternative to traditional culture-based viability testing due to its dramatically reduced handling time and reduced consumption of materials, making it preferable for use in a large-scale industrial process where regular sampling is required. The BacTiter-Glo™ kit from Promega is an all-in-one cell lysis and ATP quantification reagent, requiring just a 5 minute incubation at room temperature before automatic data acquisition in a plate reader, compared to lengthy serial dilution, plating, overnight incubation and manual counting of traditional plate count assays. A clear BMA dose-dependent decrease in cell viability was observed with a concurrent decrease in cellular ATP concentration, while time-course assays also demonstrated that BMA is able enter the cell and/or membranes freely and elicits significant toxic effects within just one minute of exposure. Expression of *acrABtoIC* or *mexCDoprJ* provided significant protection against BMA in all experiments, consistent with previous findings. Measurements of cellular ATP concentration may also shed light on the metabolic implications of RND efflux pump expression and function by providing evidence of changes in the proton motive force. Throughout these experiments, a strong correlation between cell viability and cellular ATP concentration was observed, suggesting that the ATP assay is a suitable alternative to traditional culture-based viability testing.

7.2 Measuring the effect of BMA on cell viability and cellular ATP concentration

7.2.1 Measuring the effect of increasing BMA concentrations on cell viability and cellular ATP concentration

In order to determine the toxicity limit of BMA, *E. coli* C43 Δ *acrAB* cells were exposed to increasing concentrations of BMA for one hour and both cell viability and cellular ATP concentration measured. In cells carrying the pBAD empty vector, a strong correlation ($r=0.96$, $p<0.0001$, Pearson correlation coefficient) between cell viability and ATP concentration was observed (Fig. 7.1). Both cell viability counts and the ATP assay gave an LD₅₀ value between 1.4-1.6 mM BMA (Fig. 7.1). Concentrations up to and including 1.0 mM BMA appeared to be non-toxic to *E. coli* C43 Δ *acrAB* (Fig. 7.1).

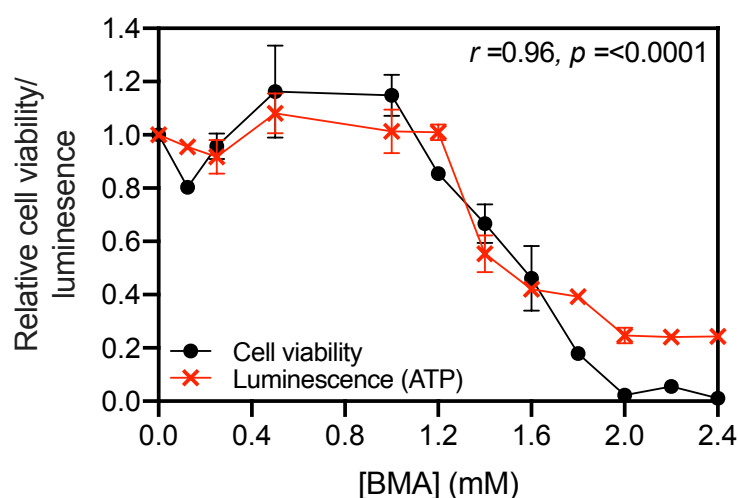


Figure 7.1. Measuring the effect of BMA on cell viability and cellular ATP concentration. *E. coli* C43 Δ *acrAB* cells carrying the pBAD empty vector were prepared to the same starting count as described in Section 2.5.1 then energised by addition of 22 mM D-glucose. BMA was added at a range of concentrations to start the assay. Samples were taken after one hour and both the cell viability and ATP assays performed as described in Sections 2.5.1 and 2.5.2, respectively. All data are normalised to an untreated control and represent the mean of three biological replicates. Error bars show standard deviation and the Pearson correlation coefficient (r) is also shown.

Expression of *acrABtoI/C* from the pBAD-*acrABtoI/CHis₈* plasmid conferred an increase in tolerance to BMA as shown by both cell viability counts and the ATP assay, with no significant change in viable count at BMA concentrations up to and including 1.6 mM, although a decline in cellular ATP concentration was observed from a BMA concentration of 1.4 mM (Fig. 7.2). An LD₅₀ between 1.8-2.0 mM BMA (Fig. 7.2), a 29% increase over cells carrying the pBAD empty vector, was observed both in viability counts and the ATP assay. A strong correlation ($r=0.85$, $p=0.001$) between cell viability and the ATP assay. A strong correlation ($r=0.85$, $p=0.001$) between cell viability and cellular ATP concentration was observed (Fig. 7.2).

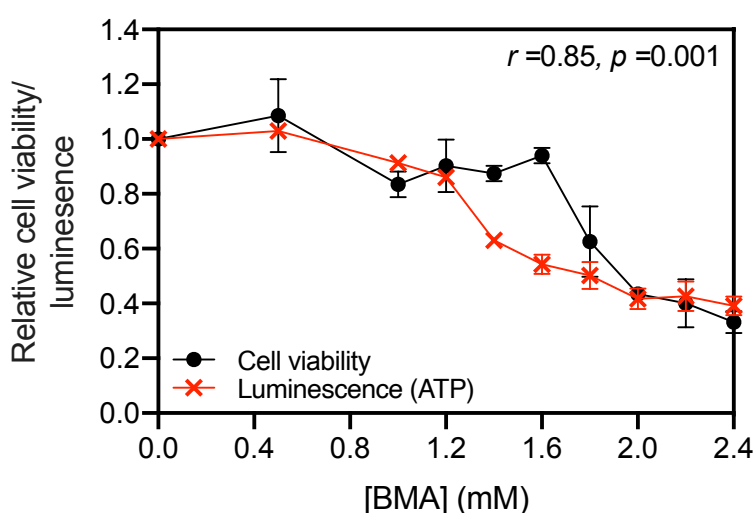


Figure 7.2. Measuring the effect of *acrAB-toI/C* expression on cell viability and cellular ATP concentration in cells treated with BMA. *E. coli* C43 Δ *acrAB* cells carrying pBAD-*acrABtoI/CHis₈* were prepared to the same starting count as described in Section 2.5.1 then energised by addition of 22 mM D-glucose. BMA was added at a range of concentrations to start the assay. Samples were taken after one hour and both the cell viability and ATP assays performed as described in Sections 2.5.1 and 2.5.2, respectively. All data are normalised to an untreated control and represent the mean of three biological replicates. Error bars show standard deviation and the Pearson correlation coefficient (r) is also shown.

7.2.2 Measuring the effect of BMA on cell viability and cellular ATP concentration over time

To characterise further BMA toxicity and its rate of entry into the cell, timecourse assays were performed in which measurements of cell viability and cellular ATP concentration were performed over the BMA exposure period. Cells carrying the pBAD empty vector suffered a 48% (± 15 SD) decrease in cell viability after just one minute of exposure to BMA (Fig. 7.3; Table 7.1). After ten minutes, cell viability had decreased by 85% (± 3 SD), then steadily decreased to a final reduction of 94% (± 2 SD) after 60 minutes (Fig. 7.3; Table 7.1). A strong correlation ($r = 0.92$, $p = 0.0007$) between viability counts and cellular ATP concentration was observed (Fig. 7.3).

