

Antibiotic resistance and *Acinetobacter*

By

Elizabeth Marie Darby

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ABSTRACT

Antimicrobial resistance is a huge global health burden, where bacterial infections are becoming more difficult to treat. In the England and Wales, bloodstream infections can be caused by a number of bacteria including either *A. baumannii* or *A. lwoffii*. This work is a comprehensive comparison between these two species of the *Acinetobacter* genus, focusing on their antibiotic susceptibility, resistance mechanisms, and ability to cause infection, where *A. baumannii* was more virulent both *in vitro* and *in vivo*, when compared with *A. lwoffii*. *A. lwoffii* was also more susceptible to antibiotic treatment and this was due to a lack of acquired resistance genes and a reduced capacity to develop resistance mutations. Efflux pumps, known to confer antibiotic resistance, were found to be fewer in *A. lwoffii* genomes compared to *A. baumannii*. Furthermore, *A. lwoffii* encoded a higher number of DNA defense systems, which, combined with a reduced efflux capacity, potentially explains the scarcity of antibiotic resistance genes in its genome and lack of resistant mutations.

This work also evaluated the *Acinetobacter* genus as a whole and found that infection-causing species often possessed a greater number of efflux pumps. AdeIJK was identified as a conserved feature across *Acinetobacter* species, indicating its significance in cellular functions and membrane modulation.

Therefore, this research shows that even two closely-related species that cause similar infections can have dramatically different susceptibility profiles to antibiotics and this is underpinned by a variety of mechanisms, including efflux. By understanding why

certain bacterial species rapidly develop antibiotic resistance and the mechanisms of this in comparison to susceptible organisms, will enable researchers to limit the development of antibiotic resistance and find novel antibiotics to treat infections and improve public health.

DEDICATION

I would like to dedicate this thesis to my parents, Michelle and Paul.

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Firstly, I would like to thank my Mum, Michelle, and Dad, Paul. Thank you for always listening to and encouraging me. I'm really grateful for your support. You're the best parents ever - even if you did abandon me part way through my PhD for sunny Dumfries! P.S: I don't expect you to read this entire thesis, so you are totally off the hook to stop reading after this!

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The following sections and associated figures were the result of collaborations. Details of the collaboration and the contribution by both parties is clarified below.

Section 2.4.4 was done in collaboration with Dr. Emma Holden at the Quadram Institute. Dr. Holden did the bioflux experiments and sent the raw image data to Elizabeth Darby. Elizabeth Darby analysed the images using ImageJ and carried out subsequent statistical analyses.

Scanning electron microscopy images from Section 4.4 were taken by Theresa Morris at the Centre for Electron Microscopy (CEM) at the University of Birmingham. The project was also set up in collaboration with Dr. Ludwig Sneider, also from the CEM. Elizabeth grew the cells and fixed them ahead of imaging and did post-image analysis.

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Glossary

accessory gene Accessory genes are found in a proportion of the members of a given species (Morneau, 2021).

antibiotic resistance When bacteria are unable to be successfully treated using antibiotics by carrying a resistance factor, which enables the bacteria to grow at concentrations of the drug that, without that factor, would inhibit growth/kill them (Balaban *et al.*, 2019).

biofilm An aggregate of microbial cells on a surface surrounded by an extracellular matrix.

capsule A polysaccharide structure that covers the bacterial cell wall.

conjugation The sharing of DNA between two bacterial cells across a conjugation pilus.

core gene Core genes are genes which are found, with high sequence conservation, across all members of the same species (Morneau, 2021).

efflux pump A membrane spanning protein complex that exports compounds from within a bacterial cell to the extracellular environment.

horizontal gene transfer The movement of genetic information between bacteria, often divided into conjugation, transduction and transformation.

pili An appendage on a bacterial cell used for adherence.

plasmid A small piece of DNA, which replicates independently from the chromosome.

prophage Prophages are the genetic material of a bacteriophage, which have been incorporated into a bacterial genome (Casjens, 2003).

tolerance A population-level phenotype where cells that are not resistant to a compound may be able to grow after being in contact with the compound due to environmental and genetic factors (Balaban *et al.*, 2019)..

transduction The process of DNA being introduced to a bacterial cell via a bacteriophage infection.

transformation The process of exogenous DNA being taken into a bacterial cell using type IV pili.

Acronyms

acb *Acinetobacter calcoaceticus baumannii* complex.

ABC ATP binding cassette.

AME aminoglycoside modifying enzyme.

AMR antimicrobial resistance.

ANI average nucleotide identity.

ARG antibiotic resistance gene.

ATP adenosine triphosphate.

BNF British National Formulary.

CARD comprehensive antibiotic resistance gene database.

Cas CRISPR-associated loci.

CRAB carbapenem resistant *Acinetobacter baumannii*.

CRISPR clustered regularly interspaced short palindromic repeats.

DNA deoxyribonucleic acid.

EPS extracellular polymeric substances.

ESKAPEE *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.*, and *Escherichia coli*.

HAE hydrophobic and amphiphilic efflux.

HGT horizontal gene transfer.

HME heavy metal efflux.

IS insertion sequence.

ISB iso-sensitest broth.

LB lysogeny broth.

LOS lipooligosaccharide.

LPS lipopolysaccharide.

MALDI-TOF matrix-assisted laser ionization time-of-flight mass spectrometry.

MATE multidrug and toxic compound extrusion.

MBEC minimum biofilm eradication concentration.

MDR multidrug resistance.

MFS major facilitator superfamily.

MIC minimum inhibitory concentration.

MLST multilocus sequence type.

MOI multiplicity of infection.

NCBI National Center for Biotechnology Information.

OD optical density.

OECD Organisation for Economic Co-operation and Development.

OMF outer membrane factor.

ORF open reading frame.

PACE proteobacterial antimicrobial compound efflux.

PAMP pathogen associated molecular pattern.

PAP periplasmic adaptor protein.

PBP penicillin binding proteins.

PMA phorbol 12-myristate 13-acetate.

PRR pattern recognition receptor.

RND resistance nodulation division.

RPMI Roswell Park Memorial Institute.

SDW sterile distilled water.

SMR small multidrug resistance.

SNP single nucleotide polymorphism.

ST sequence type.

UKHSA United Kingdom Health Security Agency.

WHO World Health Organization.

Chapter One

Introduction

1.1 Antimicrobial resistance

Antimicrobial resistance (AMR) is where microorganisms, such as bacteria and fungi, can no longer be treated successfully with drugs (Dadgostar, 2019). AMR is costly both to public health and the economy. It is estimated that 2.4 million people in Europe, North America and Australia will die from drug resistant infections in the next thirty years and this will disproportionately affect children, the elderly and immunocompromised individuals (Dadgostar, 2019; Hofer, 2019; Yang & Buttery, 2018). It is currently estimated that in Brazil, Indonesia and Russia 40-60% of infections are drug resistant and this is continuing to increase (Hofer, 2019). The global burden of AMR was modelled for the year of 2019, where there was an estimated 4.95 million deaths associated with antibiotic resistance and of these 1.27 million were directly attributed to antibiotic resistance (Murray *et al.*, 2022). Despite this, the Organisation for Economic Co-operation and Development (OECD) recommends that better hygiene, fewer prescriptions, rapid testing to determine the cause of infection and public education around AMR could avoid up to 75% of deaths caused globally by resistant

infections (OECD, 2018). Furthermore, these recommendations are relatively cheap at an estimated $2 per person, per year (OECD, 2018).

Antibiotic resistance, a component of AMR, refers directly to bacteria to surviving in the presence of clinically-relevant levels of antibiotics (Frieri, Kumar, & Boutin, 2017). In the rest of this thesis, if resistance is mentioned it refers only to antibiotic resistance.

1.2 How bacteria develop resistance

Resistance to antibiotics is not a modern phenomenon, bacteria with genes coding for proteins that provide resistance to β lactams, tetracyclines and glycopeptides have been isolated from 30,000-year-old permafrost (D'Costa *et al.*, 2011) and similarly *Staphylococcus aureus* that is resistant to methicillin has been found in European hedgehogs before clinical antibiotic use (Larsen *et al.*, 2022).

1.2.1 Intrinsic resistance

Bacteria may be intrinsically resistant to antibiotics or they can acquire resistance. Intrinsic resistance is when bacteria are naturally resistant to an antibiotic without developing mutations or acquiring novel genes that provide resistance. Intrinsic resistance can include a normal phenotype of the cell, which prevents the antibiotic from working, for example the impermeable double membrane in Gram negative bacteria (Darby, Trampari, *et al.*, 2023). Another mechanism of intrinsic resistance is the expression of efflux pump proteins, which when overexpressed can lead to clinical levels of antibiotic resistance (Webber & Piddock, 2003). In brief, efflux pumps are cell-membrane-associating proteins that export compounds from within bacterial cells to the extracellular environment. Efflux

pumps are discussed more in Section 1.5.3. Intrinsic resistance mechanisms are often found in combination with acquired resistance mechanisms which can lead to multidrug resistant (MDR) phenotypes (Shin & Park, 2017).

1.2.2 Acquired resistance - mutations reducing antibiotic susceptibility

Resistance to antibiotics may also be gained, either by the selection for mutations that render the antibiotic ineffective at targeting a given site (Weigel, Steward, & Tenover, 1998) or by the acquisition of genes (Amudhan *et al.*, 2011) and even entire genomic regions that specifically code for antibiotic resistance proteins (Holt *et al.*, 2016). Acquired resistance can furthermore be passed down vertically to subsequent bacterial generations. The mechanisms of resistance are discussed in Section 1.3.

Mutations that reduce susceptibility to antibiotics have mostly been characterised in genes which encode the target proteins of the antibiotic, for example *gyrA*, which encodes part of DNA gyrase - the target of fluoroquinolone drugs (Weigel, Steward, & Tenover, 1998). In addition to this, mutations within core metabolic genes can also confer antibiotic resistance (Lopatkin *et al.*, 2021). Often mutations within carbon and energy metabolism genes can reduce susceptibility to more than one antibiotic and in the aforementioned study a proposed mechanism for this was reduced respiration and as such reduced tricarboxylic acid cycle activity, lowering metabolic toxicity (Lopatkin *et al.*, 2021).

Mutations within the genome that confer antibiotic resistance can often be associated with fitness costs for the host bacterium due to the fact that the mutations can affect regions with important biological function, such as the ribosome (Holberger & Hayes, 2009). Despite this, antibiotic resistance can still be maintained even in the absence of antibiotic

selection pressure. It is thought that mutations, and resistance genes, can be maintained within a genome in one of three ways. The first, is that the mutation is not associated with a fitness cost in the absence of antibiotics, an example of this might be a mutation in a pre-existing resistance gene, such as a β lactamase enzyme (Melnyk, Wong, & Kassen, 2015; Vogwill & Maclean, 2015). Second, the mutation may cause a fitness defect but compensatory mutations elsewhere alleviate the stress of this. Finally, some resistance genes may be genetically linked to another region of the genome, such as in an operon which is often inherited together, where this region/ the genes nearby are necessary even in the absence of antibiotics (Melnyk, Wong, & Kassen, 2015). Interestingly, the acquisition of a plasmid, extrachromosomal deoxyribonucleic acid (DNA), with resistance genes has a smaller fitness cost than acquiring fitness point mutations (Vogwill & Maclean, 2015). Acquiring antibiotic resistance genes is mentioned in Section 1.2.3.

1.2.3 Acquired resistance – antibiotic resistance genes

1.2.3.1 Horizontal gene transfer

The acquisition of antibiotic resistance genes (ARGs) is usually mediated by horizontal gene transfer (HGT). HGT encompasses four main mechanisms that enable entire genes, such as ARGs, to be transferred quickly and efficiently between bacteria belonging to both the same and different species, shown in Figure 1.1 (McInnes *et al.*, 2020). It is important to note that HGT is not AMR specific, it mediates the transfer of any DNA.

Transduction occurs when a bacteriophage (virus that can infect bacteria) injects DNA into the bacterial cell, which can either insert into the host chromosome (known as a prophage) or be repackaged to make more phage particles. The DNA injected is mostly viral but can also contain DNA from a previous bacterial host (genetic transduction) (Arnold,

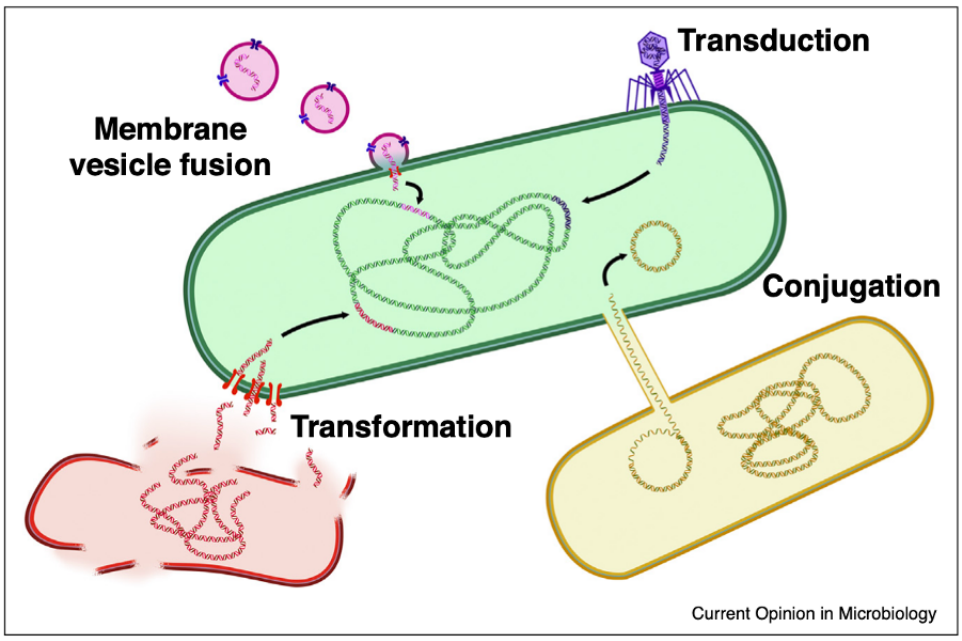


Figure 1.1: Horizontal gene transfer mechanisms. Four mechanisms of horizontal gene transfer are shown, reproduced from (McInnes *et al.*, 2020). Transduction, where bacteriophages inject bacterial DNA from a previous host into a new host. Conjugation, where plasmid DNA moves from a donor to a recipient bacterium. Transformation, where DNA from the environment is taken up by a bacterium. Finally, membrane vesicle fusion where vesicles containing DNA fuse with the recipient bacterium's cell membrane.

Huang, & Hanage, 2022). Genetic transduction can be divided into three types: generalised, specialised and lateral (Chiang, Penadés, & Chen, 2019).

Lateral transduction, unlike specialised and generalised is not a result of phages mispackaging DNA, but a natural part of bacteriophage life. Lateral transduction begins with the replication of multiple integrated prophages. Prophages are the genetic material of a bacteriophage, which have been incorporated into a bacterial genome (Casjens, 2003). Next, some prophages can exise and some are maintained to fill bacteriophage capsids (the protein shell of a virus) with prophage and sections of the bacterial chromosome, which can then be transferred and integrated into a new host (Chen, Quiles-Puchalt, *et al.*, 2018). Furthermore lateral transduction can transfer DNA at a higher frequency than generalised and specialised (Chiang, Penadés, & Chen, 2019).

Generalised and specialised transduction occurs when bacterial DNA is packaged into phage heads and is subsequently transferred to another bacterium, however in specialised transduction the process is limited to specific DNA sites (Chiang, Penadés, & Chen, 2019). Genomic islands as well as ARGs can be transferred this way (Chen, Quiles-Puchalt, *et al.*, 2018). A well characterised example of genomic islands is the AbaR family of genomic islands (Post, White, & Hall, 2010). AbaR1 is found in *Acinetobacter baumannii* AYE and on this island are 3 heavy metal resistance genes and 18 antibiotic resistance genes corresponding to decreased susceptibility to aminoglycosides, tetracyclines, β lactams and sulphonamides (Post, White, & Hall, 2010).

Lastly, a new mechanism of transduction - lateral co-transduction - has recently been described in *Staphylococcus aureus*, which is not driven by phages (Chee *et al.*, 2023). In lateral co-transduction pathogenicity islands (large regions of DNA involved in virulence) encode proteins that cause phage virions to form perfectly sized capsids that are too small for helper phage genomes but big enough for the pathogenicity island. Here, the pathogenicity

island DNA can be tightly packaged into phage capsid, where it can be injected into a new host and recircularise to its original genome size before integrating into the host genome (Chee *et al.*, 2023).

Natural transformation is the process by which exogenous DNA can be taken into a bacterial cell by naturally competent bacteria. Transformation is mediated by type IV pili, which are discussed further in Section 1.5.2.2. Around 80 bacterial species can display natural competence in the laboratory, including *Acinetobacter baylyi* and some *A. baumannii* clinical strains (Arnold, Huang, & Hanage, 2022; Ramirez & Tolmasky, 2010; Vaneechoutte *et al.*, 2006). Once the DNA is within the bacterial cell it can integrate within the host chromosome either via RecA or independently of it. RecA proteins are involved in finding homologous DNA for recombination and integration (Arnold, Huang, & Hanage, 2022).

Mobile genetic elements, such as conjugative plasmids, containing ARGs can be transferred by conjugation. Conjugation is the transfer of DNA from a donor to a recipient bacterial cell either through contact or across a mating bridge called a pilus (Cabezón *et al.*, 2015). Conjugative plasmids have the capacity to carry a number of genes, including resistance genes, into a new bacterial host and are often maintained by toxin-antitoxin systems ensuring their persistence (McInnes *et al.*, 2020). Some plasmids may be not be conjugative but mobilisable, where they can be transferred via a helper conjugative plasmid and indeed some plasmids cannot be mobilised at all.

The final mechanism of HGT is the acquisition of DNA by membrane vesicles. Membrane vesicles produced by Gram negative bacteria can fuse with different bacterial cells leading to the transfer of some of the host bacterium's DNA, for example the transfer of carbapenemase gene OXA-24 from resistant to sensitive *A. baumannii* isolates (Rumbo *et al.*, 2011).

Whilst HGT can be incredibly useful for bacteria, particularly with respect to quickly

acquiring mechanisms that allow them to survive in environments with antibiotics where they could not have survived in before, HGT can be costly. The acquired DNA is additional to what the cell had before and therefore may be energetically expensive to transcribe and translate it (Hall, Brockhurst, & Harrison, 2017). Furthermore, this DNA is not necessarily codon optimised for the host it is now a part of, and this can mean the production of misfolded proteins and the activation of a stress response (Hall, Brockhurst, & Harrison, 2017). Since HGT can be costly, cells have evolved mechanisms to control HGT. An example of this is clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated loci (Cas). CRISPR-Cas recognises particular stretches of DNA, for example from a previous bacteriophage infection, and cleaves the DNA, stopping it from integrating with the host genome (Garneau *et al.*, 2010). Additionally, restriction modification systems can cleave incoming DNA. Restriction modification systems are seen more commonly in bacteria which are frequently exposed to DNA (Oliveira, Touchon, & Rocha, 2016). Furthermore, bacteria can encode specific defense systems which eliminate costly plasmids, for example in pandemic strains of *Vibrio cholerae*, where this increases the fitness of the host (Jaskólska, Adams, & Blokesch, 2022).