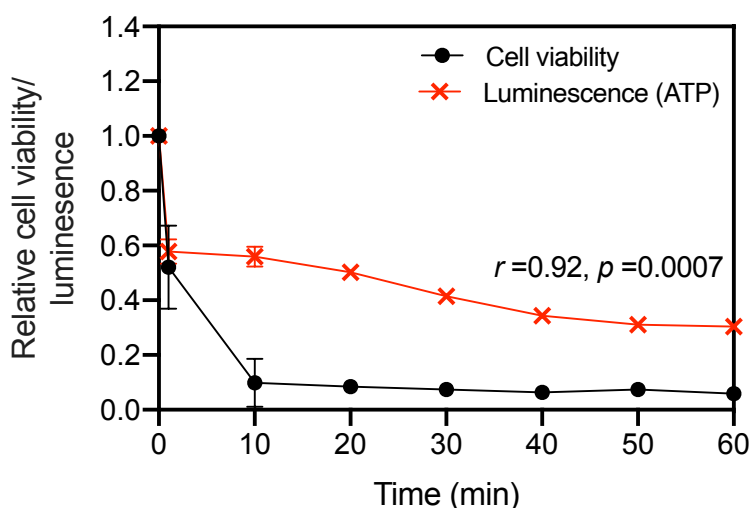


Figure 7.3. Measuring the effect of BMA on cell viability and cellular ATP concentration over time. *E. coli* C43 Δ *acrAB* cells carrying the pBAD empty vector were prepared to the same starting count as described in Section 2.5.1 then energised by addition of 22 mM D-glucose. BMA was added to a final concentration of 2 mM to start the assay. Samples were taken at desired time points and both the cell viability and ATP assays performed as described in Sections 2.5.1 and 2.5.2, respectively. All data are normalised to an untreated control and represent the mean of three biological replicates. Error bars show standard deviation and the Pearson correlation coefficient (r) is also shown.

Cells expressing *acrABtoIC* from pBAD-*acrABtoIC*His₈ were protected from BMA stress, with a mean 14% (± 10 SD) reduction in cell viability after one minute of exposure to 2 mM BMA (Fig. 7.4; Table 7.1). After ten minutes, cell viability had decreased by 48% (± 6 SD) and then remained stable, with a final reduction of 54% (± 7 SD) (Fig. 7.4; Table 7.1). The ATP assay reflected this change in viability and a very strong correlation was observed between the two data sets ($r=0.99$, $p < 0.0001$).

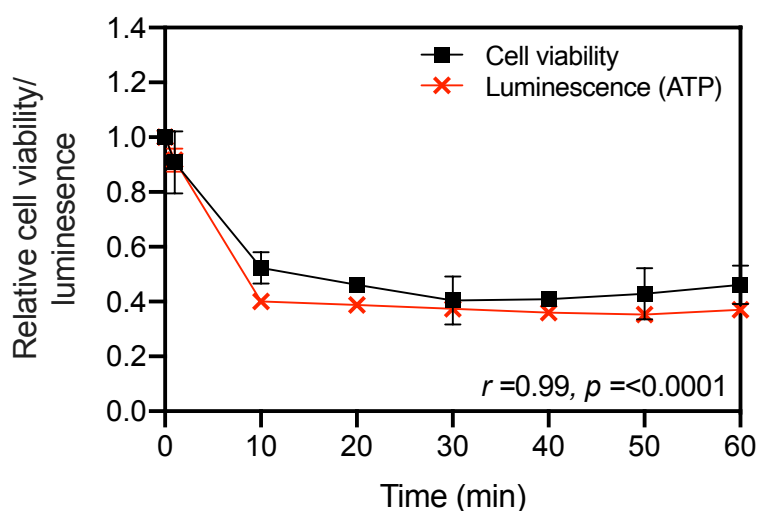


Figure 7.4. Measuring the effect of *acrAB-toIC* expression on cell viability and cellular ATP concentration in cells treated with BMA over time. *E. coli* C43 Δ *acrAB* cells carrying pBAD-*acrABtoIC*His₈ were prepared to the same starting count as described in Section 2.5.1 then energised by addition of 22 mM D-glucose. BMA was added to a final concentration of 2 mM to start the assay. Samples were taken at desired time points and both the cell viability and ATP assays performed as described in Sections 2.5.1 and 2.5.2, respectively. All data are normalised to an untreated control and represent the mean of three biological replicates. Error bars show standard deviation and the Pearson correlation coefficient (r) is also shown.

MexCD-OprJ was also able to provide significant protection against 2 mM BMA (Fig. 7.5; Table 7.1), although to a lesser extent than AcrAB-TolC (Fig. 7.4; Table 7.1), consistent with previous viability assays (see Chapter Six). After ten minutes of exposure to 2 mM BMA, cell viability had decreased by 68% (± 3 SD) but then remained

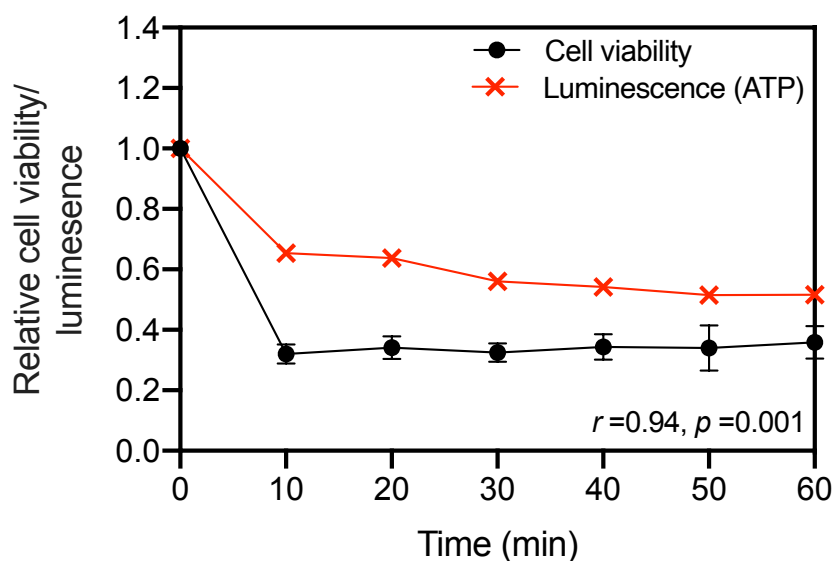


Figure 7.5. Measuring the effect of *mexCDoprJ* expression on cell viability and cellular ATP concentration in cells treated with BMA over time. *E. coli* C43 Δ *acrAB* cells carrying *pBAD-mexCDoprJHis₈* were prepared to the same starting count as described in Section 2.5.1 then energised by addition of 22 mM D-glucose. BMA was added to a final concentration of 2 mM to start the assay. Samples were taken at desired time points and both the cell viability and ATP assays performed as described in Sections 2.5.1 and 2.5.2, respectively. All data are normalised to an untreated control and represent the mean of three biological replicates. Error bars show standard deviation and the Pearson correlation coefficient (r) is also shown.

Table 7.1. The effect of efflux pump expression on the reduction in cell viability over time of cells treated with 2 mM BMA. Values show the percentage reduction in cell viability $\pm$ standard deviation and are means of three biological replicates. Samples with a statistically significant (unpaired t-test) increase in viability compared to Δ *acrAB* cells are indicated as follows: * $p \leq 0.05$, ** $p \leq 0.01$. NT – not tested.

Efflux pump	Elapsed time (min)		
	1	10	60
Δ <i>acrAB</i>	48 $\pm$ 15	85 $\pm$ 3	94 $\pm$ 2
AcrAB-TolC	14 $\pm$ 10*	48 $\pm$ 6**	54 $\pm$ 7 **
MexCD-OprJ	NT	68 $\pm$ 3*	64 $\pm$ 5 **

stable, with a final mean reduction of 64% (± 5 SD) after 60 minutes (Fig. 7.5; Table 7.1). A one minute time point was not sampled for this system owing to experimental error. Once again, a strong correlation between viability counts and the ATP assay was observed ($r=0.94$, $p=0.001$).