1.2.3.2 Common antibiotic resistance genes

Resistance genes have been characterised for a number of antibiotic classes. Table 1.1 lists examples of ARGs, their broad mechanisms of action and the class of antibiotics they confer resistance to. Further detail about the mechanisms of drug, and drug target, modification can be found in Section 1.3 below.

Table 1.1: Common antibiotic resistance genes, intrinsic and acquired

Antibiotic class	ARG example(s)	Broad mechanisms of action
Aminoglycosides	AAC(1) (acetyltransferases), ANT(6) (adenyltransferases) and APH(4) (phosphotransferases)	Enzymatic modification of the drug (Smith & Baker, 2002)
	armA (methyltransferase)	Enzymatic modification of the drug target (Doi, Wachino, & Arakawa, 2016)
β Lactams	*ampC*, *bla*TEM-1, *bla*OXA, *bla*CTX-M	Enzymatic modification of the drug (Ghafourian *et al.*, 2014)
Cationic peptides	*mcr*-1	Modification of the drug target (Li, Yin, *et al.*, 2020)
Glycopeptides	*vanA, vanC*	Modification of the drug target (Stogios & Savchenko, 2020)
Lincosamides	*lin, lnu* (nucleotidyltransferase)	Modification of the drug (Petinaki *et al.*, 2008)
	cfr, erm (methylases)	Modification of the drug target (Feßler *et al.*, 2018)
Lipopeptides	N/A	

Macrolides	*ole* (glycosyltransferase)*, *mph* (phosphotransferase) and *ere* (esterase)	Modification of the drug (Golkar, Zieliński, & Berghuis, 2018)
Oxazolidinones	*cfr* (methyltransferase), *optrA, poxtA* (ABC-F proteins)	Modification and protection of the drug target (Feßler *et al.*, 2018) (Schwarz, Zhang, *et al.*, 2021)
Phenicols	*cat* (acetyltransferase)	Inactivation of the drug and modification of its target (Schwarz, Kehrenberg, *et al.*, 2004)
	cfr (methyltransferase)	
Pyrimidines	*dfrA*1	Acquisition of drug resistant target which replaces target (Grape *et al.*, 2007)
(Fluoro)quinolones	*qnr*	Protection of the drug target (Tran & Jacoby, 2002)
Rifamycins	*rph* (phosphotransferase), rgt1438 (glycosyltransferase) and WGRif3028-2 (ADP ribosylation of drug)	Modification of the drug (Spanogiannopoulos *et al.*, 2012) (Su, Wei, *et al.*, 2014)
Streptogramins	*satA* (acetyltransferase)	Inactivation of the drug (Rende-Fournier *et al.*, 1993)

Sulfonamides	*sul*	Acquisition of drug target, which the drug has a lower affinity for (Changkaew *et al.*, 2014)
Tetracyclines	*tet*	Degradration of drug (Forsberg *et al.*, 2015)

*macrolide glycosyltransferases have not been shown to confer real-world phenotypic resistance outside of the laboratory (Golkar, Zieliński, & Berghuis, 2018).

1.3 Mechanisms of resistance

Section 1.2 described how DNA can change or be introduced into a bacterium giving rise to antibiotic resistance. Broadly, resistance can be conferred by drug target protection, the modification of the drug itself, increased efflux, reduced influx and metabolic bypass, Figure 1.2. Target protection refers to the physical protection of where the drug binds in a cell (Wilson *et al.*, 2020). The mechanisms of how these genetic changes lead to resistance are discussed below along with the mechanisms of action of each drug class, (Table 1.2).

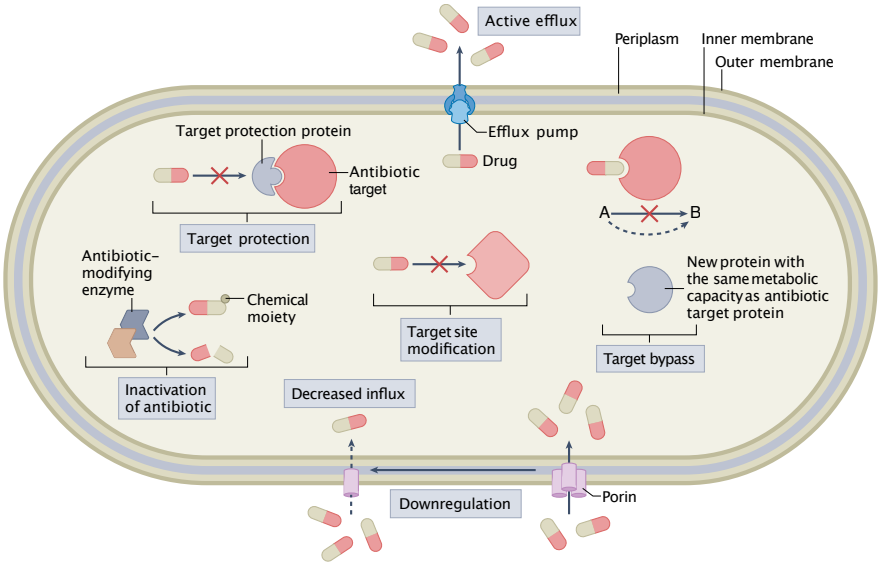


Figure 1.2: Mechanisms of antibiotic resistance. Schematic diagram of a bacterial cell depicting the major mechanisms of antibiotic resistance, reproduced from (Darby, Trampari, *et al.*, 2023). Target protection occurs when a protein physically protects the antibiotic's target, rendering the antibiotic unable to bind to it. Target modification is when the antibiotic target protein is altered so the antibiotic can no longer bind. Target bypass is when a new protein is created with the same binding capacity to the antibiotic as the original target, diluting the amount of antibiotic which can bind to the original target protein. The antibiotic itself may also be inactivated by enzymes, reducing its ability to bind to the target. Finally, the concentration of antibiotic within the bacterial cell can be reduced by increasing the export (removal) of it via efflux pumps and limiting the permeation of it into the cell by reducing influx.

Table 1.2: Major classes of antibiotic, their target and resistance mechanisms

Antibiotic class (examples)	Mechanism of action	Mechanism of resistance
Aminoglycosides (gentamicin, streptomycin, kanamycin)	Interact with the 30S ribosomal subunit of 16S rRNA causing misreading and/or truncated proteins and cell death; positively charged attach to outer membrane causing pores to increase accumulation	Aminoglycoside-modifying enzymes, for example acetyl-transferases, phosphotransferases and nucleotidyltransferases; 16S ribsomal methylases; mutations in the 16S rRNA gene; decreased influx and/or increased efflux
Beta lactams (penicillins, cephalosporins, cephamycins, carbapenems, monobactams)	Target peptidoglycan crosslinking by inhibiting penicillin-binding proteins, which crosslink the peptide chain in the cell wall, leading to lysis of the cell	Production of beta-lactamases; modification of penicillin-binding proteins; reduced permeability and increased efflux

Cationic peptides (colistin)	Bind to lipid A in lipopolysaccharide, permeabilising the outer membrane causing cell death	Modification or removal of lipid A
Glycopeptides (vancomycin)	Inhibit crosslinking and therefore synthesis of peptidoglycan by binding to D-alanyl-D-alanine in the peptide chain	Intrinsic resistance in Gram negative cells by impermeable outer membrane; in Gram positive cells, enzymes can modify and hydrolyse peptidoglycan precursors; intermediate susceptibility phenotype conferred by mutations leading to thickened membrane and low permeability
Lincosamides (clindamycin)	Target the translation of proteins, specifically 23s rRNA of the 50S ribsomal subunit, causing truncated peptide chains	Methyltransferases that modify 23s rRNA; expression of proteins that inactivate lincosamides and efflux

Lipopeptides (daptomycin)	Insert in the cell membrane and cause depolarisation, reducing the ability to create ATP and cell death	Thickening of and increasing the positive charge in the cell wall; reducing the depolarisation of membranes induced by lipopeptides
Macrolides (azithromycin, erythromycin)	Inhibit the translation of proteins by targeting 23S rRNA of the 50S ribosomal subunit, causing truncated peptide chains	rRNA methyltransferases, which methylate 23S rRNA; mutations in the ribosome; efflux; macrolide phosphotransferases and esterases; ribosomal protection by ATP-binding cassette F (ABC-F) proteins
Oxazolidinones (linezolid)	Limit translation by binding to 23S rRNA of the 50S subunit and preventing the formation of a functional 70S subunit	Modifications of the 23S rRNA, for example by methyltransferases; protection of the ribosome via ABC-F proteins
Phenicols (chloramphenicol)	Inhibit translation by binding to the A site of the 50S ribosomal subunit, inhibiting protein synthesis	Mutations within 23S rRNA of the 50S ribosomal subunit; enzymatic inactivation via acetyltransferases and efflux

Pyrimidines (trimethoprim)	Affect C1 metabolism and folate synthesis by inhibiting dihydrofolate reducatse, blocking production of tetrahydrofolate	The modification or acquisition of novel dihydrofolate reductase genes and efflux of trimethoprim
Quinolones and fluoroquinolones (ciprofloxacin)	Inhibit DNA replication by DNA gyrase or topoisomerase IV, which are involved in DNA supercoiling, strand cutting and ligating	Mutations in DNA gyrase or topoisomerase IV; the efflux of quinolones or proteins that protect DNA gyrase and topoisomerase IV
Rifamycins (rifampicin)	Inhibit transcription, specifically DNA-dependent RNA synthesis, by binding RNA polymerase	Mutations in the drug target *rpoB*; enzymatic ribosylation or inactivation of rifampicin
Streptogramins (dalfopristin)	Target protein translation by binding to 23S rRNA of the 50S ribosomal subunit at the peptidyl-transferase domain causing truncated peptides	Mutations in 23S rRNA; modification of streptogramins by acetyltransferases; efflux out of the cell

Sulfonamides (sulfamethizole)	Stop dihydrofolate acid synthesis by inhibiting dihydropteroate synthase and arresting cell growth	Mutations in the dihydropteroate synthase gene and *sul1/2* genes, which encode distinct dihydropteroate synthases that are less susceptible to sulfonamides
Tetracyclines (tigecycline, tetracycline)	Inhibit translation by binding to 16S rRNA of the 30S ribosomal subunit, preventing tRNA binding to the 30S at the A site	Efflux; protein-mediated ribosome protection; ribosomal mutations; enzymatic inactivation of the drug

Table information reproduced from (Darby, Trampari, *et al.*, 2023)

1.3.1 Drug target modification

The targets of antibiotics are often mutated in order for a cell to survive by reducing antibiotic binding and action. A well-known example of this is when bacteria acquire mutations in GyrA, the target for fluoroquinolones. By maintaining spontaneous mutations in *gyrA* that decrease drug binding, the bacteria can survive in the presence of a higher concentration of the drug (Willmott & Maxwell, 1993). The acquisition and subsequent maintenance of mutations is usually due to them being beneficial under a selection pressure, such as in the example with GryA and fluoroquinolones.

1.3.2 Drug modification and inactivation

Drug modification can often be the result of a chemical group added to the antibiotic, for example aminoglycosides can become ineffective due to the addition of phopshate groups by phosphotransferase enzymes (Wright & Thompson, 1999). Another example of this is acetyltransferase enzymes, which can acetylate chloramphenicol thereby detoxifying it (Schwarz, Kehrenberg, *et al.*, 2004). Additionally, the drug itself may be inactivated to reduce its activity, for example, via hydrolysis. The most widespread mechanism of this is the production of β lactamases. There have been over 7,000 characterised β lactamases, which can hydrolyse the β lactam ring of the antibiotics belonging to the class (Naas *et al.*, 2017).

1.3.3 Decreased intracellular accumulation

The permeability of the Gram negative outer membrane can be reduced to limit the rate at which antibiotics can enter cells (influx). The Gram negative outer membrane contains porins, beta-barrel protein channels, that allow the influx of hydrophillic compounds into a cell (Darby, Trampari, *et al.*, 2023). An example of a porin linked to antibiotic resistance is the porin CarO, which is involved in the influx of carbapenems. In *A. baumannii* CarO is often lost or interrupted by insertion sequence (IS) elements leading to carbapenem resistance (Poirel & Nordmann, 2006). Another way that bacteria reduce the intracellular accumulation of antibiotics is to actively pump them out of the cell using membrane-bound efflux pumps. This is further described in Section 1.5.3.

1.3.4 Metabolic bypass

Metabolic bypass is when bacteria provide an alternative metabolic pathway, enabling them to survive even when the drug target is compromised. This is often through the acquisition of a gene that can function like the gene target without being affected by the antibiotic. An example of this is in methicillin resistant *Staphylococcus aureus*, where it acquires exogeneous penicillin binding proteins (PBPs), the target of β lactams. These acquired PBPs are homologous to the original target but do not interact as well with β lactams (Stapleton & Taylor, 2002; Darby, Trampari, *et al.*, 2023).

1.4 Priority pathogens

The World Health Organization (WHO) published a list of priority pathogens, Table 1.3, which details species where new antibiotics are urgently needed. A number of the priority pathogens are also within the ESKAPEE group. ESKAPEE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter spp.*, and *Escherichia coli*) are defined as those posing a considerable threat to human health, independent of their increasing resistance rates (De Oliveira *et al.*, 2020; Rajput *et al.*, 2021). *Acinetobacter baumannii*, one of the subjects of this thesis is listed as "critical", the highest priority level.

Table 1.3: WHO priority pathogen list

Priority Level	Pathogen
Critical	*Acinetobacter baumannii* – carbapenem resistant
	Pseudomonas aeruginosa – carbapenem resistant
	Enterobacteriales – carbapenem resistant
High	*Enterococcus faecium* – vancomycin resistant
	Staphylococcus aureus - methicillin-resistant, vancomycin-intermediate and resistant
	Helicobacter pylori - clarithromycin-resistant
	Campylobacter spp. - fluoroquinolone-resistant
	Salmonellae - fluoroquinolone-resistant
	Neisseria gonorrhoeae - cephalosporin-resistant, fluoroquinolone-resistant
Medium	*Streptococcus pneumoniae* - penicillin-non-susceptible
	Haemophilus influenzae - ampicillin-resistant
	Shigella spp. - fluoroquinolone-resistant

Information from World Health Organization (2017)

1.5 *Acinetobacter*

1.5.1 General introduction

Acinetobacter, which comprises of 73 species to date including the well-known drug resistant organism *A. baumannii*, are water and soil dwelling, Gram negative coccobacilli (Munoz-Price & Weinstein, 2008; Parte *et al.*, 2020). The first *Acinetobacter* species was described in 1911 and *Acinetobacter* rose to prominence when outbreaks of infections were becoming increasingly common in soldiers deployed in Iraq (Beijerinck, 1911; Scott, Deye, *et al.*, 2007). *Acinetobacter* species are known to cause a wide variety of infections, from skin and soft tissue infections, to urinary tract infections and bacteraemia (Sanjeev *et al.*, 2013; Sherertz & Sullivan, 1985; Ku *et al.*, 2000). *In vitro* studies of *Acinetobacter* infections from the 1970s showed that 60-70% of clinical isolates were susceptible to ampicillin, 92.5% were susceptible to gentamicin and 57% were susceptible to chloramphenicol (Towner, 2009). This is in contrast to the multidrug resistant phenotypes seen in the clinic today, where isolates can be resistant to carbapenems, aminoglycosides, fluoroquinolones and tetracyclines (Dijkshoorn, Nemec, & Seifert, 2007). Resistance is discussed further in chapter 1.5.5.

1.5.2 *Acinetobacter* physiology

Acinetobacter express cell surface components that allow them to survive on abiotic surfaces for extended periods of time and cause infection. In *A. baumannii* the ability to persist for so long on surfaces is partly linked to biofilm formation, where strains that can form biofilm are able to survive an additional 21 days compared to their non-biofilm forming counterparts on dry surfaces (Espinal, Martí, & Vila, 2012). The outer membrane and capsule of *Acinetobacter* also protects them from antibiotic and host associated

molecules (Coyne, Rosenfeld, *et al.*, 2010; Monem *et al.*, 2020). Different components of the *Acinetobacter* cell surface are shown in Figure 1.3 and discussed below.

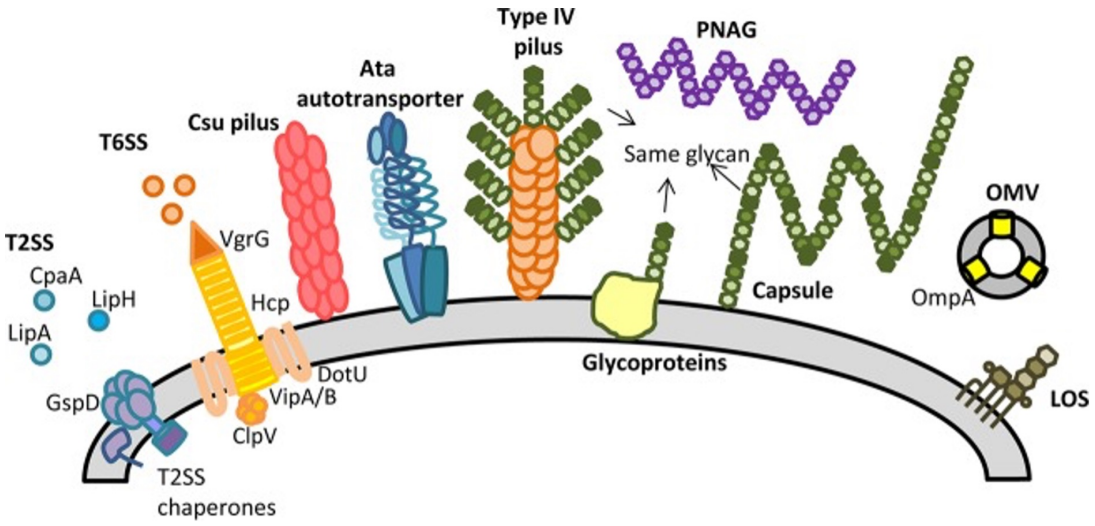


Figure 1.3: Cell surface components characterised in *Acinetobacter*. Figure reproduced from (Weber, Harding, & Feldman, 2015). The membrane schematic shows LOS, lipooligosaccharide, which is a glycolipid expressed on the cell surface, providing structural integrity to the outer membrane. Type two (T2SS) and six (T6SS) secretion systems are also shown, where they export proteins either into the extracellular environment or directly into a target cell, respectively. A Csu and type IV pilus are also found on the cell surface, where Csu pili are involved in virulence and biofilm formation and type IV pili are involved in DNA uptake and twitching motility. The autotransporter, Ata, enables *Acinetobacter* to bind to host cells via interactions with glycosylated residues. The outer membrane itself can also bleb creating outer membrane vesicles (OMV), in this instance expressing porin OmpA. Finally, surface glycoproteins and polysaccharides (PNAG - poly-*N*-acetyl-beta-(1,6)-glucosamine) are also shown.