7.3 Discussion

Initial dose-response experiments demonstrated that expression of *acrABtolC* was successful in enhancing tolerance to external BMA addition (Fig. 7.1; Fig. 7.2), consistent with previous viability assays (see Chapter Six). In time course experiments, Δ *acrAB* cells demonstrated a dramatic 47% (± 15 SD) decrease in cell viability after just one minute of exposure to BMA but were protected by expression of either *acrABtolC* or *mexCDoprJ* (Fig. 7.4; Fig. 7.5; Table 7.1). As discussed previously (see Section 6.3), solvent toxicity appears to arise from rapid partitioning into the hydrophobic environment of biological membranes where high local concentrations accumulate, disrupting membrane structure and key membrane-associated biological processes (Sikkema *et al.*, 1995; Ramos *et al.*, 2002; Eberlein *et al.*, 2018; Dyrda *et al.*, 2019). This is supported by small-angle x-ray scattering experiments performed at The University of Nottingham that demonstrated the accumulation of BMA between lipid tail groups in model lipid systems (Yeh, unpublished), while there is currently no evidence to suggest that BMA is able to enter the cytoplasm when added to the external medium. These new data suggest that BMA enters the periplasm/membranes freely and rapidly and that expression of RND pumps is an effective means of expulsion, with both AcrAB-TolC and MexCD-OprJ conferring significantly enhanced viability and ATP content throughout these experiments (Fig.

7.2; Fig. 7.4; Fig. 7.5; Table 7.1). In a large-scale industrial process, continuous product extraction using a two-phase system could be used to prevent cyclical re-entry of BMA into the membranes/periplasm, minimising toxic effects while also simplifying downstream processing (Malinowski, 2001; Luo and Lee, 2017).

Interestingly, in both dose-response and time course assays the observed reduction in colony forming units (CFU) was greater than the reduction in cellular ATP concentration in cells carrying the pBAD empty vector or pBAD-*mexCDoprJHis₈* (Fig. 7.1; Fig. 7.3; Fig. 7.5). This could potentially indicate induction of cells into a viable but nonculturable (VBNC) state, which has been widely reported in *E. coli* populations exposed to antimicrobial compounds (Pinto *et al.*, 2015; Bamford *et al.*, 2017; Robben *et al.*, 2018; Kumar and Ghosh, 2019) and hydrophobic agents (Robben *et al.*, 2018). The VBNC state is a stress response by which cells reduce their metabolic activity and enter a dormant, non-replicative state in order to evade stressors. VBNC state cells cannot be cultured by typical techniques without resuscitation, although essential cellular processes, such as ATP synthesis, are maintained. This has led to the adoption of ATP assays in quantifying the proportion of VBNC state cells in microbial populations (Lindback *et al.*, 2010; Robben *et al.*, 2018; Kumar and Ghosh, 2019; Robben *et al.*, 2019). The discrepancy between CFU counts and cellular ATP concentration was not observed in cells expressing *acrABtolC*, which had the greatest protective effect against BMA, could suggest that the protection provided by AcrAB-TolC is sufficient to mitigate cell stress and prevent induction of the VBNC state when exposed to up to 2.4 mM BMA (Fig. 7.2). Cells lacking the AcrAB-TolC pump experience greater stress and thus may be more likely to enter a dormant state. Further work is required to confirm if cells are indeed entering and are able to remain

productive in a VBNC state, but this is an important consideration when scaling up the BMA production process.

Alternatively, this discrepancy between the drop in CFU and cellular ATP concentration may be a result of the metabolic cost of RND efflux. Since both ATP synthase and RND efflux pumps catalyse transport of protons from the periplasm to the cytoplasm in order to function (Zhang *et al.*, 2017; Guo *et al.*, 2019), it is possible that continuous transport of BMA by AcrAB-TolC causes an accumulation of protons in the cytoplasm. This would lead to an acidification of the cytoplasm and dissipation of the PMF, reducing the rate of ATP synthesis and may explain the lower cellular ATP concentrations observed in the presence of AcrAB-TolC (Fig. 7.2; Fig. 7.4). Acidification of the cytoplasm has previously been reported in *Pseudomonas aeruginosa* strains overproducing RND efflux pumps and was accompanied by an increase in oxygen consumption, as well as up-regulation of the *cbb3* terminal oxidase gene (Olivares *et al.*, 2014; Olivares-Pacheco *et al.*, 2017). Similarly, work performed in *P. aeruginosa* at The University of Strathclyde has suggested that co-expression of the endogenous *cbb3* gene along with an RND efflux pump results in higher levels of tolerance to BMA than when the RND pump is expressed alone (El Bestawy, unpublished). Cbb3 is an integral membrane protein and a terminal oxidase of the respiratory chain, reducing oxygen to water and using the free energy generated to pump protons from the cytoplasm to the periplasm (Huang *et al.*, 2010). This pumping of protons in the opposite direction to an RND efflux pump could help maintain the PMF, fuelling further transport of BMA and preserving ATP synthesis. These studies suggest that increased oxygen supply, such as that provided by the superior agitation and aeration of an industrial scale reactor, could be sufficient to alleviate the fitness costs of RND pump expression (Olivares *et al.*, 2014; Olivares-Pacheco *et al.*, 2017).

Further experiments using an oxygen electrode to investigate oxygen consumption of *E. coli* expressing RND pumps could help shed light on this and develop an understanding of the fitness costs of RND pump expression.

However, a strong correlation between cell viability and cellular ATP content was observed throughout all experiments, consistent with previous reports (Tenhami et al., 2001; Kramer et al., 2008; Hammes et al., 2010; Turner et al., 2010; Finger et al., 2012; Papon et al., 2014; Vocat et al., 2015). These data suggest that ATP assays are a suitable surrogate for traditional culture-based measurements of cell viability, which are resource- and labour-intensive and therefore unfavourable in a large-scale production process where extensive sampling is required. Further experiments are required to explore the effect of other efflux pumps, esters and ester concentrations in addition to further time-resolved experiments. Utilisation of the ATP assay described here in the BMA production strain may help further our understanding of the effects of BMA accumulation and export in the relevant biological context.

Chapter 8

Conclusions and future work

8.1 Conclusions

8.1.1 Cloning and expression of genes encoding bacterial efflux pumps

Bacterial efflux pumps of interest were identified using literature searches to focus on those known to play a role in tolerance to organic solvents and other hydrophobic agents (Table 3.1). Research performed at the University of Nottingham previously identified up-regulation of genes encoding the AcrAB-TolC pump in response to BMA stress in *E. coli* (Martins, unpublished), while *P. aeruginosa* transposon mutant libraries generated at the University of Strathclyde demonstrated that disruption of the *mexABoprM* or *mexCDoprJ* genes dramatically reduced tolerance to BMA (El Bestawy, unpublished). These three efflux pumps therefore became the main focus of the work presented here. Genes encoding bacterial efflux pumps belonging to the RND, MFS, and ABC families were successfully amplified by PCR from genomic DNA and assembled into pBAD and pGNTK expression vectors. Colony PCR was used to identify positive clones, which were then confirmed to be error-free using Sanger Sequencing. By using the water lysis method of membrane isolation combined with SDS-PAGE and Western blotting to detect the inserted octahistidine tag, expression tests performed in *E. coli* BL21 and/or *E. coli* C43 Δ *acrAB* host strains showed that all cloned efflux pump genes were successfully expressed and protein localised to the membranes.

8.1.2 Characterisation of Acel and its homologues

As the most recently described family of bacterial efflux pumps, the proteobacterial antimicrobial compound efflux (PACE) family is still poorly characterised. Previous work had suggested that polyamines, which have a number of physiological functions, are the natural substrates of these efflux pumps. In the experiments presented here,

cells expressing *aceI* did not accumulate ^{14}C -labelled cadaverine to significant levels, while cells expressing the E15Q mutant or no additional efflux pump gene continued to accumulate ^{14}C -labelled cadaverine over the course of the assay. Similarly, eight PACE homologues from a range of bacterial species were screened for their ability to transport cadaverine, three of which also prevented accumulation of ^{14}C -labelled cadaverine, suggesting that this function is conserved to an extent across the PACE family and polyamines are indeed the physiological substrates of these efflux pumps. The AceI protein was purified and reconstituted into a proteoliposome system in which a cadaverine-dependent acidification of the proteoliposome lumen was observed. This combined with the observation that neutralisation of the conserved glutamate residue at position 15 abolishes transport activity suggests that AceI transports cadaverine in exchange for H^+ ions and is therefore a substrate/proton antiporter. AceI served as a valuable model system for the study of bacterial efflux pumps and is also relevant to the field of biotechnology as cadaverine is a monomer for production of industrial polyamides.

8.1.3 Characterisation of bacterial efflux pumps using the fluorescent dye Nile Red

Initial experiments intended to test the compatibility of Nile Red and BMA demonstrated an unexpected use of this fluorescent dye. When BMA was added above its solubility limit to a Nile Red solution, rapid partitioning of the lipophilic Nile Red into the organic phase and a dramatic increase in fluorescence signal was observed. Nile Red can therefore be used to test solubility of organic esters, to confirm phase separation of product in lab scale fermentations and potentially to determine where in the cell BMA accumulates upon external addition or internal production.

The optimised Nile Red efflux assay proved useful in confirming that RND efflux pumps cloned and expressed in *E. coli* laboratory strains remained functional, with activity observed for AcrAB-TolC, MexAB-OprM and MexCD-OprJ when expressed using either a p_{BAD} or p_{gntK} promoter system. Use of the *E. coli* C43 $\Delta acrAB$ host strain was necessary to eliminate the extremely high background efflux activity of the native AcrAB-TolC efflux pump; therefore this strain was used for all further experiments. While initial assays appeared to show competitive transport of BMA and Nile Red via the AcrAB-TolC efflux pump, this was later found to be a result of the additive effects of toxic levels of protein overproduction and BMA toxicity and was abolished by reducing gene expression levels. Unfortunately, measurement of competitive transport could not be achieved. These experiments did however lead to the development of a new *acrAB-tolC* expression vector that proved more appropriate for assays of BMA tolerance and highlighted the importance of appropriate expression levels.

No Nile Red efflux was observed in cells expressing any of the non-RND pumps, strengthening the hypothesis that RND efflux pumps recruit hydrophobic substrates from the periplasm, while non-RND pumps generally export cytoplasmic substrates. This is relevant in the production strain, where internal production of BMA may necessitate expression of a non-RND transporter to capture BMA from the cytoplasm.

8.1.4 Assessing the effect of isobutyrate and methacrylate esters on cell viability

Traditional culture-based viability assays revealed much about the toxicity of BMA and other organic esters. These assays clearly demonstrated the toxicity of overproduction of membrane proteins, with cells overproducing the TolC protein

demonstrating hypersusceptibility to BMA. Reducing the level of *tolC* expression was successful in reversing this phenotype and the AcrAB-TolC efflux pump conferred a 30-fold (± 7 SD) increase in cell viability when exposed to 2 mM BMA, followed by MexAB-OprM and MexCD-OprJ conferring 22-fold (± 1 SD) and 10-fold (± 4 SD) increases, respectively (Table 6.1). Each of the RND efflux pumps was found to confer significantly enhanced tolerance to all toxic esters (Table 6.1).

Viability assays also identified a number of esters with reduced toxicity compared to BMA that may make suitable alternative target products with a reduced toxicity limitation. Despite it being widely reported that $\log P_{ow}$ correlates closely with the toxicity of organic solvents, no such relationship was observed with the esters tested here. However, branched esters did appear to be significantly less toxic than unbranched esters, perhaps due to their increased steric bulk and therefore hindered penetration of membranes.

Expression of non-RND transporters often reduced tolerance to the organic esters, likely due to toxic levels of protein production as observed with TolC, suggesting that optimisation of expression levels is still needed. Unfortunately, the *p_{gntK}* promoter system intended to solve this issue had very poor reproducibility and led to anomalous cell counts, meaning appropriate expression levels will need to be tested and determined empirically for each efflux pump in future work. Of these non-RND transporters, EmrD and MdtM provided moderate protection against BMA, PIB, PMA and HMA, suggesting that these transporters either show specificity for these esters or that these esters are able to penetrate the inner membrane to reach the cytoplasm, where these pumps can capture them. Regardless, these assays highlight the importance of testing the effect of efflux pump expression in the BMA production

strain, as internal production is likely to have very different effects on the cell and will allow the non-RND pumps to be tested in a more biologically relevant context.

8.1.5 Measuring cell viability and the metabolic burden of RND efflux pumps using a luciferase-based ATP assay

Measurement of cellular ATP concentration was explored as an alternative to resource- and labour-intensive culture-based viability measurements. Dose-response assays showed that expression of either *acrAB-tolC* or *mexCD-oprJ* was effective in significantly increasing the LD₅₀ of BMA. Time course assays showed that BMA toxicity occurs quickly resulting in dramatic loss of viability after just one minute of exposure, likely due to rapid partitioning of BMA into biological membranes. However, expression of either *acrAB-tolC* or *mexCD-oprJ* significantly slowed the loss of viability and dramatically reduced the overall reduction in cell viability and ATP content.

Interestingly, cells not expressing RND pump genes showed consistently higher cellular ATP concentrations relative to their viable count than those expressing pump genes. This could indicate the metabolic burden of expression and continuous function of RND pumps, which derive their energy from the proton-motive force and thus could lead to reduced ATP synthase function, or could suggest that cells lacking the protective effect of RND efflux may enter a viable but non-culturable state as a stress response. Throughout all experiments, a very strong correlation between cell viability and cellular ATP concentration was observed, suggesting that measurement of ATP may be a suitable time and cost saving alternative to culture-based techniques.

8.2 Future work

8.2.1 ATP assays

Unfortunately, due to COVID-19 laboratory closures, work on the ATP assays was not completed. Future experiments should focus on screening the effect of all of the cloned efflux pumps on the cellular response to organic ester stress using both dose-response and time course assays. It was clear from viability assays that expression of the inner membrane proteins was generally toxic to *E. coli* and resulted in increased sensitivity to a number of the organic esters tested. As the *p_{gntK}* promoter system proved unsuitable, further work should be performed to determine empirically appropriate expression levels for each gene before continuing with viability and ATP assays with these pumps. Further dose-response and time course experiments with a range of efflux pumps and esters should shed light on the cellular response to organic ester stress and the metabolic burden of bacterial efflux pumps in the production strain.

8.2.2 Determining the effect of efflux pumps on internal production of BMA

Until recently, changes in the production strain could only be characterised by running lab-scale fermentation experiments, which were resource-, time- and labour-intensive, as well as very low throughput. However, the recent development of an agar plate-based method allows visualisation of organic ester production with much greater throughput, while also being cheap, fast and requiring just basic microbiological skills. Cells are grown overnight on a filter membrane placed on LB agar, allowing all colonies to be quickly transferred to a biotransformation medium (Fig. 8.1). This biotransformation medium contains ketoisovalerate and butanol, which serve as precursors for BMA synthesis through the second half of the production pathway (see

Section 1.5.3), as well as a photoactivated fluorescent probe. Following a one hour BMA production period on this biotransformation medium, the membrane on which the colonies have grown is once again removed and the medium is exposed to UV light to activate the probe, which then forms a highly fluorescent adduct with BMA (Fig. 8.1).