1.5.2.1 Lipooligosaccharide

Acinetobacter baumannii, like *Neisseria* and *Campylobacter*, has been shown to express lipooligosaccharide (LOS), a version of lipopolysaccharide (LPS) missing the O-antigen (Herrera, Voss, & Trent, 2021; Weber, Harding, & Feldman, 2015). LPS is a major component of the Gram negative outer membrane, where it is composed of an endotoxin (lipid A), a core oligosaccharide and O-antigen sugar molecule. LPS establishes a barrier that protects the bacterium against toxic compounds in the environment, including antibiotics (Powers & Trent, 2018; Raetz & Whitfield, 2002). Despite, the advantages of LPS/LOS in the cell surface, *Acinetobacter* has been shown to acquire mutations in the pathway for lipid A biosynthesis in the presence of colistin, rendering the cell resistant to the antibiotic by either the modification or complete loss of LOS (Hood *et al.*, 2013; Moffatt *et al.*, 2010). Removal of LOS is accompanied by compensatory mutations, which can lead to the overexpression of lipoproteins to stabilise the cell membrane (Boll *et al.*, 2016). LOS-deficiency alters the expression of cell envelope maintenance systems, including lipoprotein transport *lol*, multidrug efflux pumps, the two-component regulatory system *baeSR* and phospholipid transport *mla* (Powers et al., 2018). In *A. baumannii* cells passaged in colistin, and therefore LOS-deficient, RND efflux pumps AdeABC and AdeIJK are upregulated (Chamoun *et al.*, 2021). LOS deficiency is not specific to *Acinetobacter*, it has also been seen in *N. meningitidis* but in that instance it was not linked to polymyxin treatment (Steeghs *et al.*, 1998).

1.5.2.2 Pili

In addition to LOS, *Acinetobacter* also contain type IV pili on the cell surface. Type IV pili mediate twitching motility, biofilm formation and are involved in competence and therefore the transformation of extracellular DNA (Weber, Harding, & Feldman, 2015). Natural

competence and transformation are discussed in Section 1.2.3.1. The core and accessory type IV pili genes have been characterised in *A. baylyi* ADP1 (Leong *et al.*, 2017). Figure 1.4 shows the main genes involved in type IV pili and their roles (Leong *et al.*, 2017).

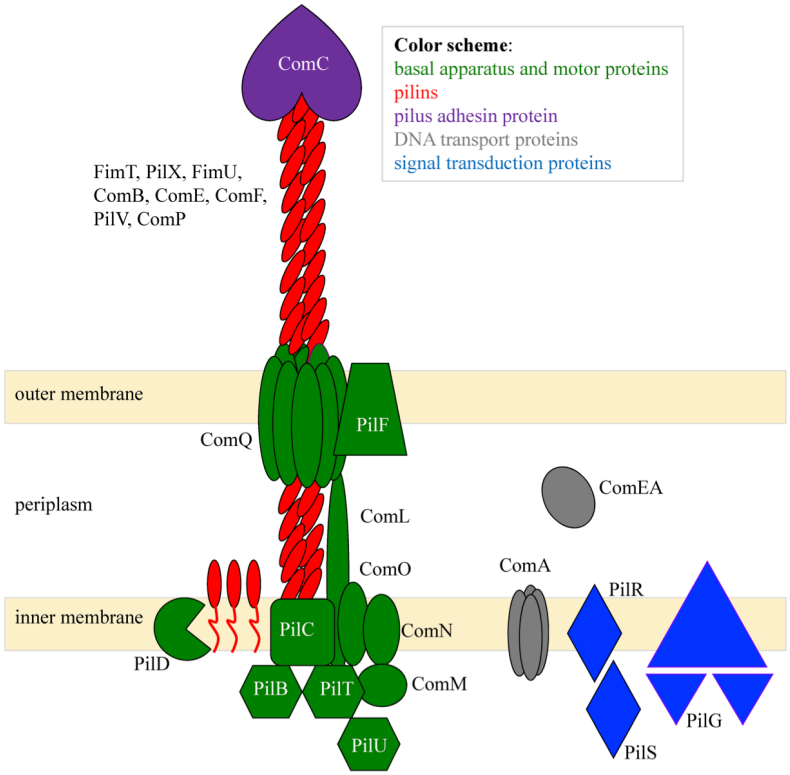


Figure 1.4: Model of the type IV pilus of *A. baylyi* ADP1, reproduced from (Leong *et al.*, 2017). Type IV pili are involved in motility (twitching) and DNA update (competence). The diagram shows the proteins involved in competence (DNA transport proteins, in grey), twitching (signal transduction proteins, in blue) and pilus assembly (in red, purple and green). It is proposed that extracellular double stranded DNA crosses the outer membrane via the pilus protein ComQ and is shuttled across the periplasm to DNA transport protein ComA. ComA unravels the DNA so it is single stranded, allowing it to pass into the bacterial cytoplasm, across the inner membrane.

In *A. baylyi* the inner membrane assembly platform (*pilC*), periplasmic connector (*comM*), which connects PilC to the secretin ComQ, and secretin ComQ (*comQ*) are core genes. Core genes are genes which are found, with high sequence conservation, across all members of the same species and accessory genes are found in a proportion of the members of the species (Morneau, 2021). Additional core type IV genes include *pilF* a pilotin that encodes a protein involved in secretin assembly, *pilU* and *pilT* which code for ATPases for pilus retraction and the genes encoding pilins *comP, comB* and *pilX*, shown in Figure 1.4.

A. baumannii is motile by both twitching and surface-associated motility, where impaired surface-associated motility but not twitching impairs virulence (Corral *et al.*, 2021). *Acinetobacter* do not encode flagella, so motility in this genus is via different mechanisms (Blaschke, Skiebe, & Wilharm, 2021). The action of twitching has been characterised and linked to the function of type IV pili and surface associated motility can be controlled by quorum sensing, light and iron availability (Blaschke, Skiebe, & Wilharm, 2021), but is generally less well understood than twitching motility (Harding, Tracy, *et al.*, 2013).

Acinetobacter also contain Csu pili, which have been shown to be involved in biofilm formation in *A. baumannii* (Tomaras, Dorsey, *et al.*, 2003). Biofilms are (often polymicrobial) aggregations of cells and a matrix that accumulate at the surface-liquid interface (Flemming & Wingender, 2010). Within biofilms, around 10% of the components are microbial cells and the remaining constituents are extracellular material, often created by the host microorganisms (Flemming & Wingender, 2010). These extracellular polymeric substances (EPS) form complex three dimensional structures within the biofilm. Polysaccharides, proteins and DNA are the most common EPS and play roles in adhesion, aggregation and cohesion of biofilms as well as providing nutrients for the microorganisms within (Davey & O'toole, 2000). *Acinetobacter* biofilms are challenging to treat medically due to their reduced susceptibility to antibiotics and biocides (Eze, Chenia, & El Zowalaty,

2018). Furthermore, *Acinetobacter* can use their Csu pili to colonise a range of abiotic surfaces such as implanted medical devices (Pakharukova *et al.*, 2018). Not only are biofilms more difficult to treat medically, but they are also the perfect environment for genetic exchange between different bacteria through HGT due in part to the availability of extracellular DNA (Abe, Nomura, & Suzuki, 2020; Penesyan *et al.*, 2019). It has been shown that *Pseudomonas*, which is not normally competent, is capable of natural competence in a biofilm environment and therefore can more easily acquire foreign DNA (Nolan *et al.*, 2020).

Another component of the *Acinetobacter* outer membrane is the presence of glycoproteins. Protein glycosylation is found throughout the entire *Acinetobacter* genus but there is diversity in the glycoproteome of each species. In strains of *A. baumannii* there can be different pathways to generate glycans, but despite this the glycoproteins produced have similar characteristics (Scott, Kinsella, *et al.*, 2014). The absence of glycoproteins is linked to a reduced ability to form biofilm and reduced virulence in *A. baumannii* (Iwashkiw *et al.*, 2012).

1.5.2.3 Capsule

Acinetobacter produce an extracellular capsule to protect the cell further from host-associated killing, such as complement proteins (Weber, Harding, & Feldman, 2015). A capsule is required for *A. baumannii* to survive in human serum and rat soft-tissue infection models (Russo *et al.*, 2010). A capsule is also needed for the optimal expression of bioemulsifiers, which degrade hydrocarbons and can have industrial applications, in *A. lwoffii* (Desai & Banat, 1997; Nakar & Gutnick, 2003). The capsule in *A. baumannii* is also modified in the presence of sub-inhibitory translation-inhibiting antibiotics, where exopolysaccharide production increases, which in turn increases the capsule-derived defence

against complement proteins and increases virulence (Geisinger & Isberg, 2015). Furthermore, *A. baumannii* lacking an exopolysaccharide capsule have increased susceptibility to peptide antibiotics (Geisinger & Isberg, 2015).

1.5.2.4 Protein secretion systems

Bacteria secrete proteins for communication, adhesion to surfaces (biofilm formation), nutrient acquisition and virulence (Meuskens *et al.*, 2019). *Acinetobacter* can export proteins via type I, type II, type IV, type V (autotransporters) and type VI secretion systems as well as outer membrane vesicles.

Type I secretion systems in *A. baumannii* have been shown to export biofilm associated proteins (Bap), which aid biofilm formation and maturation (Harding, Pulido, *et al.*, 2017; Harding, Hennon, & Feldman, 2018) and are also essential for survival and replication within macrophages (Sycz *et al.*, 2021). The type II secretion system of *A. baumannii* can export virulence factors and the inactivation of it reduces *in vivo* fitness in a murine model and *in vitro* fitness when cultured with long chain fatty acids alone (Johnson, Waack, *et al.*, 2016; Jackson-Litteken *et al.*, 2022). Therefore, type II secretion systems in *A. baumannii* are important for fitness and may contribute to fatty acid metabolism (Johnson, Waack, *et al.*, 2016). Autotransporters or type V secretion systems, such as the *Acinetobacter* trimeric autotransporter (Ata) are integral for the adhesion of *A. baumannii* strains to collagen in the extracellular matrix and biofilm formation (Bentancor *et al.*, 2012; Weber, Harding, & Feldman, 2015). Type IV secretion systems are used by bacteria to secrete toxins that kill competing bacteria, for example *A. baumannii* M2 can outcompete *E. coli* using its type IV secretion system (Carruthers *et al.*, 2013). The nucleotide conservation of type VI secretion systems in *Acinetobacter* is high and the expression is tightly controlled (Weber, Miyata, *et al.*, 2013). In *A. baumannii* ATCC

17978, type VI secretion is controlled by a plasmid that encodes repressors and H-NS silencing (Weber, Ly, *et al.*, 2015). This plasmid also contains antibiotic resistance genes, with activity against β lactams, aminoglycosides and trimethoprim (Weber, Ly, *et al.*, 2015). Multidrug resistant (MDR) *A. baumannii* strains have been shown to have inactivated their type VI secretion systems. It is hypothesised this is due to antibiotic pressure being high enough for there to be no need to harbour often repressed type VI secretion systems, when energy could be used elsewhere (Wright, Haft, *et al.*, 2014).

Outer membrane vesicles, which are blebs of the outer membrane released from the cell, are involved in both virulence and horizontal gene transfer (Section 1.2.3.1). *A. baumannii* has been shown to release outer membrane vesicles with ARGs such as *bla*OXA-24 and proteins involved in biofilm formation, quorum sensing, oxidative stress tolerance and a number of virulence factors, such as Omp38 (Fulsundar *et al.*, 2015; Li, Zhang, *et al.*, 2015; Rumbo *et al.*, 2011).

Finally, preliminary work has been done to look into type VI secretion systems, which can mediate HGT by the translocation of DNA into bacterial (and indeed, eukaryotic) cells by cell-to-cell contact (Christie, Whitaker, & González-Rivera, 2014). In *A. baumannii* ACICU plasmid encoded type VI secretion proteins have been characterised (Elhosseiny & Attia, 2018; Iacono *et al.*, 2008).

1.5.3 Efflux pumps

Whilst not shown in Figure 1.3, efflux pumps are also key components in the cell membranes of *Acinetobacter* species. As mentioned in chapter 1.3.3 efflux pumps are membrane-spanning proteins that can export substrates from within a bacterial cell to the extracellular environment (Chitsaz & Brown, 2017). Efflux pumps can have broad

substrate ranges, for example they may export dyes, antibiotics, heavy metals and toxic host molecules such as bile salts (Blair & Piddock, 2016; Colclough, Alav, *et al.*, 2020). Efflux pumps can play direct and indirect roles in drug resistance. For example, overexpression of an efflux pump can lead to phenotypic drug resistance (Swick *et al.*, 2011). Furthermore, efflux can also aid the development of drug resistance by allowing the cell time to acquire resistance mutations by keeping the antibiotic levels low enough to allow for survival, an example of this is selection for single nucleotide polymorphisms (SNPs) in *grlA* a target of ciprofloxacin (Papkou *et al.*, 2020). Although efflux pumps are commonly linked to antibiotic resistance, they also carry out broad functions to aid the homeostasis of cells. For example, efflux pumps have been linked to the export of host-derived antimicrobial agents and virulence determinants (Henderson *et al.*, 2021). The pumps are divided into families based upon their structure, function and mechanism of action. The families of efflux pumps found in *Acinetobacter* are shown in Figure 1.5. Broadly, efflux pumps function using two forms of energy, either adenosine triphosphate (ATP) or ionic gradients. The ATP binding cassette (ABC) family of efflux pumps use the former, where all other families use the latter (Chitsaz & Brown, 2017), Figure 1.5. The resistance nodulation division (RND) family of efflux pumps are most well known for their ability to export antibiotics, where some of these pumps can export structurally different antibiotics leading to multidrug resistance, although it is important to note not all antibiotics exported by efflux pumps confer clinical levels of resistance (Piddock, 2006). RND systems are broadly split into two categories based upon the substrates they export – hydrophobic and amphiphilic efflux (HAE) and heavy metal efflux (HME) pumps (Klenotic *et al.*, 2020).

The most well-studied RND pump is AcrAB-TolC found in *E. coli*. AcrAB-TolC is a tripartite system built around an inner membrane H^+/drug antiporter - AcrB (Klenotic *et al.*, 2020). The substrates are acquired from either the periplasmic space or the outer leaflet of the

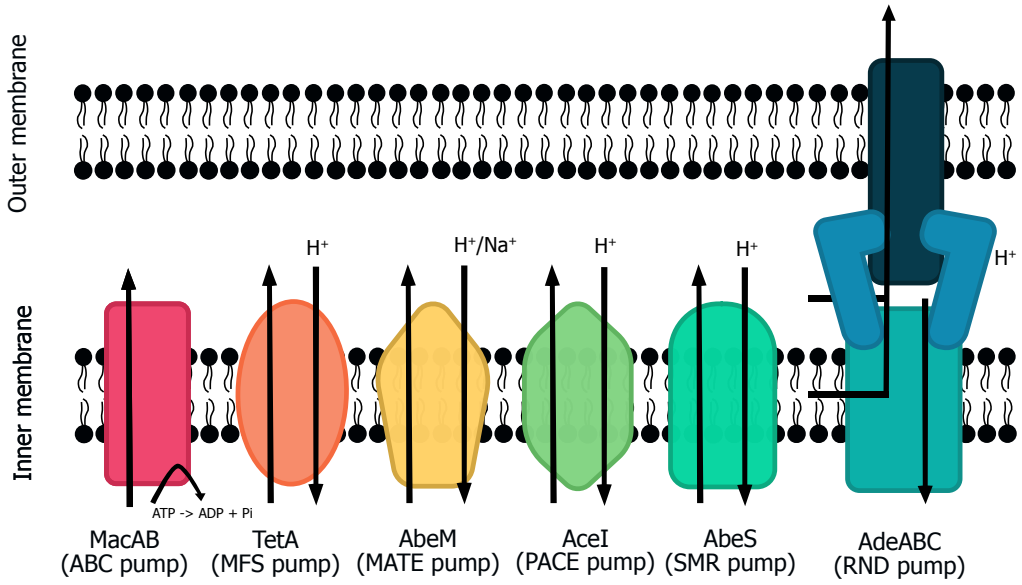


Figure 1.5: Classes of efflux pump in *Acinetobacter*. Figure based upon Abdi *et al.* (2020). *Acinetobacter* species can encode efflux pumps from six families (shown above). RND, ABC and MFS pumps can form tripartite complexes (although RND is only shown as such). Tripartite complexes include a pump protein in the inner membrane, as well as periplasmic adaptor proteins located between the pump protein and outer membrane channel, which spans the outer membrane. The upwards arrows show the direction of substrate export, either from the cytoplasm or either the cytoplasm or periplasm for RND pumps. ABC pumps use ATP for energy, whereas the other pumps use ionic gradients, shown by downwards arrows. Example *Acinetobacter* efflux pumps are listed above the pump family type e.g. MacAB, which is ABC pump.

inner membrane and pumped out of the cell via the outer membrane factor (OMF) channels, TolC, and periplasmic adaptor proteins (PAP), AcrA (Alav, Kobylka, *et al.*, 2021; Chen, Shi, *et al.*, 2022; Horiyama, Yamaguchi, & Nishino, 2010; McNeil *et al.*, 2019). The RND transporters function as trimers, which contain three functionally interdependent protomers, cycling consecutively through the Loose (L), Tight (T) and Open (O) conformational states during co-operative catalysis (Murakami, Nakashima, Yamashita, Matsumoto, *et al.*, 2006; Seeger *et al.*, 2006).

1.5.3.1 Non-RND systems

ATP binding cassette (ABC) transporters hydrolyse ATP to ADP and inorganic phosphate to export compounds either to the periplasm or outside of the cell. An example of an ABC pump in *Acinetobacter* is MacAB, which exports macrolide antibiotics, in addition to virulence factors, lipopolysaccharides and siderophores (Okada *et al.*, 2017). MacAB has also been implicated in the envelope stress response in mature biofilms, where it helps *A. baumannii* to maintain its cell membranes in high osmolarity conditions (Robin *et al.*, 2022). In addition to being found in *Acinetobacter*, MacAB has been characterised in other species, such as *E. coli*, where it forms a tripartite complex with the outer membrane channel TolC (Fitzpatrick *et al.*, 2017).

Major facilitator superfamily (MFS) efflux pumps can be tripartite, like RND systems, or just a single inner membrane protein and in both instances they use the proton motive force (Pasqua, Bonaccorsi di Patti, *et al.*, 2021). *Acinetobacter* can encode a number of different MFS pumps, for example TetA and FloR (which are found on genomic island AbaR1), as well as AbaF, CraA and EmrAB (Abdi *et al.*, 2020). TetA exports tetracyclines, FloR exports florfenicol, AbaF exports fosfomycin, CraA exports chloramphenicol and EmrAB exports colistin and also aids survival in osmotic stress conditions (Abdi *et al.*, 2020; Lin, Lin, &

Lan, 2017).

In addition to MFS and ABC pumps, *A. baumannii* can also encode the multidrug and toxic compound extrusion (MATE) pump, AbeM. MATE transporters use either a H^+ or Na^+ electrochemical gradient to move substrates outside of the cell (Radchenko *et al.*, 2015). AbeM from *A. baumannii* has been cloned into *E. coli* lacking the major RND pump AcrAB, showing that it can export hydrophilic substrates, such as ciprofloxacin and norfloxacin, as well as gentamicin, triclosan, acriflavine, rhodamine 6G, ethidium bromide, kanamycin, trimethoprim, doxorubicin and chloramphenicol (Su, Chen, *et al.*, 2005). AbeM is, to date, the only MATE pump characterised in *Acinetobacter*.

AceI, in *A. baumannii*, is the founding protein of the proteobacterial antimicrobial compound efflux (PACE) pump family. PACE transporters are comprised of an inner membrane protein that, again, functions by using the proton motive force (Hassan, Liu, *et al.*, 2018). AceI actively exports the biocide chlorhexidine, which is found commonly in alcohol-based skin disinfectants (Hassan, Elbourne, *et al.*, 2015; Hennig *et al.*, 2017). PACE transporters are found across a range of Gram negative pathogens, including *Pseudomonas* and *Klebsiella* and are usually highly conserved, indicating they provide an important function to the host (Hassan, Liu, *et al.*, 2018).

The final, non-RND family in *Acinetobacter* is the small multidrug resistance family (SMR) of efflux pumps. SMR transporters utilise the proton motive force and are situated in the inner membrane of the cell (Srinivasan, Rajamohan, & Gebreyes, 2009). QacE is an example of a horizontally acquired SMR pump that can also be found on AbaR1 in *A. baumannii* along with the MFS pumps listed above and a study has shown that 40% of *A. baumannii* isolates encode QacE (Fournier *et al.*, 2006; Rajamohan, Srinivasan, & Gebreyes, 2010). QacE exports quaternary ammonium compounds (Coyne, Courvalin, & Périchon, 2011). Another SMR pump found in *Acinetobacter* is AbeS, where AbeS is

homologous to EmrE from *E. coli* and provides low-levels of resistance to chloramphenicol, quinolones, erythromycin and novobiocin (Coyne, Courvalin, & Périchon, 2011). A comprehensive list of all the characterised efflux pumps in *Acinetobacter* to date and their respective families can be found in Table 1.4 below (Kornelsen & Kumar, 2021).

Table 1.4: Efflux pumps characterised in *Acinetobacter*

Efflux pump family	Example	Reduced susceptibility to	Characterised in
MFS	CraA	Chloramphenicol	*A. baumannii*
	AmvA	Erythromycin	*A. baumannii*
	AbaF	Fosfomycin	*A. baumannii*
	AbaQ	Quinolones	*A. baumannii*
	TetA	Tigecycline	*A. baumannii*
	CmlA	Chloramphenicol	*A. baumannii*
MATE	AbeM	Fluoroquinolones	*A. baumannii*
ABC	A1S_0536	Erythromycin	*A. baumannii*
	A1S_1535	Gentamicin, chloramphenicol	*A. baumannii*
	MacAB	Potentially macrolides and tigecycline	*A. baumannii*
SMR	AbeS	Chloramphenicol, fluroquinolones, novobiocin, erythromycin	*A. baumannii*
	QacE	Quaternary ammonium compounds	*A. baumannii*
PACE	AceI	Chlorhexidine	*A. baumannii*
	A1S_1503	Acriflavine	*A. baumannii*
RND	AdeABC	Aminoglycosides, trimethoprim, chloramphenicol, fluoroquinolones, tetracyclines, pentamide	*A. baumannii*

AdeDE	Amikacin, ceftazidime, chloramphenicol, ciprofloxacin, erythromycin, meropenem, rifampicin, tetracycline	*A. pittii*
AdeFGH	Chloramphenicol, trimethoprim, tetracycline-tigecycline, clindamycin, fluoroquinolones	*A. baumannii*
AdeIJK	β lactams, chloramphenicol, tetracycline, erythromycin, licosamides, fluoroquinolones, fusidic acid, novobiocin, rifampicin, trimethoprim	*A. baumannii*
AdeXYZ	N/A	*A. baylyi*
CzcABCD	Heavy metals such as copper	*A. baumannii*
AbeD	Ceftriaxone, gentamicin, rifampicin, tobramycin, benzalkonium chloride	*A. baumannii*
ArpAB	Amikacin, tobramycin	*A. baumannii*
AcrAB	Colsitin, tobramycin, acriflavine	*A. nosocomialis*

Table reproduced from Kornelsen & Kumar (2021).

1.5.3.2 RND systems

The resistance nodulation division (RND) efflux pumps are the most clinically relevant pumps for antibiotic resistance due to their capacity to export a broad range of structurally distinct antibiotics (Colclough, Alav, *et al.*, 2020). AdeABC was the first RND pump characterised in *A. baumannii*, and it is now estimated that around 80% of strains encode the complete system and all three components are found in a higher proportion of clinical isolates compared to environmental isolates (Coyne, Courvalin, & Périchon, 2011). Furthermore, AdeABC has a broad substrate range and can export aminoglycosides, β lactams, chloramphenicol, tetracyclines, erythromycin, ethidium bromide and fluoroquinolones (Coyne, Courvalin, & Périchon, 2011; Magnet, Courvalin, & Lambert, 2001). AdeABC is a tripartite system, where AdeA is the periplasmic adaptor protein, AdeB is the multidrug transporter protein and AdeC is the outer-membrane-spanning protein (Magnet, Courvalin, & Lambert, 2001). AdeABC is not constitutively expressed and a multidrug resistance phenotype is only seen upon overexpression of the genes (Coyne, Courvalin, & Périchon, 2011).

AdeABC expression is positively regulated by AdeRS, a two-component system, where AdeS is a signaling regulator, which gets induced by an unknown signal and AdeR is a response regulator that can bind to the promoter sequence of AdeABC increasing expression (Chang *et al.*, 2016; Colclough, Alav, *et al.*, 2020). In addition to AdeRS, the two-component system BaeSR also acts as a positive regulator for AdeABC in *A. baumannii*, AdeRS is thought to cause initial expression of AdeABC and BaeRS then causes over-expression to levels where drug resistance can be seen (Colclough, Alav, *et al.*, 2020; Lin, Lin, & Lan, 2015).

In addition to AdeABC, *A. baumannii* also expresses AdeIJK, shown in Figure 1.6. AdeIJK is also positively regulated by the two-component system BaeSR (Colclough, Alav,

et al., 2020). AdeIJK is constitutively expressed in *A. baumannii* providing intrinsic resistance to ticarcillin, cephalosporins, fluoroquinolones, tetracyclines, aztreonam, lincosamides, rifampicin, chloramphenicol, novobiocin, fusidic acid, anti-folates and cotrimoxazole but not aminoglycosides. In addition, AdeIJK can work synergistically with AdeABC to extrude tigecycline (Coyne, Courvalin, & Périchon, 2011; Yoon, Balloy, *et al.*, 2016). In strains lacking AdeC, AdeAB can form a complex with the AdeK outer membrane protein, in a similar way to how TolC in *E. coli* can work with AcrAB and AcrEF (Leus *et al.*, 2020). AdeIJK is also found in other *Acinetobacter* species, for example *A. calcoaceticus, A. nosocomialis, A. junii, A. haemolyticus, A. parvus, A. radioresistens, A. ursingii, A. johnsonnii* and *A. lwoffii*, where the highest percentage identity to the *A. baumannii adeIJK* sequences is seen in *A. calcoaceticus* and *A. nosocomialis* (Rosenfeld *et al.*, 2012). Furthermore, the aforementioned species' also encode *adeN*, a TetR-like regulator, which represses AdeIJK expression (Rosenfeld *et al.*, 2012).

AdeIJK has broad roles in cell homeostasis. For example, it modulates the lipids in the cell membrane by direct efflux therefore protecting *Acinetobacter* from arachidonic and omega 3 fatty acids, which are host-derived antibacterial fatty acids (Jiang, Hassan, *et al.*, 2019). Furthermore, motility and AdeIJK are linked, whereby the deletion of the OMF, AdeK, results in a loss of twitching but not surface associated motility (Corral *et al.*, 2021). Finally, the interruption by IS elements of repressor gene *adeN*, and resultant over expression of *adeIJK*, increases virulence in *A. baumannii* and reduces the ability to form biofilm (Saranathan *et al.*, 2017).

Another RND pump characterised in 90% of *A. baumannii* clinical isolates is AdeFGH. AdeFGH has a low nucleotide identity to AdeABC and AdeIJK but is more like BpeEF-OprC and CeoAB-OpcM from *Burkholderia* species (Coyne, Rosenfeld, *et al.*, 2010). AdeFGH can confer a multidrug resistance phenotype when it is over expressed, to fluoroquinolones, chloramphenicol, trimethoprim, clindamycin, tetracyclines, tigecycline

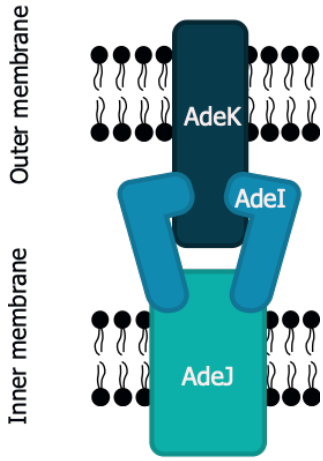


Figure 1.6: Schematic of RND efflux pump AdeIJK. AdeIJK is a tripartite pump, where AdeI is the periplasmic adaptor protein, AdeK is the outer membrane protein channel and AdeJ is the RND transporter protein, where compounds bind. AdeIJK is one of the main RND efflux systems in *Acinetobacter*. Figure and legend based upon (Damier-Piolle *et al.*, 2008).

and sulfamethoxazole, however under laboratory conditions the pump is not expressed (Coyne, Courvalin, & Périchon, 2011). AdeFGH can also export SDS, acridine orange, safranin and ethidium bromide (Coyne, Rosenfeld, *et al.*, 2010). AdeFGH is regulated by a helix-turn-helix LysR-type regulator found upstream called AdeL (Coyne, Rosenfeld, *et al.*, 2010).

In addition to the three RND pumps mentioned, there are another two Ade systems that have been characterised, although they are not commonly found in *A. baumannii*. AdeDE has been characterised mainly in *Acinetobacter pittii*, where it exports aminoglycosides, carbapenems, ceftazidime, fluoroquinolones, erythromycin, tetracycline, rifampicin and chloramphenicol (Bergogne-Bé, Zin, & Towner, 1996; Chu, Chau, & Houang, 2006; Coyne, Courvalin, & Périchon, 2011). AdeDE is proposed to work with another outer membrane protein for example AdeC or AdeK but research to prove this has yet to be published (Coyne, Courvalin, & Périchon, 2011). The second system, AdeXYZ, has >97% DNA sequence identity to AdeIJK and is again found in *A. pittii* and *A. baylyi* where, due to sequence similarity, it is likely to export similar substrates to AdeIJK but the exact profile is currently unknown (Chu, Chau, & Houang, 2006; Coyne, Courvalin, & Périchon, 2011). The remaining RND efflux pumps are CzcABCD, a heavy metal ion transporter characterised in *A. baumannii*, AbeD also characterised in *A. baumannii* that exports ceftriaxone, gentamicin, rifampicin, tobramycin and benzalkonium chloride, ArpAB an amikacin and tobramycin exporter and lastly AcrAB a tobramycin, acriflavine and colistin exporter found in *A. nosocomialis* (Kornelsen & Kumar, 2021).

ArpAB is involved in a phenotypic colony phase variation between opaque and translucent colonies in *A. baumannii* AB5075 (Tipton, Farokhyfar, & Rather, 2017). Immediately upstream of *arpAB* is *arpR* a divergently transcribed TetR regulator that can repress the expression of *arpAB*. This study also showed that colonies in the opaque phase were less motile (surface motility) and less virulent in a *Galleria mellonella* model of

infection (Tipton, Farokhyfar, & Rather, 2017).

1.5.4 Infections

Acinetobacter can cause a broad range of infections including bacteraemia, implant-device associated infections, skin and soft tissue infections and even pneumonia and urinary tract infections (Wong *et al.*, 2017). Many cases of *Acinetobacter* infections are nosocomial, but community acquired infections are also reported. *Acinetobacter* infections disproportionately affect those with weaker immune systems (infants and adults >75 years old) (United Kingdom Health Security Agency, 2021). The United Kingdom Health Security Agency (UKHSA) produced an *Acinetobacter* surveillance report in 2021, where they documented the main causative agents of *Acinetobacter*-derived bacteraemia in England. *A. lwoffii* was the leading causative agent, responsible for 30% of *Acinetobacter* hospital acquired bacteraemia in 2020 and *A. baumannii* was the next most prevalent, causing 21% (United Kingdom Health Security Agency, 2021), Table 1.5. The same report documented the incidence of *Acinetobacter* blood-stream infections in England as between 1.25 - 1.66 per 100,000 people. In the UK, *Acinetobacter* does not fall under mandatory healthcare associated infection surveillance, so often specific epidemiological data is limited. In the Queen Elizabeth Hospital, in Birmingham, United Kingdom, *A. lwoffii* is mainly isolated from burns, wounds and the skin (O'Shea, personal communication, 2023). *Acinetobacter* infections often have the highest incidence in low to middle income countries such as Bangladesh and Thailand, where *Acinetobacter* infections are the most common HAIs (Gandra *et al.*, 2020). Specifically, in Thailand between 2011 and 2014 there were 123.5 cases per 100,000 people of HAIs, of which *Acinetobacter* species represented 3% (3.705 per 100,000) (Rhodes *et al.*, 2019). In addition to human infections, *A. baumannii* and *A. lwoffii* cause the most *Acinetobacter*-derived animal infections (Maboni *et al.*,

Table 1.5: Number of *Acinetobacter*-derived blood stream infections in England, UK. Data from United Kingdom Health Security Agency (2021).

Species	2016		2017		2018		2019		2020	
	No.	%	No.	%	No.	%	No.	%	No.	%
***Acinetobacter* spp.**	**873**	**100**	**870**	**100**	**849**	**100**	**937**	**100**	**882**	**100**
A. baumannii / calcoaceticus	175	20	170	20	150	18	175	19	188	21
A. haemolyticus	8	1	15	2	10	1	6	1	6	1
A. johnsonii	31	4	29	3	36	4	28	3	31	4
A. junii	30	3	29	3	29	3	23	2	20	2
A. lwoffii	311	36	282	32	279	33	317	34	265	30
A. nosocomialis	1	<1	1	<1	3	<1	7	1	6	1
A. parvus	4	<1	4	<1	6	1	5	1	6	1
A. pittii	10	1	17	2	37	4	18	2	23	3
A. radioresistens	17	2	19	2	16	2	28	3	33	4
A. ursingii	51	6	79	9	81	10	92	10	105	12
Acinetobacter spp., other named*	58	7	26	3	26	3	40	4	41	5
Acinetobacter spp., sp. not recorded	177	20	199	23	176	21	198	21	157	18

* Including A. *beijerinckii, A. bereziniae, A. dijkshoorniae, A. dispersus, A. guillouiae, A. gyllenbergii, A. proteolyticus, A. schindleri, A. towneri, A. variabiliss.*

2020). *A. lwoffii* and *A. baumannii* are both found in hospitals and are resistant to desiccation, irradiation and biocides, Section 1.5.5, which explains their ability to persist in settings such as intensive care wards (Jawad *et al.*, 1998; Manian *et al.*, 2011; Rathinavelu, Zavros, & Merchant, 2003).

A. lwoffii is a commensal of the human skin and is often found in the oropharynx (Regalado, Martin, & Antony, 2009). *A. lwoffii* is also thought to have protective roles, where it induces a pro-inflammatory immune response that guards against developing asthma in a murine model (Alashkar Alhamwe *et al.*, 2022). Additionally, *A. lwoffii* has been shown to modulate the immune system via the skin protecting it from allergens (Fyhrquist *et al.*, 2014). For example, heat-killed *A. lwoffii* administered to skin induces a T helper cell response and the production of interleukin 10 (IL-10), an anti-inflammatory cytokine, protecting the host against systemic allergic sensitisation and inflammation (Debarry *et al.*, 2010; Alashkar Alhamwe *et al.*, 2023). In addition, the immunomodulatory

role in asthma is also due to the production of IL-10 (Alashkar Alhamwe *et al.*, 2022). Work in mice has shown that the presence of *A. lwoffii* reduces inflammation through a reduction of both macrophages and eosinophils, with a shift to recruitment of a specific type of M2 macrophage cells, where M2 possess anti-inflammatory properties (Kang *et al.*, 2022). Conversely, *A. lwoffii* can cause a variety of infections, often in immunocompromised human hosts (Ku *et al.*, 2000) and fish (Cao *et al.*, 2018; Zhang, Dou, *et al.*, 2023; Dadar, Adel, & Zorriehzahra, 2016). Whilst *A. baumannii* is not part of the normal flora of human skin it can easily colonise both the skin and upper respiratory tract and it is thought to be able to do this, in part, by the production of acinetobactin, which sequesters iron away from commensal species (Knauf *et al.*, 2022).

A. lwoffii therefore is naturally found both in the skin but can also survive in aquatic environments (fish infections) (Regalado, Martin, & Antony, 2009; Cao *et al.*, 2018). In addition to this environmental strains of *A. lwoffii* can be used in bioremediation, such as for the degradation of hydrocarbons and phenol (Al-Hadithi, Al-Razzaq, & Fadhil, 2017; Xu *et al.*, 2021). Therefore, this species can survive in a wide range of environments such as high salt environments (skin, salt water) and heavy metal environments for bioremediation.

The *Acinetobacter calcoaceticus – (Acinetobacter) baumannii* (*acb*) complex also causes infections. The members of this complex include *A. baumannii* and closely related species (*A. calcoaceticus, A. lactucae, A. nosocomialis, A. pittii*, and *A. seifertii*) (Püntener-Simmen *et al.*, 2019). These species can all cause human infection and are grouped together due to the difficulty in distinguishing between them using traditional clinical methods, for example matrix-assisted laser ionization time-of-flight mass spectrometry (MALDI-TOF). Common treatment options for an infection that is highlighted as *acb* complex include ceftazidime (cephalosporin, β lactam), carbapenems (β lactam), sulbactam (β lactamase inhibitor), piperacillin/tazobactam (β lactam), cefepime (cephalosporin, β lactam), aminoglycosides, fluoroquinolones, polymyxins (lipopeptide),

minocycline (tetracycline), doxycycline (tetracycline) and tigecycline (tetracycline) (Michalopoulos & Falagas, 2010). The British National Forumlary (BNF) recommends β lactams for both community and hospital acquired blood stream infections, with penicillin firstly and meropenem if the organism displays resistance (BNF, 2023).