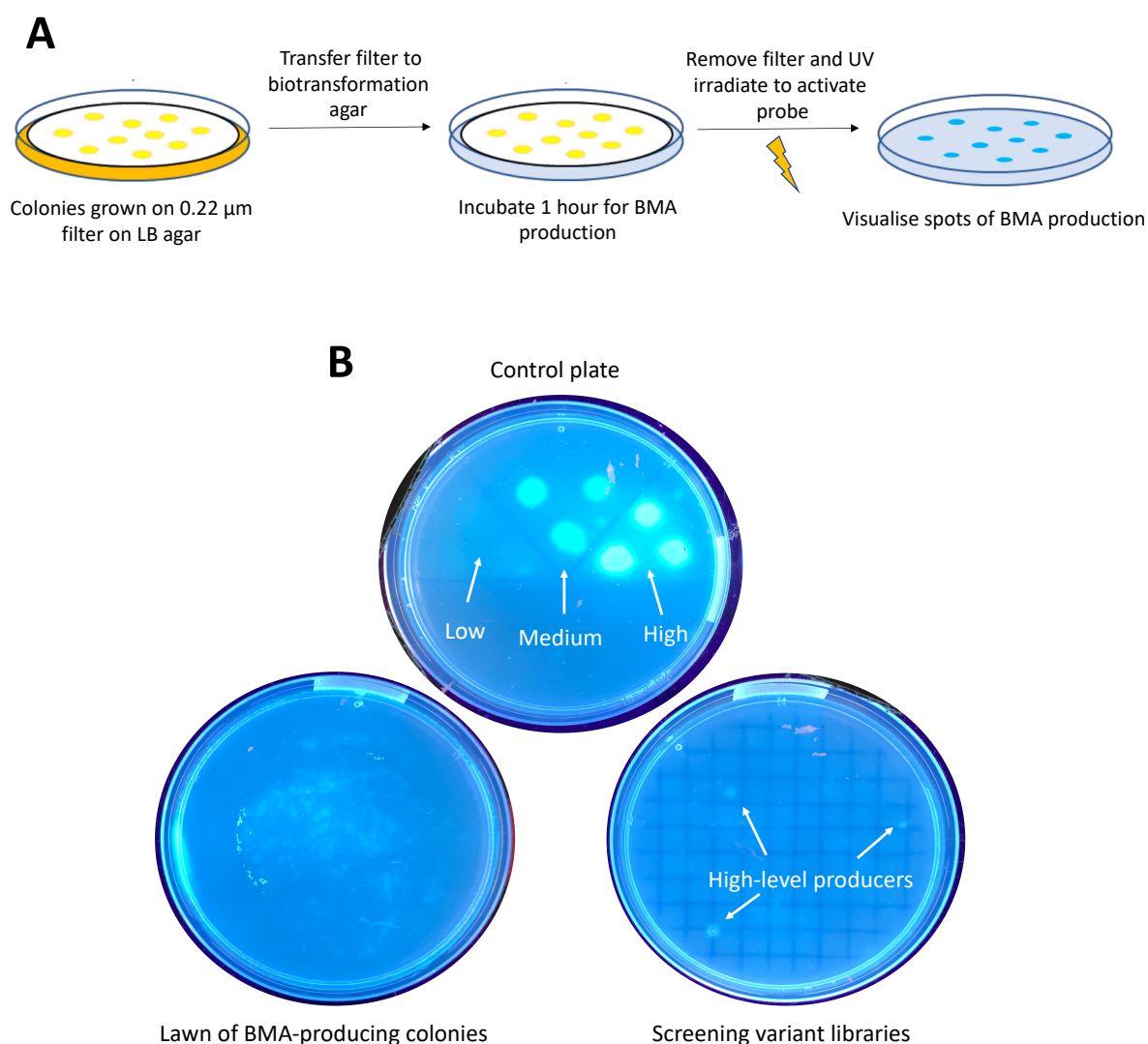


Figure 8.1. Plate-based method for quantifying BMA production. Cells are first grown on LB agar overnight on top of a 0.22 μ m filter to allow for simple transfer of colonies, then transferred onto a biotransformation medium containing BMA precursors and a photoactivated fluorescent probe. Following a one hour production period, the membrane is removed and the probe activated using UV light. The probe then reacts with BMA to form a highly fluorescent adduct that can be visualised under UV light. An overview of the protocol is shown (A) as well as example control plates showing low, mid and high-level producers, a plate with a lawn of BMA-producing colonies and a plate used to screen variant libraries for high-producing individual colonies (B).

This allows colonies with increased BMA production to be identified and then picked from the membrane for further characterisation. It is hoped that by expressing both the production pathway and an efflux pump of interest in *E. coli*, this plate-based assay can be used to identify those pumps that are able to export BMA, therefore resulting in greater extracellular yields. Strains carrying both the production pathway and each of the cloned efflux pumps have already been constructed, although COVID-19 laboratory closures have prevented this work. These experiments would therefore be a top priority in the future in order to characterise the effect of efflux pump expression in the production strain, which is particularly important for the non-RND transporters. As discussed previously, transport of internally produced BMA may require an inner membrane efflux pump to transport from the cytoplasm to the periplasm, followed by an RND pump for full extrusion from the cell. As such, following optimisation of expression, non-RND pumps should be screened both alone and in combination with an RND pump using this method to determine if there is a synergistic effect on BMA tolerance and therefore production.

8.2.3 Ligand binding assays

Assays of ligand binding to purified protein can be valuable in determining the affinity of transport proteins for a substrate and in further characterising protein-ligand interactions. As such, both AcrA and AcrB have been successfully purified for future study as the interaction between these proteins appears to play a vital role in ligand binding and protein function (Zgurskaya and Nikaido, 1999) (Fig. 8.2).

Intrinsic tryptophan fluorescence of proteins can be exploited to identify conformational changes in proteins induced by ligand binding. Tryptophan residues are selectively excited using a wavelength of 295 nm and addition of a ligand to which