As mentioned previously, infections may also be caused by other *Acinetobacter* species. If an infection was determined to be caused by an antibiotic-sensitive *Acinetobacter* species, cephalosporins (ceftazidime/ cefepime) and carbapenems (e.g. imipenem) are recommended initially (Tas, Oguz, & Ceri, 2017). To determine antibiotic resistance, hospitals can measure the minimum concentration of a given antibiotic needed to stop the bacteria from growing. In addition to an often increased susceptibility to antibiotics, non-*acb Acinetobacter* can also be less virulent. For example, a study in *Galleria mellonella* found that *A. baylyi* and *A. lwoffii* are less virulent than *A. baumannii* (Peleg *et al.*, 2009), the mechanisms for these differences are currently unknown. Murine models have also been used to mimic *A. baumannii* infections such as sepsis and meningitis but *A. baumannii* strains often exhibit low virulence in mice so high titres of bacteria need to be administered (McConnell, Actis, & Pachón, 2013).

Research is currently being done to understand the mechanisms of *A. baumannii* infection in the blood. It is already clear that there is strain variation when it comes to the ability of *A. baumannii* isolates to survive and grow in human serum, owing to differential sensitivities to human complement proteins (Kamuyu *et al.*, 2022). Decreased sensitivity to complement in *A. baumannii* is mediated by the expression of CipA, a plasminogen-binding protein. CipA bound plasminogen leads to the degradation of fibrinogen and complement protein C3b (Koenigs *et al.*, 2016). C3b is central to all classical component pathways and therefore the opsonisation of bacteria and tagging for phagocytosis are impaired (Lookeren Campagne, Wiesmann, & Brown, 2007).

Furthermore, some clinical isolates of *A. baumannii* can infect, replicate and persist

within macrophage vacuoles (phagosomes/phagolysosomes) and this phenotype is dependent on type I secretion systems, which was previously discussed in Section 1.5.2.4. The ability to colonise macrophages is not specific to *Acinetobacter* and other pathogens such as *Salmonella*, *Staphylococcus aureus* and *Mycobacterium tuberculosis* can also behave as intracellular pathogens (Mitchell, Chen, & Portnoy, 2017). Survival in macrophages is challenging for pathogens, especially given that intracellular bacteria are often engulfed within phagolysosomes that are extremely bactericidal, acidic and hydrolytic environments (Nguyen & Yates, 2021). Similarly to *S. aureus*, *A. baumannii* is able to survive within phagolysosomes, at least in part, due to the expression of catalase enzymes, which degrade reactive oxygen species (Sato *et al.*, 2019). Macrophages are not the only cells which *Acinetobacter* can interact with, for example Figure 1.7 shows *A. baumannii* attached to a human alveolar epithelial cell (McConnell, Actis, & Pachón, 2013). *A. baumannii* has also been shown to evade killing by human neutrophils, where neutrophils can also phagocytose bacteria during an infection (Bhuiyan *et al.*, 2016). When compared with other Gram negative pathogens, such as *Pseudomonas aeruginosa*, *A. baumannii* does not as readily induce the formation of neutrophil extracellular traps, which can lead to the lysis of bacterial cells (Kamoshida *et al.*, 2015). Despite this, traps can be induced by the production of reactive oxygen species, which are also produced in response to a bacterial infection by phagocytes (Garcia-Patino, Garcia-Contreras, & Licona-Limon, 2017). It is now understood that neutrophils are essential immune cells in the clearance of an *A. baumannii* infection, where they are rapidly recruited and elicit bactericidal effects (Faassen *et al.*, 2007; Lazaro-Diez *et al.*, 2017).



Figure 1.7: Scanning electron micrograph of *A. baumannii* cells attached to a human alveolar epithelial cell. *A. baumannii* coccobacilli are shown attached to the surface of lung carcinoma cell line, A549 cells. Figure reproduced from (McConnell, Actis, & Pachón, 2013).

1.5.5 Resistance

Some *Acinetobacter* can persist and cause prolonged infections in part because they can exhibit high levels of antibiotic and biocide resistance. *Acinetobacter* may also be resistant to irradiation and desiccation.

1.5.5.1 Antibiotic Resistance

Antibiotic resistance phenotypes seen in *Acinetobacter* are usually from the *acb* complex, where carbapenem resistant *Acinetobacter baumannii* (CRAB) is such a problem it is listed on the WHO priority pathogen list, Figure 1.3. CRAB is found globally and is a leading cause of morbidity and mortality (Higgins, Dammhayn, *et al.*, 2010; Isler *et al.*, 2019). In CRAB bacteraemia a mortality rate of 57.5% is seen after 30 days (Lee, Kim, *et al.*, 2022). As such, new treatment options are being explored for CRAB, for example cefiderocol, a novel β lactam (Isler *et al.*, 2019). Antibiotic resistance is seen in many classes of drugs, the most common *Acinetobacter* mechanisms are outlined below.

β lactam

β lactam drugs are often used first to treat *Acinetobacter* infections, such as cephalosporins and carbapenems (Michalopoulos & Falagas, 2010). β lactamases are enzymes that hydrolyse the β lactam ring, inactivating the antibiotic (Strynadka *et al.*, 1992). These enzymes can be broadly divided by class based upon sequence similarity. Classes A, C and D are serine β lactamases, whereas class B are metal β lactamases. Class A serine β lactamases include penicllinases and cephalosporinases, class B metallo-β-lactamases act upon carbapenems, class C act upon cephalosporins and class D β lactamases (prefix OXA) include extended spectrum β lactamases (Evans & Amyes, 2014; Ali *et al.*, 2018). Examples include TEM-1 and CTX-M, which are class A, NDM-1, which is class B, AmpC, which is class C and OXA-51, which belongs to class D (Rahman *et al.*, 2018; George, Jacoby, & Munoz-Price, 2005).

A. baumannii intrinsically expresses an AmpC-type cephalosporinase at low levels, which does not affect drug susceptibility until the presence of an insertion sequence, IS*Aba*1, upstream of the bla_{ampC} gene, which causes a reduced susceptibility to cephalosporins (Poirel, Bonnin, & Nordmann, 2011). Insertion sequences (and transposons) are mobile genetic

elements that move between replicons and can also be found on plasmids (Salgado-Camargo *et al.*, 2020). Antibiotic resistance genes are often linked to insertion sequences (Partridge *et al.*, 2018). AmpC production has also been documented in a small number of *A. lwoffii* strains from India but the mechanism was not determined (Singla *et al.*, 2014).

A. baumannii also expresses OXA-51 (a β lactamase found on the chromosome). OXA-51 normally provides low level carbapenemase resistance, which can be increased by the presence of IS*Aba*1 or IS*Aba*9 insertion sequences upstream (Turton, Ward, *et al.*, 2006; Figueiredo, Poirel, Papa, *et al.*, 2009). *A. lwoffii* also carries a class D β lactamase (OXA-134), but it is not always expressed and therefore does not always contribute to reduced carbapenem susceptibility (Figueiredo, Poirel, Seifert, *et al.*, 2010). Carbapenemase-carrying plasmids are the leading cause of carbapenem resistant *A. baumannii* infections (Mosqueda *et al.*, 2014).

A. baumannii can also harbour class A β lactamases, such as TEM-1, which confers resistance to sulbactam (Krizova *et al.*, 2013). TEM enzymes are named after a patient, Temoniera, who carried the *E. coli* strain where the first TEM was found (Datta & Kontomichalou, 1965; Ruiz, 2018). In addition, in class A, are CTX-M enzymes, which provide resistance to cefotaxime. CTX is the acronym for cefotaxime and it was found in Munich, hence CTX-M (Cantón, González-Alba, & Galán, 2012). Globally, *A. baumannii* isolates have been identified encoding CTX-M genes, which again reduces the number of available drugs to treat patients (Nagano *et al.*, 2004; Shakil & Khan, 2010; Potron *et al.*, 2011). At the time this thesis was written, there were no reported cases of *A. lwoffii* containing either TEM or CTX-M genes.

Finally, are the class B β lactamases, such as NDM. NDM, New Dehli β lactamase, is named after where it was first discovered (Walsh *et al.*, 2011). NDM-1 has, to date, been described in 3 *A. lwoffii* strains on plasmids. In addition to this, NDM-1 has been

found in many different isolates of *A. baumannii* (in addition to *Escherichia, Klebsiella* and *Enterobacter* species) (Caporaso *et al.*, 2010; Pfeifer *et al.*, 2011; Boulanger *et al.*, 2012; Decousser *et al.*, 2013). NDM-1 confers resistance to all β lactam antibiotics apart from monobactams, therefore the expression of it in *Acinetobacter* can vastly change treatment options for infections (Baraka *et al.*, 2020). The incidence of NDM-1-encoding *A. baumannii* seems far greater than the incidence in *A. lwoffii*, but this may be biased by the lack of papers published on *A. lwoffii* and fewer genomes sequenced.

Aminoglycosides

Aminoglycoside modifying enzymes (AMEs) are expressed in *A. baumannii* leading to phenotypic resistance. AMEs can be either phosphotransferases, acetyltransferases or adenyltransferases and all three have been characterised in *A. baumannii* (Poirel, Bonnin, & Nordmann, 2011). In addition to AMEs are RNA methylase enzymes such as ArmA, which are also found in *A. baumannii* and modify the 16S rRNA (modifying the target of aminoglycoside drugs) (Nie *et al.*, 2014; Zhu, Wang, *et al.*, 2009).

Fluoroquinolones

Resistance to fluoroquinolones (and quinolones) often occurs due to mutations in the genes, which encode the proteins where the drugs bind. In *A. baumannii* a S86L mutation in GyrA and an S80L mutation in ParC have been shown to increase the minimum inhibitory concentration of ciprofloxacin (Vila, Ruiz, Goni, *et al.*, 1995; Vila, Ruiz, Goñi, *et al.*, 1997). Resistance to quinolones is also mediated by efflux pumps, which is discussed more in Section 1.5.3. Resistance to quinolones in non-*baumannii Acinetobacter* is uncommon in the clinic (Espinal, Roca, & Vila, 2011).

Polymyxins

Polymyxin resistance often arises from mutations within the antibiotics target, lipid A. Mutations in *pmrA* and *pmrB* genes, which encode the two component system increasing transcription of PmrCAB and responsible for the modification of lipid A, have been shown to increase the minimum inhibitory concentration of colistin in *A. baumannii* (Charretier *et al.*, 2018). Colistin resistance in *A. lwoffii* has been increasing in England, Table 1.7, but the mechanism has yet to be identified. In Italy, colistin resistance has also been characterised in an *A. lwoffii* hospital isolate and, in this instance, it was attributed to the presence of *mcr*-1, which is a plasmid-borne colistin resistance gene first documented in *E. coli* (Caselli *et al.*, 2018). The way *mcr*-1 confers resistance is also by modifying lipid A, the target of colistin (Hinchliffe *et al.*, 2017) but it is still contested whether *mcr-1* can actually cause phenotypic resistance to colistin outside of laboratory testing (Zhu, Liu, *et al.*, 2021).

Tetracyclines

Cycline resistance is mostly mediated by *tet* efflux genes and *tet* antibiotic resistance genes. Tet efflux pumps are highly efficient transporters providing high level resistance to tetracyclines (Wang, Guo, *et al.*, 2014). In *Acinetobacter* the tetracycline efflux genes are often found on AbaR resistance islands, Section 1.2.3.1. An example of a non-efflux *tet* gene is *tetX*, where it catalyses the oxidation of tetracycline, destroying it, conferring high level tetracycline and tigecycline resistance (Forsberg *et al.*, 2015; Fang, Chen, *et al.*, 2020).

In summary, there is more evidence for antibiotic resistance in *A. baumannii* than there is in *A. lwoffii*, which agrees with local epidemioloigcal data for England (United Kingdom Health Security Agency, 2021).

1.5.5.2 Biocide resistance

In addition to multidrug and extensive drug resistance, *Acinetobacter* species can also be resistant to biocides. This is important in nosocomial pathogens such as *Acinetobacter* because biocides, such as benzalkonium chloride and chlorhexidine, are used to prevent bacterial infections by contamination and are widespread in healthcare-associated, industry and domestic settings (Liu, Fu, *et al.*, 2017). Resistance to biocides is mediated by resistance genes, such as *qac* genes, which encode efflux pumps to export biocides (Liu, Fu, *et al.*, 2017). *Acinetobacter* can also exhibit tolerance, where cells do not encode a resistance factor but can grow once the compound is removed, an example mechanism of this is dormancy (Balaban *et al.*, 2019). Dormancy is where cells have reduced metabolism and ATP and therefore, depending on the mechanism of action, it can take longer for an antibiotic/biocide to get into a cell and interfere with its target (Balaban *et al.*, 2019). Tolerance is often seen in biofilm lifestyles because within a biofilm there is a proportion of the cells which are slow or not growing, contributing to the need for higher concentrations of drugs (Ciofu *et al.*, 2022).

1.5.5.3 Desiccation tolerance

The ability of bacteria to survive on dry surfaces, such as those found in hospitals is advantageous and members of the *Acinetobacter* genus are extremely tolerant to desiccation and nutrient starvation. *Acinetobacter* is exemplary at colonising healthcare environments and this has lead to outbreaks of infection, for example during U.S. military operations in Iraq and Afghanistan where in excess of 700 military personnel were infected or colonised with *A. baumannii* (Scott, Deye, *et al.*, 2007; Moultrie, Hawker, & Cole, 2011; Farrow III, Wells, & Pesci, 2018). *A. radioresistens* strains have been found to survive an average of 157 days in conditions mimicking UK hospital surfaces (Jawad *et al.*, 1998). In addition the common causative agents of bacteriaemia, *A. lwoffii* and *A. baumannii*, can

survive for an average of 3 and 20 days respectively (Jawad *et al.*, 1998). In *A. baumannii* desiccation tolerance is linked to the two component regulator BfmRS, which is necessary for the expression of stress-related proteins in stationary phase (Farrow III, Wells, & Pesci, 2018). BfmRS has also been linked to biofilm formation, pili, motility, antibiotic resistance and virulence (Tomaras, Flagler, *et al.*, 2008; Geisinger, Mortman, *et al.*, 2018; Clemmer, Bonomo, & Rather, 2011; Gebhardt *et al.*, 2015; Liou *et al.*, 2014).

1.5.5.4 Irradiation resistance

Not only is *A. radioresistens* tolerant to desiccation it can also survive radiation exposure. Three isolates of *A. radioresistens* have been shown to survive gamma rays (Nishimura, Ino, & Iizuka, 1988). As with desiccation tolerance, cultures are least susceptible at stationary phase (Saha & Chopade, 2009). According to the WHO, food may be irradiated up to 10 KGy with no associated human health hazard. The study by Saha & Chopade (2009) found that despite *A. radioresistens* being able to survive up to 4x the radiation of other *Acinetobacter* species, 8 KGy was sufficient to inhibit growth of all *Acinetobacter* tested.

1.6 Aims of this thesis

A. baumannii and *A. lwoffii* are the most common causes of *Acinetobacter* bacteraemia in England (Table 1.5). However, *A. baumannii* has developed extensive drug resistance (Table 1.6) while *A. lwoffii* has remained generally drug sensitive (Table 1.7) (United Kingdom Health Security Agency, 2021). Both species occupy the same niches, cause clinically similar infections and are treated with similar antibiotics. Little is known about *A. lwoffii* and why it has not developed multidrug resistance like *A. baumannii*. The broad aim of this thesis is to understand why *A. baumannii* displays drug resistance and *A. lwoffii* seems not to and

Table 1.6: Resistance profiles of *A. baumannii* (*acb*) complex bacteraemia in England, UK, reproduced from United Kingdom Health Security Agency (2021).

Antimicrobial agent	2016			2017			2018			2019			2020		
	S (%)	S, IE (%)	R (%)	S (%)	S, IE (%)	R (%)	S (%)	S, IE (%)	R (%)	S (%)	S, IE (%)	R (%)	S (%)	S, IE (%)	R (%)
gentamicin	91	0	9	86	0	14	86	0	14	88	0	12	90	0	10
ciprofloxacin	85	2	14	84	3	13	88	0	12	74	12	14	50	35	15
imipenem	81	0	19	82	0	18	85	0	15	85	2	12	88	0	13
meropenem	90	2	7	87	2	11	89	1	9	90	2	8	89	2	9
colistin	100	0	0	100	0	0	94	0	6	90	0	10	80	0	20
piperacillin/tazobactam	77	7	16	73	12	15	78	1	21	70	5	25	76	3	21

S = susceptible; S, IE = susceptible (susceptible, increased exposure); R = resistant

Table 1.7: Resistance profiles of *A. lwoffii* blood stream infections in England, UK, reproduced from United Kingdom Health Security Agency (2021).

Antimicrobial agent	2016			2017			2018			2019			2020		
	S (%)	S, IE (%)	R (%)	S (%)	S, IE (%)	R (%)	S (%)	S, IE (%)	R (%)	S (%)	S, IE (%)	R (%)	S (%)	S, IE (%)	R (%)
gentamicin	100	0	0	99	0	1	100	0	0	98	0	2	100	0	0
ciprofloxacin	98	0	2	97	0	3	99	0	1	78	17	5	65	30	4
imipenem	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0
meropenem	100	0	0	99	1	1	99	0	1	98	1	2	99	0	1
colistin	100	0	0	100	0	0	83	0	17	100	0	0	100	0	0
piperacillin/tazobactam	96	0	4	95	2	3	97	0	3	92	1	7	92	0	8

S = susceptible; S, IE = intermediate (susceptible, increased exposure); R = resistant.

how we can use that to understand the evolution and selection of AMR in bacteria more broadly.

This thesis will investigate antibiotic resistance and infection-causing potential across the *Acinetobacter* genus with a focus on the two most common causes of *Acinetobacter*-derived bacteraemia, *A. baumannii* and *A. lwoffii*.

The overall aim of Chapter 3 is to determine how *A. lwoffii* and *A. baumannii* differ with respect to susceptibility to antibiotics. The hypothesis is that *A. lwoffii* is more susceptible to antibiotics than *A. baumannii* and potential reasons why this may be the

case are listed below.

- There are fewer antibiotic resistance and efflux pump genes within the genomes of *A. lwoffii* than *A. baumannii*, which make it less likely for *A. lwoffii* to demonstrate drug resistance
- *A. lwoffii* has DNA defence mechanisms that make it less likely to participate in horizontal gene transfer

To that end the following objectives will be met.