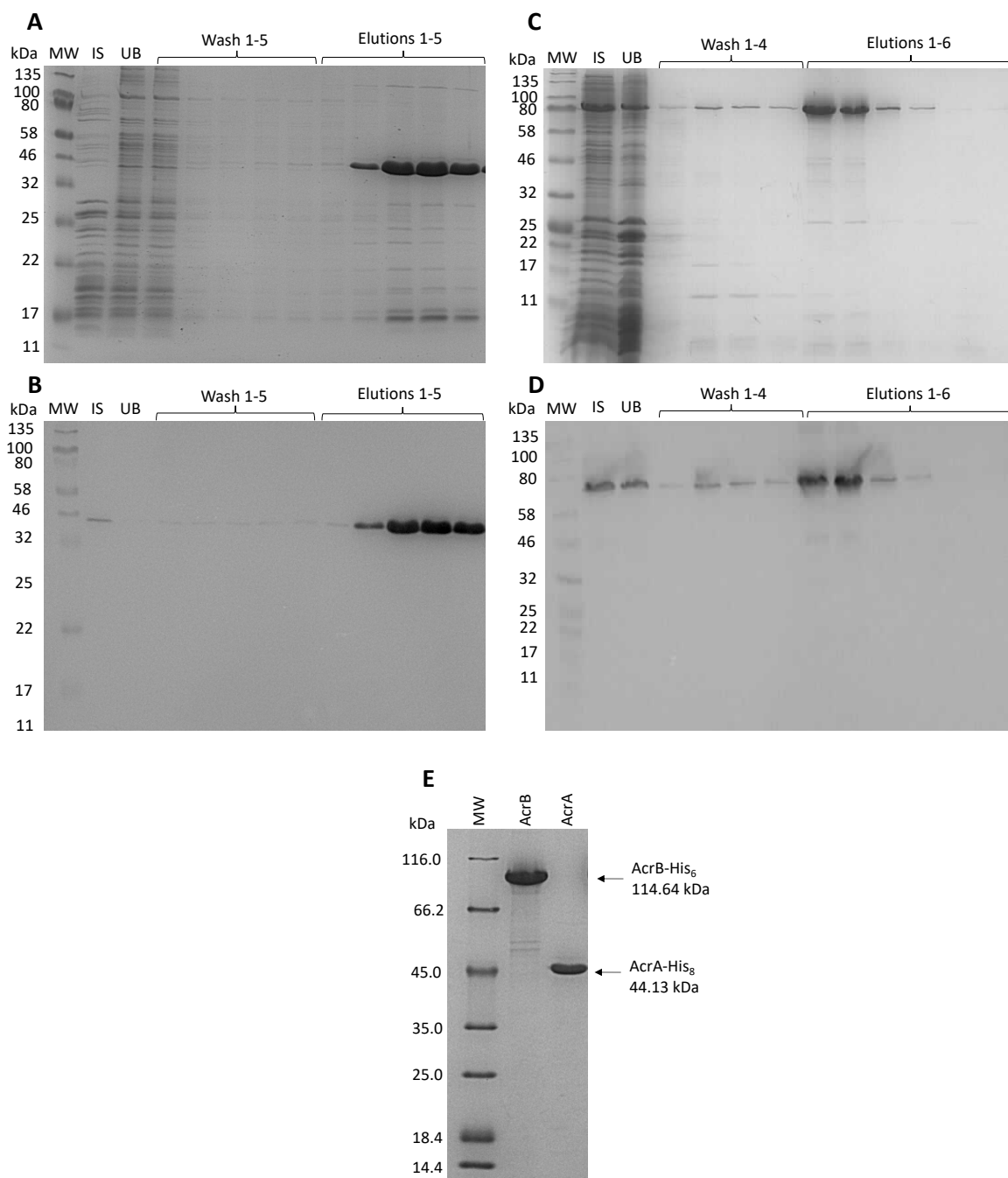


Figure 8.3 Purification of AcrA and AcrB. SDS-PAGE (A) and Western blot (B) of AcrA purification and SDS-PAGE (C) and Western blot (D) of AcrB purification. SDS-PAGE analysis of the final concentrated proteins is shown in (E). Membranes were prepared as described in Section 2.6.3 from cells carrying the pBAD-*acrA*His₈ or pET24a*acrB*His₆ plasmid, which were grown and harvested as described in Section 2.2.6. SDS-PAGE and Western blotting analysis were performed as described in Section 2.6.1 using HisProbe-HRP to identify His-tagged proteins. Purification procedures are described in Section 2.6.6.2. Lanes designated 'MW' were loaded with a molecular weight marker and purification gels show the insoluble (IS) and unbound (UB) fractions. Protein molecular weights shown were calculated from the relevant protein sequence using Benchling Biology Software.

the purified protein of interest binds will induce conformational changes, resulting in changes in the local environment of those tryptophan residues and therefore a fluorescence change (Vivian and Callis, 2001; Akbar *et al.*, 2016). Titration of organic esters could therefore be used to characterise transporter binding kinetics and understand the affinity of different efflux pumps for these substrates.

Another fluorescence-based assay uses the fluorescent dye 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM). By subjecting the protein of interest to increasing temperatures, the protein denatures to reveal buried cysteine residues with which the CPM dye reacts resulting in increased fluorescence (Alexandrov *et al.*, 2008). These data can be used to determine a melting temperature (T_m) of the protein. In the presence of a binding ligand however, proteins are generally stabilised and will therefore have an increased T_m . This assay has however been the subject of debate as it yields a very narrow range of melting temperatures for diverse proteins that are not consistent with T_m s determined by alternative methods (Wang *et al.*, 2015). This is thought to result from nonspecific interaction of the CPM dye with hydrophobic regions of proteins, raising questions around its use in the study of membrane proteins. This assay was tested with purified AcrAB, as described in Section 2.7.1, but addition of BMA up to a concentration of 3 mM appeared to result in a moderate destabilising effect (data not shown), perhaps due to the interaction of BMA with the hydrophobic DDM micelle core causing destabilisation of the solubilised protein. Further work is needed to determine if this assay is suitable for characterising binding of BMA to detergent-solubilised membrane transport proteins, but its ease of use and use of standard laboratory equipment makes it an appealing method.

Alternatively, circular dichroism (CD) monitors the differential absorption of left and right circularly polarised light to reveal information about the secondary structure

of a protein (Siligardi *et al.*, 2014). For example, an inner membrane transporter is primarily alpha helical, which will yield a characteristic CD trace. By performing a temperature ramp on the protein of interest, the loss of secondary structure can be monitored in real time and used to determine a protein melting temperature. As mentioned previously, the presence of a binding ligand generally has a stabilising effect on proteins and therefore binding of a substrate, such as BMA, should result in an increased T_m .

8.2.4 Structural studies of efflux pumps

The efficacy of AcrAB-TolC in enhancing tolerance to BMA and other organic esters has also led to structural investigation of the interaction between AcrB and BMA. Cryogenic electron microscopy (cryo-EM) carried out at The University of Leeds by the Meunch group used AcrB purified in styrene-maleic acid copolymer (SMA) in the presence of butyl 2-(bromomethyl)acrylate in the hope of identifying a BMA binding site. In the presence of this brominated BMA analogue, non-structural density was observed in the periplasmic binding pocket of AcrB that is not present in the published AcrB structure (Fig. 8.3). Initial *in silico* docking experiments suggest that this density is significantly larger than the BMA analogue but this could be due to movement of the substrate between multiple binding sites within the same substrate binding pocket, which has been reported for AcrB substrates previously (Nakashima *et al.*, 2011). Regardless, further rounds of purification and data acquisition are required in order to increase the resolution of the cryo-EM images and hopefully identify a BMA binding site.

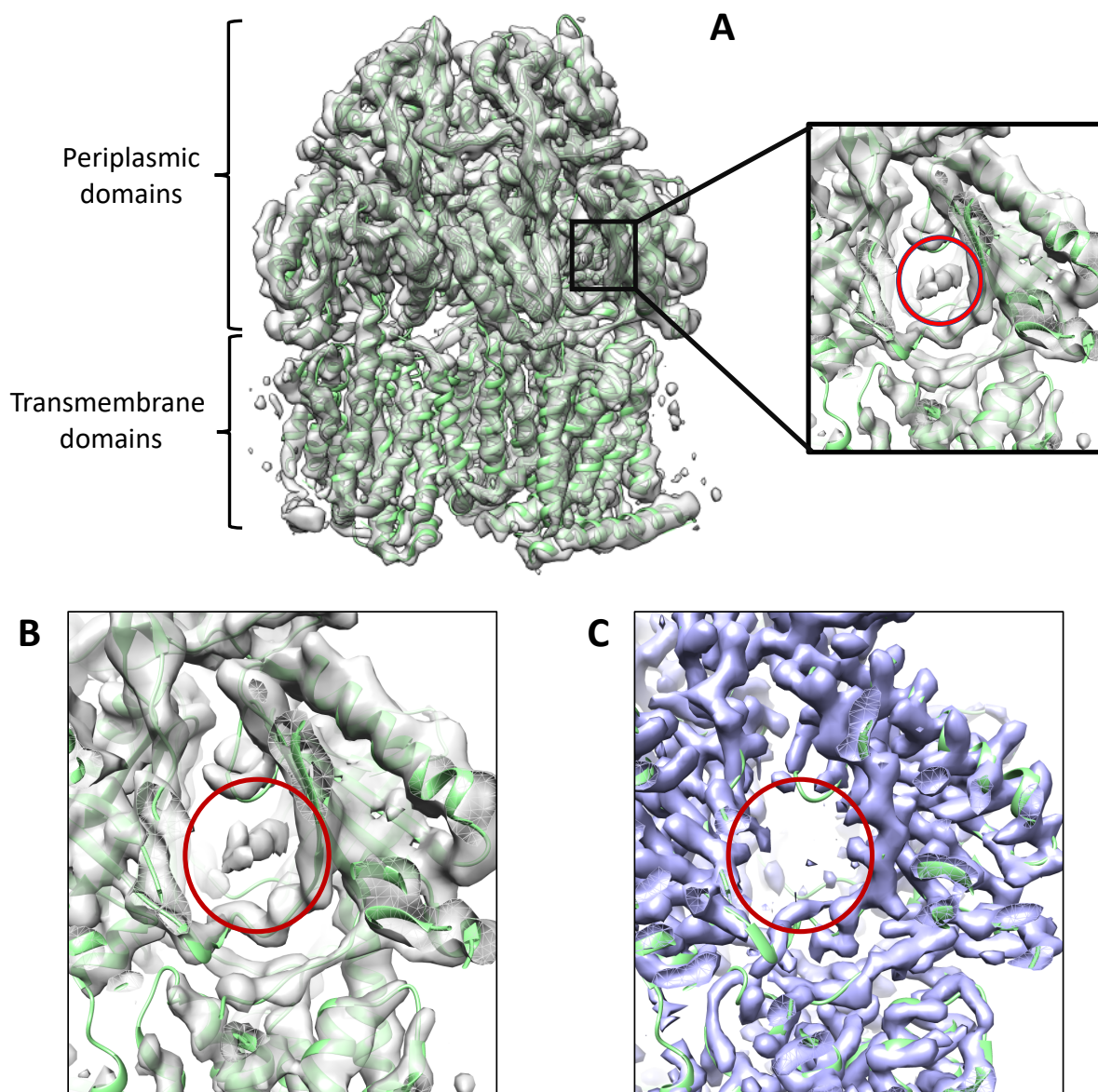


Figure 8.3. Identification of non-structural density in the AcrB periplasmic binding pocket. View of the AcrB functional trimer in the presence of butyl 2-(bromomethyl)acrylate (A) and a detailed view of the non-structural density observed in the substrate binding pocket at an average resolution of 4.6 Å (B), compared to the same site in the published AcrB structure (no substrate bound) at an average resolution of 3.2 Å (C) (PDB code 6BAJ, Qiu *et al.*, 2018). A red circle marks the location of the non-structural density. Structures are aligned with the AcrB crystal structure, shown in green (PDB code 4C48, Du *et al.*, 2014). Data analysed by Rachel Johnson, University of Leeds, Leeds, UK (2019).

8.2.5 Directed mutagenesis

Successful identification of a BMA binding site would also allow for directed mutagenesis of AcrB to enhance transport of BMA and other organic esters. Mutagenesis has previously been used to generate AcrB variants with up to 47% and 400% improved efflux of n-octane and α -pinene, respectively (Foo and Leong, 2013), and *E. coli* strains tolerating 25% higher exogenous n-butanol concentrations than those expressing wild-type AcrB (Fisher et al., 2014). Mutagenesis could therefore be used to enhance transport of BMA while also minimising unwanted transport of pathway intermediates.

8.2.6 Measuring accumulation of radiolabelled BMA

Measurements of the transport of radiolabelled substrates are extremely valuable, as demonstrated by the assays performed in this study on the PACE family proteins (see Chapter Four). Rather than monitoring the movement of a surrogate fluorescent substrate, transport of any given compound can be measured directly if it is synthesised with a radiolabel, commonly ^{14}C or ^3H . Although ^{14}C -BMA has been synthesised for these purposes, BMA is volatile, adsorbs to filter membranes, dissolves common laboratory plastics and partitions readily into cell membranes, making measurements of uptake/efflux with this radiolabelled substrate incredibly difficult. Further work should focus on optimising assays of ^{14}C -labelled BMA transport as, although much development is required, these assays would be invaluable in developing our understanding of BMA transport.

8.2.7 Cloning more genes encoding bacterial efflux pumps

Although this work largely focused on three systems, AcrAB-TolC, MexAB-OprM and MexCD-OprJ, there are a number of naturally solvent tolerant and/or solventogenic bacteria such as *Pseudomonas putida* and *Alkanivorax borkumensis* that represent a rich pool of promising solvent efflux pumps that is as yet unexplored (see Table 3.1). For example, solvent tolerance in *P. putida* has previously been attributed to a number of RND efflux systems, including TtgABC, TtgDEF, TtgGHI and SrpABC (Rojas et al., 2001; Kieboom et al., 1998), while *A. borkumensis* possesses a range of RND efflux systems of the hydrophobe/amphiphile efflux-1 (HAE-1) family (Dunlop et al., 2011). We have shown here that heterologous RND efflux pumps cannot only be cloned and expressed in *E. coli*, but also retain their efflux activity and can indeed confer enhanced tolerance to BMA. It is therefore worthwhile exploring a more diverse selection of efflux pumps from a broader range of bacterial species in the hope of identifying other pumps that are effective in exporting BMA and other organic esters from the cell.

8.3 The future of sustainable polymers

While producing plastics and other polymers from renewable feedstocks provides obvious benefits such as improved sustainability and a reduced environmental impact, as well as economic benefits by reducing reliance on the volatile crude oil market, plastic pollution remains a major global issue. It is estimated that eight million tonnes of plastic waste enter the oceans each year, where they cause severe damage to ecosystems and both disrupt and contaminate food chains that we rely on (UKRI, 2020). While recycling of plastics has become commonplace, current recycling methods require large inputs of energy and virgin polymer, while also yielding

materials of lower quality and value (Hopewell et al., 2009). Similarly, many biodegradable polymer alternatives are of lower quality than traditional polymers and struggle to match the characteristics required of these materials, such as optical clarity and mechanical strength (Reddy et al., 2003; Thakur et al., 2018; El-malek et al., 2020). The cost of producing 'biopolymers' remains relatively high compared to traditional materials due to high research and development costs, small scale of production and higher product recovery costs (Thakur et al., 2018; El-malek et al., 2020). It is therefore important that development of these technologies continues and that these approaches be used together in order to improve the sustainability of the plastics industry.

Recent efforts have focused on the use of cheaper alternative feedstocks, such as agricultural and food waste, in the hopes of reducing the production costs of biological and biodegradable polymers (Ten and Vermerris, 2013; Isikgor and Becer, 2015; Tsang et al., 2019). Another approach has been the investigation of novel degradation and recycling of traditional polymers, such as the discovery and engineering of enzymes responsible for the degradation of polyethylene terephthalate (PET) (Palm et al., 2019; Knott et al., 2020; Tournier et al., 2020) and even the discovery of waxworms that are able to consume and digest high density polyethylene (HDPE) (Yang et al., 2014; Bombelli et al., 2017; Kundungal et al., 2019). Each of these technologies face multiple hurdles before they can truly replace current manufacturing and waste management practices, but by combining these approaches to create and dispose of polymers in more sustainable, responsible ways, significant progress can be made in reducing the impact that polymers have on the planet.

8.4 Concluding remarks

Significant progress has been made in the characterisation of Acel and other members of the PACE family of bacterial membrane transport proteins. The work presented here adds to existing evidence that short chain diamines, such as cadaverine, are in fact the physiological substrates of the PACE family efflux pumps and that these proteins function as substrate/proton antiporters.