1. Determine the susceptibility profiles of both *A. baumannii* and *A. lwoffii* to a range of antibiotics
2. Find the presence of genes which encode for proteins enabling bacteria to display a reduced susceptibility to antibiotics
3. Find signatures of the horizontal acquisition of resistance genes within the genomes of both species
4. Determine the presence of mechanisms to prevent the addition of foreign DNA within the species' genomes
5. Determine if *A. lwoffii* and *A. baumannii* can evolve mutations leading to antibiotic resistance, and if they do, if they select for similar mutations

Chapter 4 aims to compare the biology, with a focus on virulence, between *A. lwoffii* and *A. baumannii* using both experiments and genomics.

The objectives of this chapter are to:

1. Compare the biology of *A. baumannii* and *A. lwoffii* under laboratory conditions

2. Measure the biofilm formed by strains of either species both under static and laminar flow conditions

3. Compare the virulence both *in vitro* and *in vivo* of either species

4. Use the available whole genome sequences to relate biofilm and virulence phenotypes with their genetic backgrounds

Finally, the aim of Chapter 5 is to determine the genomic differences of RND efflux pumps not only in *A. lwoffii* and *A. baumannii*, but across the *Acinetobacter* genus as a whole. By ascertaining which RND systems are present, it is possible to begin to find ways to inhibit efflux to increase (and in the case of *A. baumannii* restore) susceptibility to antibiotics. To this end, the following aims will be met.

1. Develop a method to search genome sequences for RND proteins

2. Determine the number of types of RND pumps across *Acinetobacter*

3. Compare the RND transporters of *A. baumannii* and *A. lwoffii*

Importance

By researching efflux and resistance genes in across *Acinetobacter*, and with a focus on *A. lwoffii* and *A. baumannii*, it might be possible to determine why *A. lwoffii* infections are mostly drug-sensitive and how likely it is that in the future *A. lwoffii* may develop resistance. Furthermore, it can help in the understanding of how best to reduce the rate of resistance and ensure *A. lwoffii* remains a treatable nosocomial pathogen. This work may also provide information on how *A. baumannii* is developing (and has developed) resistance and guide how to best treat both infections in a clinical setting.

Chapter Two

Materials and Methods

2.1 General methods

2.1.1 Bacterial strains used in this work

A. lwoffii 5867 (GCA_900444925.1), *A. baumannii* AYE (CU459141.1) and *A. baylyi* ADP1 ATCC 33304 (CR543861.1) were used as wild-type reference strains both in the laboratory and for bioinformatics analyses. *A. baumannii* AYE is a clinical strain responsible for an outbreak in France in 2001 (Vallenet *et al.*, 2008). *A. lwoffii* 5867 is an NCTC strain with a refseq available on the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/assembly/GCA_900444925). *A. baylyi* ADP1 was originally isolated from soil and is used as a model organism because it is naturally transformable and susceptible to antibiotics (De Berardinis *et al.*, 2008). In addition to the above strains a number of *A. baumannii* and *A. lwoffii* wild-type strains, listed in Table 2.1, were used to characterise either species.

Table 2.1: List of strains used in this work. Strains coloured in brown are of environmental origin (including animal), those in blue are human-associated, strains in black have an unknown isolation source and type strains are in **bold**.

Code	Description	Source/Reference
AL04	*A. lwoffii* clinical isolate	Katie Hardy, Heartlands Hospital, UK
AB05	*A. baumannii* AYE	(Fournier *et al.*, 2006)
AB06	*A. baumannii* UKA2	(Turton *et al.*, 2005)
AB07	*A. baumannii* NCTC 13302	Matthew Wand, UKHSA
AB12	*A. baumannii* ATCC 17978 Δ*adeIJK*	Ayush Kumar, University of Manitoba
AB16	*A. baumannii* NCTC 12156	(Bouvet & Grimont, 1986)
AB17	*A. baumannii* MPT156	Matthew Wand, UKHSA
AB18	*A. baumannii* All8	Matthew Wand, UKHSA
AB19	*A. baumannii* W1	Matthew Wand, UKHSA
AB20	*A. baumannii* NCTC 10303	(Stenzel & Mannheim, 1963)
AB21	*A. baumannii* NCTC 13302	Matthew Wand, UKHSA
AB22	*A. baumannii* NCTC 13423	(Turton *et al.*, 2006)
AB23	*A. baumannii* NCTC 13424	(Turton *et al.*, 2004)
AB24	*A. baumannii* UKA1	Matthew Wand, UKHSA
AB25	*A. baumannii* UKA2b	Matthew Wand, UKHSA
AB26	*A. baumannii* UKA9	Matthew Wand, UKHSA
AB27	*A. baumannii* UKA15	Matthew Wand, UKHSA
AL28	*A. lwoffii* 5867	Purchased from UKHSA
AL29	*A. lwoffii* NCTC 10309	(Stenzel & Mannheim, 1963)
AL31	*A. lwoffii* NCTC 5866	(Audureau *et al.*, 1940)

AL32	*A. lwoffii* MW156, isolated from endoscope rinsewater	Matthew Wand, UKHSA
AL33	*A. lwoffii* MW158, isolated from endoscope rinsewater	Matthew Wand, UKHSA
AY36	*A. baylyi* ADP1 ATCC 33304	Purchased from American Type Culture Collection
AL44	*A. lwoffii* E9	Benjamin Evans, University of East Anglia
AB49	*A. baumannii* ATCC 17978 Δ*adeABFGHIJK*	(Kornelsen *et al.,* 2021
AL50	*A. lwoffii* isolated from agriculture	Jolinda Pollock, SRUC
AL51	*A. lwoffii* isolated from agriculture	Jolinda Pollock, SRUC
AL52	*A. lwoffii* isolated from agriculture	Jolinda Pollock, SRUC
AL53	*A. lwoffii* isolated from agriculture	Jolinda Pollock, SRUC
AL54	*A. lwoffii* isolated from agriculture	Jolinda Pollock, SRUC
AL55	*A. lwoffii* isolated from agriculture	Jolinda Pollock, SRUC
AB57	*A. baumannii* ATCC 17978	(Piechaud *et al.,* 1951)
AB58	*A. baumannii* ATCC 17978 Δ*adeABFGHIJK* + pVRL2Z	This work
AB59	*A. baumannii* ATCC 17978 Δ*adeABFGHIJK* + pVRL2Z:Abayl*adeXYZ*	This work
AB60	*A. baumannii* ATCC 17978 Δ*adeABFGHIJK* + pVRL2Z:Abaum*adeIJK*	This work
AL61	*A. lwoffii* NCTC 11883	(Audureau *et al.*, 1940)
AL62	*A. lwoffii* NCTC 7976	(Audureau *et al.*, 1940)

2.1.2 Bacterial growth, storage and identification

Bacterial strains were stored at -80°C in cryogenic bead tubes and cultured in lysogeny broth (LB) (Merck) at 37°C, unless stated otherwise. When required for plasmid stability, selective antibiotics were added to cultures using the antibiotics in Table 2.3, Section 2.3.1. To verify the cryogenic stocks, all strains were routinely subject to 16S rRNA PCR using previously published primers (Muyzer, 1995) and subsequent Sanger sequencing of the amplicon (Eurofins). The PCR used primers 535 and 536, Table 2.2, the DNA was purified from agarose gels using a QIAquick gel extraction kit (Qiagen) and sequences were then searched on BLASTn (NCBI) to determine species level identification. In addition, all wild type *Acinetobacter* strains were subject to whole genome sequencing by Illumina (MicrobesNG). All sequencing was short-read and had >30x coverage. The raw data was trimmed to ensure high quality using Trimmomatic (Bolger, Lohse, & Usadel, 2014) and assembled using SPAdes (Bankevich *et al.*, 2012) by MicrobesNG. All subsequent bioinformatics steps were done by the author, as described in Section 2.2.

Table 2.2: Primer list

Primer	Amplifies	Sequence	Tm (°C)
p508	*adeJ*	ACAGTTACTAAATCTGGTGC	51
p509	*adeJ*	CTTTTAATCGAACCTCAGCA	52
p535	16S	CCTACGGGAGGCAGCAG	65
p536	16S	ATTACCGCGGCTGCTGG	67
p660	*adeI*	TTTAACTTTAAGAAGGAGATATACAAATGATGTCGGC TAAGCTTTGGG	51
p661	*adeK*	AGTGGTGGTGGTGGTGGTGCTTATTGCTTTTTTAAGT TCAGCACTAGATGGTTGA	51
p688	*adeY*	AACAAATGAATGGTCTGGATGGCT	54
p689	*adeY*	ACGGCTAAGCGGTTCAGAAA	54
p735	*adeX*	TGATATCGAATTCCTGCAGCCCGGGATGACGTCGGCT AAGCTTTGG	59
p736	*adeZ*	AATTGGAGCTCCACCGCGGTGGCGGCCGCTTATTTCT TTTTCAGATCTGCACTTGAAGGCTGATG	59

16S rRNA primers from (Muyzer, 1995). All other primers designed in this work.

2.2 Bioinformatics methods

2.2.1 Downloading sequences of *A. baumannii* and *A. lwoffii*

All available *A. baumannii* (6,127) and *A. lwoffii* (41) whole genome sequences on NCBI were downloaded (20/03/2022). Furthermore, all wild-type *A. baumannii* and *A. lwoffii* strains used in the laboratory were whole genome sequenced by Illumina (MicrobesNG) and those sequences were also added to the datasets. This gave a total of 6,137 *A. baumannii* sequences and 49 *A. lwoffii* sequences.

2.2.2 Quality checking

Assembly quality was measured using Quast v.5.0.2 (Mikheenko *et al.*, 2018) and sequences were binned according to N50 and Ns per 100 Kbp values. Sequences with N50 values >30,000 and <165 Ns per 100 Kbp were high enough quality to pass. N50 is the measure of the shortest contig that is at least 50% of the length of the genome. The cut-off was chosen because although N50 values >15,000 are deemed to be of a fine quality, a more discriminatory cut-off of 30,000 is preferred in the literature (Ellington *et al.*, 2017). The cut-off for Ns per 100 Kbp was informed by Quast, which highlights sequences with outlying values for Ns per 100 Kbp and it was kept consistent across the two species.

Following Quast, assemblies were checked for their average nucleotide identity (ANI) to reference sequences for either species (*A. baumannii* AYE CU459141 and *A. lwoffii* 5867 GCA_900444925.1) using fastANI v.1.31 (Jain *et al.*, 2018). A cut off of greater than or equal to 95.5% was used according to established criteria (Vijayakumar, Biswas, & Veeraraghavan, 2019; Walter *et al.*, 2020; Chan *et al.*, 2012). The next step highlighted duplicate assemblies, by using MASH (v.2.2.2) to find identical sequences (Ondov *et al.*, 2016). Identical sequences

(M = 0) were determined using mash sketch and dist to find mash distance of the sequence files. The mash output file was then analysed using a custom R script (https://github.com/C-Connor/MashDistDeReplication/blob/master/MashDistDeReplication.R) to print a file listing the duplicate genomes, which were then removed from subsequent analyses. Once the assemblies of low quality, or those belonging to different species, were removed there was a total of 38 *A. lwoffii* whole genome sequences and 4,906 *A. baumannii* whole genome sequences.

The final quality checking step was CheckM (v.1.1.3) (Parks *et al.*, 2015). CheckM checks for genome completeness and contamination. Cut off values of >95% completeness and <5% contamination were used based upon previously published research (Zou *et al.*, 2019; Parks *et al.*, 2015). The final number of sequences for each species was, 38 *A. lwoffii* and 4,809 *A. baumannii*.

2.2.3 Categorising the sequences based upon multilocus sequence type

All *A. baumannii* sequences were subject to categorisation by multilocus sequence type (MLST). MLST uses alleles found at 7 housekeeping genes in an organism to group species based upon similarity. The command line program MLST v.2.16.1 (Seemann, 2022) was invoked using both the Oxford and Pasteur schemes. Both the Pasteur and Oxford MLST schemes for *A. baumannii* are imperfect, the Pasteur scheme is less able to discriminate closely related sequences and the Oxford scheme is affected by homologous recombination of alleles (Castillo-Ramirez & Graña-Miraglia, 2019). There is not currently an *A. lwoffii* MLST scheme so the 38 sequences could not be typed.

2.3 Antibiotic resistance in *A. lwoffii* and *A. baumannii*

2.3.1 Determining the susceptibility of strains to antibiotics, biocides and dyes

The minimum inhibitory concentration of a range of antibiotics, biocides and dyes were determined using either the agar doubling dilution method according to CLSI guidelines (CLSI, 2020) or the broth microdilution method according to EUCAST guidance (EUCAST, 2023). A comprehensive list of compounds tested and their manufacturers can be found in Table 2.3, below. All MIC experiments were carried out using iso-sensitest broth (ISB) (Merck).

Table 2.3: List of compounds used in this work for susceptibility testing

Compound (Manufacturer)	Cat number	Solubility
Aztreonam (Fisher)	455410010	DMSO
Benzalkonium Chloride (Sigma)	12060	SDW
Carbenicillin (Fisher)	15414429	SDW
Cefotaxime (Fisher)	10084487	SDW
Chloramphenicol (Fisher)	10368030	70% Methanol
Chlorhexidine digluconate (Sigma)	C9394	SDW
Ciprofloxacin (Fisher)	13531640	SDW
Clindamycin hydrochloride (Generon)	A10227-50	DMSO
Crystal violet (Fisher)	10646972	SDW
Erythromycin (Fisher)	10338080	70% Ethanol
Ethidium bromide (Fisher)	10042120	SDW

Fusidic acid (Sigma)	F0881	SDW
Gentamicin (Fisher)	10224873	SDW
Meropenem (TCI Chemicals)	M2279	SDW
Moxifloxacin (Sigma)	PHR1542	60% DMSO
Nalidixic Acid (Fisher)	10373622	SDW
Novobiocin (Fisher)	15403619	SDW
Oxacillin (Sigma)	28221	SDW
Rifampicin (Fisher)	10533325	SDW
Tetracycline disodium salt (Fisher)	10460264	SDW
Ticarcillin (Sigma)	T5639	SDW
Triclosan (Sigma)	72779	70% Methanol

*SDW = sterile distilled water

2.3.2 Determining the presence of antibiotic resistance genes and efflux pumps

The next steps compared the genomes of *A. lwoffii* and *A. baumannii*. Initially, this focused on antibiotic resistance genes and evidence of horizontal gene transfer, but it also included genes associated with virulence, biofilm, and twitching. The presence of efflux and antibiotic resistance genes was determined using ABRicate (v.0.8.13) (Seeman, 2020a) and the Comprehensive Antibiotic Resistance Database (CARD) using cut off values of 50% coverage and 50% identity. ABRicate was run a second time using a custom database "AciRND" of known RND genes found in the *Acinetobacter* genus, these genes can be found at: https://github.com/emd803/Gene-Databases/tree/main. The same cut off values were used to find homologous genes in the different members of the genus.

The number of whole genome sequences differed substantially between *A. baumannii* and *A. lwoffii*, therefore to directly compare the number of efflux pump and antibiotic resistance genes, a random permutation sample was taken. Here, 38 (the same as the total number of *A. lwoffii* sequences) were sampled from the *A. baumannii* dataset and their gene presence information was extracted. The mean was calculated for the sample of 38 *A. baumannii* sequences and iterated 100 times, giving an average number of genes for a sample the same size as the *A. lwoffii* population. This number was compared to the mean number of genes in the *A. lwoffii* population (n=38) using an unpaired T-test with Welch's corrections to highlight any statistical significance. The random permutation test was invoked using a custom R script created by Dr. Chris Connor, University of Birmingham (unpublished).

2.3.3 Searching for evidence of horizontal gene transfer

To test the hypothesis that *A. lwoffii* does not accept exogenous DNA in the same way *A. baumannii* can, the genomes were searched for the presence of horizontal gene transfer. This included natural transformation via type IV pili, CRISPR-Cas systems, restriction modification systems, the presence of plasmids and prophages within the genomes and the total levels of homologous recombination in the genomes of both species.

2.3.4 Defining the presence of twitching genes

Another custom database, "twitching", was created using core and accessory genes linked to type IV pili, twitching and natural transformation in *A. baylyi* (Leong *et al.*, 2017). The database can be found at: https://github.com/emd803/Gene-Databases/tree/main. All whole genome sequences from both species were searched for the presence of homologs of

the *A. baylyi* genes with >50% identity and coverage. The number of twitching genes per genome for both species was compared using an unpaired T-test with Welch's corrections.

2.3.5 Sub-surface twitching motility of *Acinetobacter*

To try to match genotype to phenotye, the twitching motility of *Acinetobacter* strains was determined using an established protocol (Biswas, Machen, & Mettlach, 2019). Briefly, 25 mL no-salt LB plates [0.5% (*w/v*) yeast extract (Sigma Aldrich), 1% (*w/v*) tryptone (Sigma Aldrich) and 1% (*w/v*) agar (Sigma Aldrich)] were poured and left to solidify. A small well was made in the centre of the plates using a sterile p1000 pipette tip and 5 μL of culture (5 colonies suspended in 100 μL of PBS) was added to the bottom of the well. Plates were incubated for 16 hours and the twitching halo was stained with 1% (*w/v*) crystal violet (Sigma Aldrich) and the diameter was measured. This experiment was independently repeated four times. Twitching halos from *A. baumannii* and *A. lwoffii* were compared using an unpaired T-test with Welch's corrections.

2.3.5.1 Presence of *Acinetobacter* plasmids

To find the presence of *Acinetobacter* plasmids a replication (*rep*) gene database was used, derived from information in Lam *et al.* (2023), again, using ABRicate. Stricter cut off values of 80% coverage and 80% identity were used in order to identify only true *rep* genes and again searched all of the *A. baumannii* and *A. lwoffii* sequences. To determine the difference in the number of *rep* genes per sequence from either species a two-tailed T test with Welch's corrections was performed.

Since both species contained plasmids, 42 plasmids from *A. baumannii* (representing plasmids from each known lineage (Salgado-Camargo *et al.*, 2020)) and a random 42

plasmids, from NCBI, found in *A. lwoffii* were analysed. Firstly, the plasmid sequences were annotated using Prokka (v.1.14.6) (Seemann, 2014) and subject to functional categorisation using EggNOG Mapper (v.1.0.3) (Huerta-Cepas *et al.*, 2017). Subsequently, ABRicate was used to find the presence of resistance genes using the ResFinder database (Bortolaia *et al.*, 2020) and the presence of metal and biocide genes was determined using the BacMet database via BLASTp (v.2.9.0) (National Centre for Biotechnology Information, 2021) on the command line. Panaroo was also invoked to find core genes in plasmids belonging to *rep* groups R3-T25 and R3-T45 (Tonkin-Hill *et al.*, 2020).

2.3.5.2 CRISPR

To highlight CRISPR-Cas systems, the command line program CCtyper v.1.4.4 used (Russel *et al.*, 2020), which identifies and characterises CRISPR and Cas loci. The CRISPR_Cas.tab output file was searched for the presence of CRISPR sequences.

2.3.5.3 Prophages

The presence of prophages within the genomes was estimated using PHASTER (Arndt *et al.*, 2016). The presence of prophages has been linked to the acquisition of antibiotic resistance and virulence-associated genes. At the time of writing PHASTER was not available as a command-line program and therefore a random 10 *A. baumannii* and 10 *A. lwoffii* were manually searched using the web-interface of PHASTER. The presence of intact prophage DNA was used to indicate that a strain had previously been vulnerable to that phage infection.

2.3.5.4 Quantifying the amount of recombination in *A. lwoffii* and *A. baumannii* genomes

Since recombination level is indicative of horizontal gene transfer, a random 38 *A. baumannii* and all 38 *A. lwoffii* sequences were aligned to reference sequences using Snippy core (Seeman, 2020b), CU459141.1 *A. baumannii* AYE and GCA_900444925.1 *A. lwoffii* 5867 respectively. The Snippy core alignment was cleaned with snippy-clean_full_aln and input into Gubbins (Croucher *et al.*, 2015) for recombination analyses, using Fasttree (Price, Dehal, & Arkin, 2010) as the phylogenetic tree builder. Gubbins outputs a per branch statistic file, in which the number of recombination blocks, bases in recombinations and total genome length values were computed. The average number of each of these was compared against either species and a T-test was performed (Graphpad Prism).

2.3.5.5 DNA defence systems

DNA defence systems were searched for using DefenseFinder with the default parameters (Tesson *et al.*, 2022). DefenseFinder includes restriction modification systems, CRISPR-Cas and anti-phage systems. DNA defence system number was directly compared using a Mann Whitney statistical test in Graphpad Prism.

2.3.6 Selection of antibiotic resistant *Acinetobacter* mutants

Broth evolution experiments were conducted to determine if *A. baumannii* and *A. lwoffii* could evolve spontaneous mutations allowing their survival in three clinically relevant drugs (meropenem, ciprofloxacin and gentamicin).

Briefly, 5 mL of nutrient broth (Millipore, Sigma) was prepared and on day 0 single

colonies from AB18 or AL28 were inoculated into the tubes. The following conditions were set up: a negative (broth only) sterility control with no bacteria, two tubes without drug to simulate normal genetic drift/ mutations in AB18 and AL28 and then five tubes of AB18 and AL28 in the presence of increasing concentrations of each antibiotic. At days 0 and 1 the concentration was 1/4 MIC, days 2 and 3 was 1/2 MIC, days 4 and 5 was MIC and day 6 was a fold above MIC. A 1% transfer (50 μL of culture in 5 mL nutrient broth) was passaged each day and, if required, an antibiotic was added. At the end of the experiment, cells were spread plated on LB and mutants were picked at random for subsequent testing.

Mutant colonies were subject to whole genome sequencing by MicrobesNG to generate short-read data at >30% coverage and the presence of mutations was determined using the command-line tool Snippy (Seeman, 2020b). Subsequently, to determine if there were any changes in susceptibility to antibiotics, agar dilution MICs were carried out as per Section 2.3.1.

2.4 Investigating the biological differences between *A. baumaniii and A. lwoffii*

2.4.1 Measuring the growth of strains for 12 hours at 25°C, 30°C and 37°C

Overnight cultures of strains were diluted to an optical density at a wavelength of 600_{nm} (OD_{600}) of 0.1 and 100 μL of adjusted culture was added to a 96 well microtitre plate. The OD_{600} was measured for each well every ten minutes for 12 hours in a FLUOstar Omega (BMG Labtech) and then plotted on a graph. The average of three technical replicates was plotted for each biological replicate. All experiments had four biological replicates.

Generation time was calculated using the R package Growthcurver (Sprouffske & Wagner, 2016). Generation time was compared using unpaired T tests with Welch's corrections (Graphpad, Prism).

2.4.2 Scanning electron microscopy of *A. lwoffii* and *A. baumannii*

Overnight cultures of strains were diluted 1:50 for *A. baumannii* and 1:10 for *A. lwoffii* and grown to mid-log phase. Cells were centrifuged at 3,000 x*g* and washed with phosphate buffered saline (PBS). Cells were then resuspended in 2.5% glutaraldehyde and left for one hour before imaging. Cells were imaged by Theresa Morris, CEM Birmingham, at 25,000x, 10,000x and 5,000x magnification on a Thermo Fisher Apreo 2 SEM. Cell length analysis was done in ImageJ (Collins, 2007), where a random 100 cells were chosen and their length was measured. Cell length data was plotted as a box and whisker plot on Graphpad Prism. A one-way ANOVA with Tukey's multiple comparisons was used to calculate statistical significance (Graphpad Prism).

2.4.3 Measuring static biofilm formation using crystal violet

Strains were grown overnight in low-salt LB broth [0.5% (*w/v*) NaCl, 1% tryptone (*w/v*) (Sigma Aldrich), 0.5% yeast extract (*w/v*) (Sigma Aldrich)] and diluted to a CFU of ~$1x10^7$, where 200 μL was added to a 96 well microtitre plate and incubated for 48 hours at 37°C. Subsequent biofilms were washed with sterile distilled water and stained with 200 μL crystal violet (0.1% *w/v*, Sigma) for 15 minutes. The stain was washed with sterile distilled water and solubilised with 200 μL of 70% ethanol. The optical density at OD_{600} was measured to quantify the amount of biofilm stained in each well. The mean of three technical replicates were used to form one biological replicate value, which was repeated on four independent

occasions. Median and interquartile range values were calculated in Graphpad Prism and a Welch's unpaired T test was used to compare biofilm formation between species (Swift, 1997).

2.4.4 Measuring biofilm formation under laminar flow

The work in this section was done by Dr. Emma Holden at the Quadram Institute as part of a collaboration with the team of Professor Mark Webber. Biofilm formation under laminar flow was measured using the Bioflux system (Benoit *et al.*, 2010). Flow cells were primed with LB without salt at 5 dyne/cm^2 and seeded with approximately 10^7 cells. Attachment of the biofilms was promoted at room temperature for three hours and the plate was then incubated at 30°C with a flow rate of 0.3 dyne/cm^2. Biofilm images were captured using an inverted light microscope at 4x, 10x and 20x magnification after 12, 24 and 48 hours. Three images at distinct, random locations were taken. After 48 hours, the flow cells were pulsed at 5 dyne/cm^2 for 5 seconds to capture images of attached biofilm only. Experiments were performed with two technical and two biological replicates. Images taken were analysed using ImageJ (Collins, 2007), where the percentage of black pixels in the field of view was calculated as a proxy for biofilm growth. The median percentage coverage was then plotted on a graph with error bars showing the range of data on Graphpad Prism (Swift, 1997).

2.4.5 Searching for biofilm-associated genes

ABRicate was also invoked using a custom database of known *Acinetobacter*-specific biofilm associated genes, which was created based upon gene information from Hamidian *et al.* (2022). The database can be found at: https://github.com/emd803/Gene-Databases/tree/main. This custom database "vandb" was also searched using ABRicate where homologous

genes were identified that had >50% identity and coverage to the reference. The same database also included virulence genes and was used to highlight these in Section 2.4.11. The number of biofilm genes in each sequence per species was compared using an unpaired T-test with Welch's corrections (Graphpad, Prism).

2.4.6 Measuring the antibiotic susceptibility of biofilms

The concentrations of antibiotics required to eradicate established biofilms were determined using a method based upon a published protocol (Ravi, Aslam, & Veeraraghavan, 2019). Briefly, ~$1x10^7$ CFU/mL were grown overnight in ISB at 37°C using a Nunc peg lid in a 96 well plate (Thermo Fisher). The following day a fresh 96 well plate containing doubling dilutions of antibiotics were created and the peg lid was removed from the ISB-only and placed in the antibiotic plate. The plates were incubated for 16 hours at 37°C and bacterial growth was visually determined to define at which concentration growth was inhibited. The experiment was independently repeated three times. Antibiotics and biocides used are listed in Table 2.3.

2.4.7 Measuring the growth of strains in the presence of human serum

Growth in the presence of serum was done according to (Kamuyu *et al.*, 2022). Briefly, overnight cultures were diluted to 10^6 cells in LB and grown in either LB-only, 50% LB and 50% heat-inactivated serum or 50% LB and 50% normal human male serum (Merck, H4522). To heat-inactivate, serum was heated to 56°C for 30 minutes. Growth was measured for 24 hours at 37°C in the FLUOstar Omega (BMG Labtech). Again, generation time was calculating using Growthcurver (Sprouffske & Wagner, 2016).

2.4.8 Survival in human serum

In addition to growth, the survival of strains in the presence of serum was determined. Cells were grown to mid-log phase and diluted to 1x10^6 CFU/mL. 10 μL of cells were added to 90 μL of either normal human serum (Merck, H4522) or LB and incubated with gentle rocking (20 rpm) at 37°C. At 0 hours, 45 minutes, 1.5 hours, 3 hours and 24 hours, 10 μL was removed from each condition, serially diluted in PBS and spot plated to enumerate CFUs.

2.4.9 Determining if *A. baumannii* or *A. lwoffii* can attach to, get inside and or replicate within human macrophages

Human monocyte THP-1 cell line (ATCC TIB-202) was cultured in Roswell Park Memorial Institute (RPMI) Medium with Glutamax (Thermo Fisher) with 10% fetal bovine serum (Thermo Fisher) at 37°C and 5% CO_2. THP-1 monocytes were differentiated into macrophages by plating the cells and supplementing the medium with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) 48 hours prior to infection. Macrophages were infected according to Sycz *et al.* (2021), where a multiplicity of infection (MOI) of 100 was used. Attachment, uptake, and growth/proliferation (after 6 hours) were quantified. Attachment was determined by subtracting the number of intracellular bacteria (invasion) from the total number of bacteria associated with macrophages (and within macrophages). Extracellular bacteria were killed using gentamicin at either 100 μg/mL or 1,000 μg/mL for AB05. A one-way ANOVA with Tukey's multiple comparisons was used to compare the CFU/mL of strains in each experiment (Graphpad, Prism).

2.4.10 *Galleria mellonella* model of *A. lwoffii* and *A. baumannii* infection

The virulence of clinical and non-clinical *A. baumannii* and *A. lwoffii* was determined using a *Galleria mellonella* model of infection. The protocol used was based upon (Peleg *et al.*, 2009). Briefly, *G. mellonella* larvae were infected by intrahemocoelic injection with 20 μL of culture, which contained 1x10^6 CFU/20 μL, through the last left pro-leg. The number of dead (melanazied) larvae was recorded every 24 hours for four days after inoculation, where 10 larvae were used for each condition. This experiment was repeated independently three times. Kaplan-Meier mortality curves were plotted and a Mantel Cox test was used to determine statistically significant results in Graphpad Prism (Swift, 1997).

2.4.11 Searching for virulence-associated genes

ABRicate was also invoked using a custom database of known *Acinetobacter*-specific virulence (and biofilm) associated genes, which was created based upon gene information from Hamidian *et al.* (2022). The database can be found at: https://github.com/emd803/Gene-Databases/tree/main. Homologous genes were identified that had >50% identity and coverage to the reference. The same database was used to find biofilm genes in Section 2.4.5. The number of virulence genes per sequence for each species was compared using an unpaired T-test with Welch's corrections in Graphpad Prism.

2.5 Broad genomic characterisation of the *Acinetobacter* genus

In order to determine how *A. lwoffii* and *A. baumannii* are related to the rest of the *Acinetobacter*, further analyses were conducted to include all 64 members, as of 05/07/2021, of the genus. Up to 4 Refseqs for each species were downloaded from NCBI, sequence list in doi:10.1099/mgen.0.000964, supplementary S2.

2.5.1 Presence of efflux pump genes

Using the "AciRND" database mentioned in Section 2.3.2, the genomes were searched for RND genes. AciRND can be found at: https://github.com/emd803/Gene-Databases.

2.5.2 Development of novel BLASTp search to find RND proteins

Amino acid sequences of characterised HAE proteins from 15 different Gram negative bacterial species and HME RND proteins from 7 different species were aligned in separate files using MAFFT (v.7) (Rozewicki *et al.*, 2019). The consensus sequence in >80% of the aligned sequences was taken from either alignment file to create a conserved residue file (appendix B), which BLASTp could use to search for other RND proteins within genomes from both *Acinetobacter* and other Gram negative species (National Centre for Biotechnology Information, 2021).

When validating the method to determine the number of RND proteins in other Gram negative species the following reference sequences were searched: *A. baumannii* AYE CU459141.1, *C. jejuni* NCTC 11168 GCA_900475265.1, *E. coli* K12 MG1655 NC_000913.3,

H. influenzae NCTC 8143 GCA_001457655.1, *K. pneumoniae* ATCC 43816 CP064352.1, *N. gonorrhoeae* FA1090 NC_002946.2, *P. aeruginosa* PAO1 GCA_000006765.1, *S. enterica* SL1344 GCA_000210855.2 and *S. flexneri* 5a M90T CP037923.

2.5.3 Defining *Acinetobacter* species that cause human infection

If an *Acinetobacter* species had been published to cause a human infection and appeared on Google Scholar before 05/07/2021, it was classified as being able to cause human infection. This method is not foolproof because there may be instances where a species can cause infection but it has not been published and therefore has been omitted in this search however, common causative agents should be captured.

2.5.4 Phylogenetic tree of *Acinetobacter*

The phylogenetic tree of *Acinetobacter* was created using a core gene alignment generated by Panaroo (v.1.2.3) as an input for Fasttree (v.2.1.10) (Tonkin-Hill *et al.*, 2020). Fasttree was implemented using the generalised time reversible model of evolution (Price, Dehal, & Arkin, 2010).

2.5.5 Creating a heatmap to display RND genes and an *Acinetobacter* phylogeny

The heatmap displaying the number of RND proteins and presence of RND genes was created using R packages gheatmap in ggtree, ggplot2 and treeio, where the phylogenetic tree and a matrix with the metadata were visualised (R Core Team, 2021; Wang, Lam, *et al.*, 2020; Wickham, 2016; Yu, 2020).

2.6 Are AdeXYZ and AdeIJK homologous systems?

To determine if AdeXYZ and AdeIJK are homologous RND systems and differ only in nomenclature, the genomic context, structure and function of AdeIJK/XYZ was assessed.

2.6.1 Genomic context and similarity of *adeIJK/XYZ*

To determine if *adeIJK* is found in the same genomic context in three species of *Acinetobacter*, *A. baumannii* AYE (CU459141.1), *A. lwoffii* 5867 (GC_900444925.1) and *A. baylyi* ADP1 (CR543861.1), 10 Kb up and down stream of *adeIJK* was extracted and visualised in Easyfig (v.2.2.5), with tBLASTx homology annotated (National Centre for Biotechnology Information, 2021; Sullivan, Petty, & Beatson, 2011).

2.6.2 Recombination and SNPs surrounding *adeABC, adeFGH* and *adeIJK*

To assess whether *adeABC, adeFGH* or *adeIJK* were found in a recombination hotspots, whole genome alignments of *A. baumannii* (random n=100 assemblies, from Section 2.2.1) were created using Snippy (4.6.0) (Seeman, 2020b) and Gubbins (v.3.1.3) (Croucher *et al.*, 2015), where Gubbins highlighted areas of recombination. Recombination predictions were visualised in Phandango (Hadfield *et al.*, 2018).

2.6.3 Modelling AdeIJK and AdeXYZ

Modelling analyses were conducted by Prof. V. N. Bavro, University of Essex. The distal binding pocket (DBP) properties the experimental eravacycline-bound structure of AdeJ

(PDB ID 7M4P, chain B), corresponding to the T-conformer, as well as the structures of AcrB T-conformers occupied by minocycline (PDB ID 4DX5.pdb chain B; (Eicher *et al.*, 2012)) and levofloxacin (PDB ID 7B8T, chain C; (Ornik-Cha *et al.*, 2021)) were analysed.

2.6.4 Cloning *adeIJK* from *A. lwoffii, A. baumannii* and *adeXYZ* from *A. baylyi*

The *adeIJK* efflux pump genes were cloned using NEB HiFi cloning, where primers 660 and 661 (Table 2.2) amplified *A. baumannii adeIJK*. Similarly, *adeXYZ* was amplified using primers 735 and 736 from *A. baylyi* ADP1. The operons were cloned into a pVRL2 vector (Lucidi *et al.*, 2018) with a gentamicin resistance marker. Vectors were digested with XmaI (New England Biolabs) and NotI-HF (New England Biolabs) restriction endonucleases. Cloning was confirmed by internal PCR checks for the RND gene in the *adeIJK/adeXYZ* operons using primers 508, 509 and 688, 689, Table 2.2, and subsequent whole plasmid sequencing of the insert (Plasmidsaurus). Cloned vectors were initially transformed into *E. coli* NEB 5α (New England Biolabs) for checking, then electroporated into *A. baumannii* ATCC 17978 Δ*adeAB* Δ*adeFGH* Δ*adeIJK* for phenotypic analyses. Electroporation conditions were as follows 2.5 Kv, 200 Ω, 20 μF.

2.6.5 Finding the substrate profiles of *adeIJK* and *adeXYZ*

Similarly to Section 2.3.1, the susceptibility of antibiotics was measured for the cloned strains to determine if the substrate profiles of *adeIJK* and *adeXYZ* were similar. Antibiotics used are listed in Table 2.3. Overnight bacterial cultures were made in the presence of appropriate selection (100 μg/mL gentamicin for pVRL2). Cultures were diluted to an optical density at 600_{nm} of 0.01 and grown for 16 hours in a 96 well plate in increasing concentrations of

compounds.

Chapter Three

Antibiotic resistance in *A. baumannii* and *A. lwoffii*

3.1 Background

According to the most recent UKHSA data, *A. lwoffii* is generally susceptible to a wide range of antibiotics, in stark contrast to *A. baumannii*, which is often resistant to multiple, structurally different antibiotics (United Kingdom Health Security Agency, 2021), Figures 1.6 and 1.7 (Chapter 1).

3.2 Aims and hypotheses

The aim of this chapter is to explore the differences in antibiotic susceptibility between both species and determine potential mechanisms behind this. The hypothesis is that *A. lwoffii* strains would be more susceptible to antibiotics than *A. baumannii* and there may also be differences in their genetic makeup to account for this. Therefore, the susceptibility of both

species to a range of antibiotics was determined, as well as the ability to evolve antibiotic resistance in the laboratory for three clinically relevant antibiotics. In order to explain some of the phenotypes observed for *A. lwoffii* and *A. baumannii*, whole genome sequences from both species were analysed, including the sequences of strains used in the laboratory experiments as well as sequences downloaded from NCBI.