The major focus of this research was the identification and characterisation of bacterial efflux pumps able to confer enhanced tolerance to butyl methacrylate, an acrylic polymer precursor. Three RND efflux pumps, AcrAB-TolC of *E. coli* as well as MexAB-OprM and MexCD-OprJ of *P. aeruginosa*, were found to confer significantly enhanced tolerance to BMA and a range of other organic esters, demonstrating their potential applications in an ester production strain. Future work should focus on producing these efflux systems in the BMA production strain, both alone and in combination with non-RND transporters, to determine if these pumps increase production of BMA. The MFS transporters MdtM and EmrD conferred enhanced tolerance to a subset of the esters tested and may be a good starting point for future experiments. Increased production and therefore greater yields of BMA will result in a more economically viable, sustainable route to renewable acrylic monomers from simple sugars. This will ultimately contribute to reducing reliance on crude oil and its derivatives in this industry, thus dramatically reducing the environmental impact of the manufacture of acrylic polymers. This research therefore aligns with 'Kaiteki', a core philosophy of our sponsors at Mitsubishi Chemicals, which refers to 'the sustainable well-being of people, society and our planet Earth' (Mitsubishi Chemical Holdings Corporation, 2020).

Appendices

Appendix A - DNA sequences of cloned genes confirmed by Sanger sequencing

arcABHis₈-tol/CStrep

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121	cgtaagtctg	ccgccgatcg	tgatgtcgcc	tttgaaaaaa	ttaatgaagc	gcgcagttcca
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241	aacggcatca	actctaacgc	gaccagtgcg	tccttgtagt	taactcaatc	catttttgat
301	atgtcgaat	ggcgtgcgtt	aacgctgcag	gaaaaagcag	cagggattca	ggacgtcacg
361	tatcagaccg	atcagcaaac	cttgatcctc	aacaccgcga	ccgcttattt	caacgtgttg
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961	gttaactcgc	aggtgaaaca	ggcacagtac	aactttgtcg	gtgccagcga	gcaactggaa
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emrABtoIChis₈

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emrDHis₈

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mdlBHis₈

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*msbA*His₈

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1801 taa

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*mdtL*His₈

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mdtMHis8

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acrBHis6

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ace/His₈

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ace/E15QHis₈

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A1S_1503His₈

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Fbal_3166His₈

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Mlut_15630His₈

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241 tcgaccgtgg cgttactttg gaacattgtg tttactccg tgtttgaaac gctggagcgt
301 cgcttaggta tcacaggtcg tccgtggtgg gttcgcgtag cgcacgcggg cggttttgaa
361 ggtgggctga tcgttttctt ggtgccagct gtagcactga ttctgggcat tgggtgggc
421 gaagcctttc tgattgaagc cggtttgcgt gtgttctttc tgggtgtacg ggccgtgtat
481 gcttacgcct tcgattctgt atttggtctc ccgattccg ccgctcgcgc agaggcaacc

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541 tgcagagggtg gccgcggcag ccatcaccat catcaccatt aa

PFL_4558His₈

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1 atgaattctc agaaatccct taacgaacgc atcttccagg cggtaggttt tgaactgctg
61 gcgggtgctga tctgcacccc gctgctgtcc tggatcatgg acaagcccat ggccgacatg
121 ggccctggtga ccatcggcac cggcctgttg gccctggcct ggaacgtggt gttcaacggc
181 ctgttcgacc ggttgctcaa gcgcttgtag tgggtccaca acggttgac ccgggtcctg
241 catgcgttga tgttcgaagg cgggctggtg gcagtgggag taccgatgat tgcctgggtg
301 ctgaatatca gcctgctgca ggcgttcac ctggacatcg gcgtgctgct gttcttcctg
361 ccgtacacct atgtctatca ctgggtctac gacgtgctgc gggacaagtg gctgcaacag
421 cgccctggctc acgggtcgac ctgcagagggt ggccgcggca gccatcacca tcatcaccat
481 taa
```

PSPTO_3587His₈

```
1 atgaattcac agaaagactc actgaacatg acgcatcgcg aacaaccaac gcgccagcaa
61 ccgcccgtgg tccataagac aattcgcgaa cgcgcctgc acgcgactct gttcgaagtg
121 ggaggcggtta ttttagtggc accactctta gcttgatca tgaaccagtc gctggccatg
181 attgggatta tgacggcat gatcagcacc gtcgcatgc tgtggaacat ggtgtacaat
241 gcattgttcg accgtcttcg cagtcgcttt ggcttcgcta tgaccgcaat gaccgtatt
301 ctgcacgcca tcggttttga agccggcctc atcctggcgg tagtccatt ggccgctgg
361 tggctgtcaa tctccttaat gcaggcgctt tggcttgata ttggtctgtt actgatgttc
421 ttgccatata ccctgttgtt taactgggct tatgataatc ttcgcgaacg cctggttcag
481 cgtcgtatgg cgcgttgtag agtcttaacc tgcagagggt gccgcggcag ccatcaccat
541 catcaccatt aa
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STY3166His₈

```
1 atgaattcat ctaaggtttt tagcttcatg cagcacgacg ccattcagcg ccgttcgctg
61 ccggagcgca ttttccatgc cgtctgcttt gaggtattg caacagcgat cctggcggcc
121 accacggcat ggctgatgca acgctctgtt ctggagatgg gtggcctgac cattcttctc
181 gcgaccacg ccatgatctg gaatatcatt tacaatgctc tgttcgatcg cctctggccc
241 gctcatcagg tgcgccgtac agcaaaagtg cgcgcgttac atgcattagg attcaggtcc
301 gggtttatcg tcattggcgt ctgatcggt gcctgggttc tgaacgtatc cctcctgcaa
361 gccttcaccc tggaaattgg cttctttctg tttttctgc cttacactat gctttacaat
421 tgggcctacg acgttctgcg ccaacgcatt gttaccgcc gtcagcagcg tgtcagtgcc
481 acctgcagag gtggccgcgg cagccatcac catcatcacc attaa
```

TmargHis₈

```
1 atgaattcac gcaaccaagc ggtggagtct aaacaggacg gtgtcgcgct tcgtacctgg
61 cgtgaccgtg tccgccaggt gttagcgctc gaggcggcg ggctggtgtt gattaccctt
121 ccgttcagct gggcctctgg cgcgccgctc ctggattcgt tgggcctgct ggcacttctt
181 gctctgattg cggcactgtg gaacgcgttg tataacacg tgttcgaccg cctcgaggct
241 cgtttcctgc accgccgtgc agatctgcgt cctccgctgt ggcgtgtagc gcacgcactg
301 ggttttgagg gcgactgct tttccttacg ttgccggtga ttgtcgcgtg gaccggtatg
361 ggttggttgg aagcgttggg tgcgtatctg ggcctggcct tggcgatata cgtatacgcg
421 tacgttttca actggtgtta tgaccgttta tttccattg gccctaccgg cgaacagacc
481 tgcagagggt gccgcggcag ccatcaccat catcaccatt aa
```

VP1155His₈

```
1 atgaattcca acgagcgcat ttttcacgcg gtgttggttg agctgatggc gttggccatc
61 attgtaccgg cggccgcgct gattaccggc aaaggcagta gtgatctggc gttagtgggt
121 attgggctgt cactgtacac agtggtttgg aattacattt ataacctgta cttcgataag
181 tggttttgct cgaaccgcgc ggaccgtagc ttggcaatgc gtctgggtca tacggtcggc
241 ttcgaagggg gtctgatctt tatctccatc cccgtcatcg catggtttct ggaaatcacg
301 tttttgcgcg ccctgatgct ggaagcgggc ttcctggtct tctttctgtt ctacgcgacc
361 ggttttaact ggctgtatga taaagtacag ccattcggga agatgcgcaa actgctggtc
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421 acctgcagag gtggccgagg cagccatcac catcatcacc attaa

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