3.3 Whole genome sequences

There were far fewer *A. lwoffii* sequences available than *A. baumannii* on NCBI, presumably due to the difference in research interest between the two organisms. However, as many assembled genomes of *A. lwoffii* as possible were downloaded, and the sequences were supplemented with whole genome sequences from the laboratory's collection of *A. lwoffii* strains. The laboratory collection of *A. lwoffii* strains was expanded as much as possible to compare the two species. The number of sequences downloaded, and passed through each quality step is shown in Figure 3.1. Despite the small dataset size, the *A. lwoffii* sequences came from a range of sources, including veterinary (particularly sheep infections), human and environmental. Furthermore, there is currently not a multilocus sequence typing (MLST) scheme for *A. lwoffii* and applying the MLST schemes from *A. baumannii* was unsuccessful, therefore it was not possible to measure the diversity of the strains this way.

However, for *A. baumannii* there were thousands of genome sequences available and two MLST schemes to cateogorise the data. Since both the Pasteur and Oxford MLST schemes are typically used, all *A. baumannii* sequences were typed using both schemes. Using the Pasteur scheme, there were 273 different STs found, including 213 sequences unable to be typed. The top 5 Pasteur STs were ST2 (n=2,832), ST1 (n=268), ST25

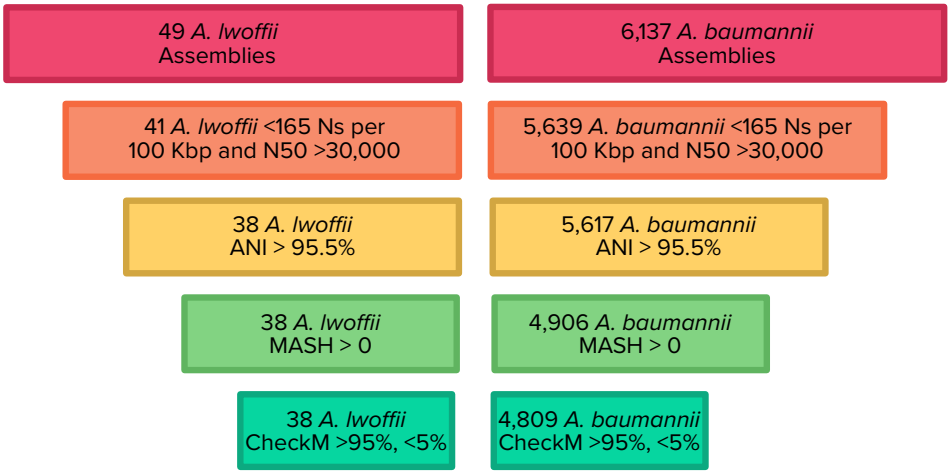


Figure 3.1: Quality checking workflow for whole genome sequences. *A. lwoffii* genomes are listed on the left and *A. baumannii* genomes on the right. The number of sequences that passed each step are listed in the boxes alongside any cut-off values. Assemblies were downloaded from NCBI and checked using Quast (orange step) (Mikheenko *et al.*, 2018), FastANI (yellow step) (Jain *et al.*, 2018), MASH (pale green) (Ondov *et al.*, 2016) and CheckM (darker green) (Parks *et al.*, 2015).

(n=130), ST79 (n=89) and ST78 (n=76).The Oxford scheme found 281 different ST groups, more than Pasteur, but it could not type 3,520 of the sequences (72%). The top 5 Oxford STs were ST350 (n=93), ST229 (n=80), ST281 (n=61), ST447 (n=44) and ST349 (n=43). The Pasteur scheme typed more of the *A. baumannii* sequences and was therefore the more useful scheme in this work given the large spread of the dataset.

3.4 *A. lwoffii* is more susceptible to a range of antibiotics than *A. baumannii*

The susceptibility to a range of clinically relevant antibiotics was measured using an agar doubling dilution method (CLSI, 2020; Andrews, 2001). Antibiotics from nine different classes were chosen to try and understand the susceptibility profiles as broadly as possible, this included first generation and subsequent generation antibiotics e.g., ampicillin and meropenem. Unfortunately, polymyxins were not included because they require testing with polypropylene plastics and the agar plates were made of polystyrene, which would have produced inaccurate results due to the drug binding to the plates. The strains selected for susceptibility testing were, at the time, all of the available laboratory isolates of *A. baumannii* and *A. lwoffii*. *A. lwoffii* is as, or more, susceptible than *A. baumannii* to all compounds tested, shown in Tables 3.1 and 3.2. Furthermore, according to the available EUCAST breakpoints (EUCAST, 2023), *A. lwoffii* was clinically susceptible to the compounds tested, whereas some *A. baumannii* were clinically resistant, e.g. AB05, which was resistant to ciprofloxacin and gentamicin - drugs used clinically to treat *Acinetobacter* infections. These data agree with previously published data indicating clearly that *A. lwoffii* is more susceptible to antibiotics than *A. baumannii* (United Kingdom Health Security Agency, 2021).

Table 3.1: MIC values (μg/mL) for *A. baumannii*

Strain	AMP	AZT	CEF	CHL	CIP	CLI	ERY	FUS	GEN	MER	MOX	NAL	NOV	OXA	RIF	TET
AB05	512	>8	>32	256	**>32**	128	32	128	**1024**	0.5	8	>1024	16	1024	16	128
AB16	512	>8	16	128	**2**	64	128	64	**16**	0.25	0.25	8	8	256	4	2
AB17	512	>8	32	256	**>32**	64	32	128	**16**	0.5	8	>1024	16	1024	16	128
AB18	64	>8	16	128	1	64	16	128	4	0.25	0.06	8	8	256	4	1
AB19	8	>8	32	128	**>32**	64	32	128	**16**	0.25	8	>1024	8	128	4	8
AB20	32	>8	16	128	**2**	64	16	128	4	0.25	0.12	16	8	512	4	2
AB21	1024	>8	>32	128	**>32**	64	64	64	**1024**	**>16**	8	1024	16	>1024	4	>128
AB22	1024	>8	>32	256	**>32**	128	64	128	**64**	1	>8	>1024	16	>1024	4	>128
AB23	1024	>8	>32	128	**>32**	32	1024	64	**>1024**	**>16**	>8	>1024	8	>1024	4	>128
AB24	1024	>8	>32	128	**>32**	64	16	128	**16**	**>16**	4	>1024	8	>1024	4	>128
AB25	1024	>8	>32	256	**>32**	128	64	128	**128**	**8**	>8	1024	32	>1024	4	>128
AB26	32	>8	32	256	1	64	16	256	4	0.25	0.12	16	16	512	4	4
AB27	1024	>8	16	128	**>32**	32	8	64	**1024**	**16**	4	1024	8	>1024	4	>128

AMP = ampicillin, AZT = aztreonam, CEF = cefotaxime, CHL = chloramphenicol, CIP = ciprofloxacin, CLI = clindamycin, ERY = erythromycin, FUS = fusidic acid, GEN = gentamicin, MER = meropenem, MOX = moxifloxacin, NAL = nalidixic acid, NOV = novobiocin, OXA = oxacillin, RIF = rifampicin, TET = tetracycline. EUCAST clinical resistance breakpoints are available only for CIP (>1 μg/mL), GEN (>4 μg/mL) and MER (>2 μg/mL). Values clinically resistant are shown in bold.

Table 3.2: MIC values (μg/ mL) for *A. lwoffii*

Strain	AMP	AZT	CEF	CHL	CIP	CLI	ERY	FUS	GEN	MER	MOX	NAL	NOV	OXA	RIF	TET
AL04	<1	1	2	2	0.06	2	<0.5	16	<2	0.03	0.5	2	8	16	0.5	0.5
AL28	<1	4	1	1	0.06	1	0.5	4	<2	0.12	<0.03	2	4	8	0.5	0.25
AL29	<1	>8	2	1	0.06	2	0.5	8	<2	0.03	<0.03	<1	8	32	0.5	0.25
AL31	<1	8	2	1	0.12	1	0.5	8	<2	0.03	<0.03	4	8	32	0.5	0.25
AL32	<1	4	1	1	0.03	4	0.5	8	<2	0.12	<0.03	2	8	16	0.5	0.25
AL33	<1	8	1	1	0.06	4	0.5	8	<2	0.12	<0.03	2	8	16	0.5	0.25

AMP = ampicillin, AZT = aztreonam, CEF = cefotaxime, CHL = chloramphenicol, CIP = ciprofloxacin, CLI = clindamycin, ERY = erythromycin, FUS = fusidic acid, GEN = gentamicin, MER = meropenem, MOX = moxifloxacin, NAL = nalidixic acid, NOV = novobiocin, OXA = oxacillin, RIF = rifampicin, TET = tetracycline. EUCAST clinical resistance breakpoints are available only for CIP (>1 μg/mL), GEN (>4 μg/mL) and MER (>2 μg/mL).

3.5 There are fewer ARGs and efflux pump genes in *A. lwoffii* sequences

3.5.1 Finding resistance and efflux pump genes using the CARD database

The minimum inhibitory data from Section 3.4 indicated that *A. lwoffii* is more sensitive to a broad range of antibiotics than *A. baumannii*. In order to relate the susceptibility data with the presence of ARGs (as found by the Comprehensive Antibiotic Resistance Database) in the genome sequences, the MIC values from Tables 3.1 and 3.2 were compared to the presence of genes in Table 3.3. Note that efflux pump related genes are discussed below and not included in Table 3.3. Finally, the presence of ARGs was also determined for all available whole genome sequences, not just the laboratory strains, Tables 3.4 and 3.5.

Table 3.3 shows the complement of resistance genes found in the laboratory strains. Generally, *A. lwoffii* contained OXA-type β lactamases but this did not always translate to reduced β lactam susceptibility when comparing it to the data in Table 3.2. For example, AL04 is one of the least susceptible *A. lwoffii* to oxacillin (32 μg/mL) and it contains one β lactamase - OXA-284. This specific β lactamase was not found in any other laboratory isolates of *A. lwoffii* and only in one other sequence deposited on NCBI: GCA_002321025.1. The presence of OXA-type β lactamases but not reduced susceptibility could be due to them not being expressed. Sometimes, resistance genes require insertion sequences upstream of them, where the insertion sequence promoter also leads to expression of the resistance gene (Poirel, Decousser, & Nordmann, 2003). AL28 (GCA_900444925.1) was the most susceptible to oxacillin (8 μg/mL) and also encoded a single β lactamase, OXA-283. OXA-283 and OXA-284 are very similar β lactamase proteins, both belonging to the OXA group OXA-

134. The presence of OXA-type β lactamases even in susceptible organisms like *A. lwoffii* is common. It has been suggested that several *Acinetobacter* species, including *A. baumannii* and *A. lwoffii* encode an intrinsic OXA-type beta lactamase (Périchon *et al.*, 2014).

Table 3.3: The presence of antibiotic resistance genes in laboratory *A. baumannii* (AB) and *A. lwoffii* (AL) isolates. Strains in bold have associated susceptibility data.

Strain	# ARGs	ARG types
AL04	3	OXA284, *golS*, *mdsB*
AL44	2	OXA282, *ugd*
AL51	3	OXA282, *golS*, *mdsB*
AL52	2	OXA283, *ugd*
AL54	1	OXA285
AL55	1	OXA283
AL28	2	OXA-283, *rsmA*
AL29	5	APH(3")-Ib, APH(6)-Id, OXA285, *sul*2, *udg*
AL31	1	OXA283
AL32	1	OXA276
AL33	1	OXA276
AB06	10	AAC(3)-Ia, ADC-30, APH(3")-Ib, APH(3')-Ia, APH(6)-Id, *lpsB*, OXA83, TEM181, *aadA*27, *sul*1
AB07	10	AAC(3)-IIe, ADC-1, APH(3")-Ib, APH(3')-Via, APH(6)-Id, *lpsB*, OXA24, OXA66, *aadA*27, *sul*1

AB17	18	AAC(3)-Ia, AAC(6')-Ian, ADC-11, ANT(2")-Ia, ANT(3")-IIa, APH(3")-Ib, APH(3')-Ia, APH(6)-Id, *lpsB*, OXA10, OXA69, VEB1, *aadA*27, *arr*2, *catI*, *dfrA*1, *dfrA*10, *sul*1
AB18	4	ADC-2, *lpsB*, OXA89, *aadA*27
AB19	6	AAC(6')-Ib7, ADC-11, *lpsB*, OXA69, *aadA*27, *sul*1
AB20	5	ADC-159, *lpsB*, OXA365, *aadA*27, *sul*2
AB22	11	AAC(3)-Ia, ADC-30, ANT(3")-IIa, APH(3")-Ib, APH(3')-Ia, APH(6)-Id, *lpsB*, OXA66, TEM181, *aadA*27, *sul*1
AB23	15	ADC-30, OXA23, *lpsB*, APH(3")-Ib, APH(6)-Id, APH(3')-Ia, *blaZ*, *mphE*, *msrE*, *armA*, *sul*1
		ANT(3")-IIa, *catB*8
		AAC(6')-Ib7

AB25	10	AAC(3)-Ia, ADC-30, APH(3")-Ib, APH(3')-Ia, APH(6)-Id, *lpsB*, OXA83, TEM181, *aadA*27, *sul*1
AB26	4	ADC-79, *lpsB*, OXA100, *aadA27*
AB27	10	AAC(3)-Iie, AAC(6')-Ian, ADC-79, APH(3")-Ib, APH(6)-Id, *lpsB*, OXA23, OXA64, *aadA*27, *sul*2
AB05	18	AAC(3)-Ia, AAC(6')-Ian, ADC-11, ANT(2")-Ia, ANT(3")-Iia 2x, APH(3")-Ib, APH(3')-Ia, APH(6)-Id, *lpsB*, OXA10, OXA69, VEB1, *aadA*27, *arr*2, *catI*, *dfrA*1, *dfrA*10, *sul*1

Furthermore, aminoglycoside (specifically gentamicin) resistance in the lab strains and the presence of aminoglycoside resistance genes was compared. The most susceptible *A. baumannii* strains were AB18, AB20 and AB26. AB18 and AB20 only encode one ARG with action against aminoglycosides: *aadA27*. AB20 also encodes an ANT-type and two AAC-type genes in addition to *aadA27*. The least susceptible strain was AB23 and also encoded aminoglycoside ARGs: APH(3")-Ib, APH(6)-Id, APH(3')-Ia, ANT(3")-IIa and AAC(6')-Ib. This strain encoded more genes than the more susceptible strains. For *A. lwoffii* all strains tested had MICs below the level of detection for gentamicin, indicating

they were highly susceptible. Again, a few aminoglycoside resistance genes were found, such as APH-type and AAC-type. It could be that these enzymes are not active against gentamicin but other aminoglycoside drugs.

To get a broader picture of the presence of resistance genes in these two species, the whole genome sequences of 38 *A. lwoffii* and 4,809 *A. baumannii* (laboratory sequences and those downloaded from NCBI) were searched for antibiotic resistance and efflux pump genes again using the Comprehensive Antibiotic Resistance Database (CARD) in ABRicate. To compare if the average number of ARGs differed between the two species, the dataset size disparity had to be accounted for. In order to do this the *A. baumannii* dataset was subject to a random permutation script, which sampled 38 *A. baumannii* sequences (the same size as the *A. lwoffii* dataset), 100 times at random. The mean of the 100 iterations (30) was compared with the mean number of genes highlighted in *A. lwoffii* (9). These numbers were then directly compared using an unpaired T-test with Welch's corrections, where p=<0.0001. Indicating that the total number of resistance and efflux pump genes in *A. baumannii* is significantly higher than in *A. lwoffii*.

Despite the difference in number of genes, both species encoded similar types of ARGs, where most corresponded to reducing susceptibility to β lactams and aminoglycosides. However, the number of distinct resistance genes differed, for example 198 different ARGs reducing susceptibility to β lactams were found across the *A. baumannii* sequences whereas only 11 different ARGs for β lactams was found across *A. lwoffii*. A similar pattern was seen for the other drug classes and efflux pumps, Figure 3.2. This is unsurprising given that the *A. baumannii* dataset is larger and therefore contains more distinct resistance genes for each drug class. In Figure 3.2 separate bars were included where efflux-related genes were removed because antibiotic resistance is not their primary function.

Tables 3.5 and 3.4 show the ARGs (and efflux pump genes) found in 50% of more

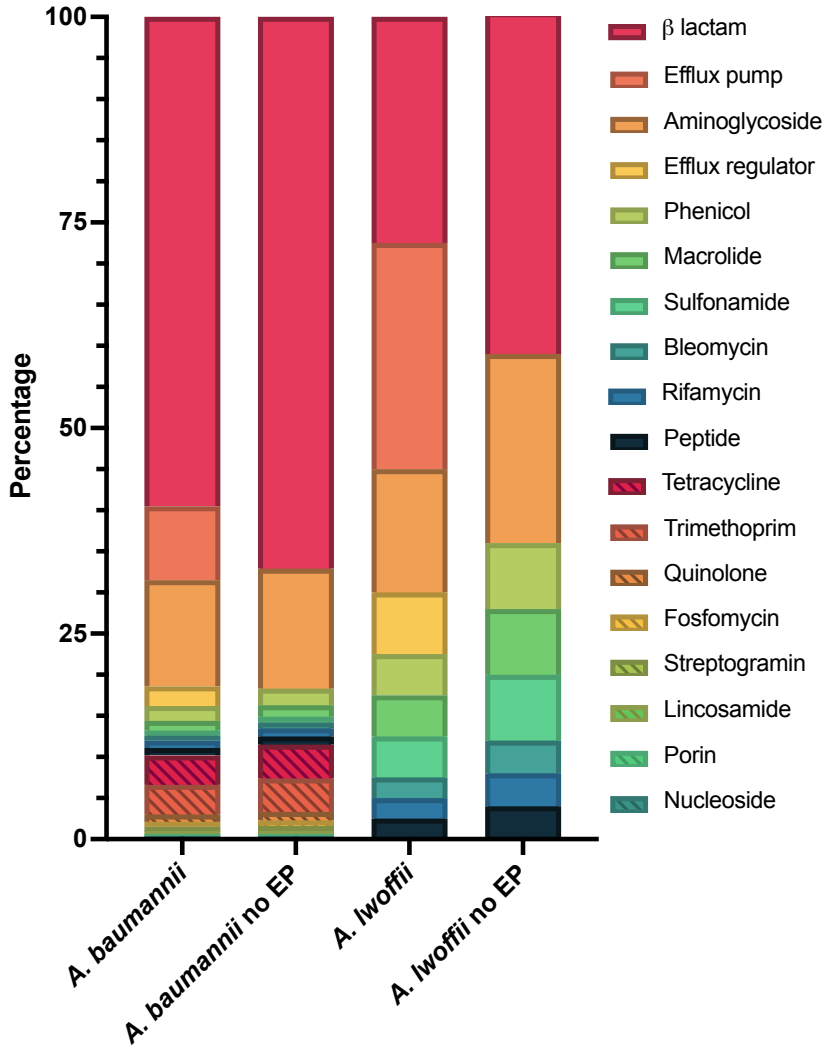


Figure 3.2: Predicted resistance gene types in *A. baumannii* and *A. lwoffii* whole genome sequences. Stacked bar charts show the proportion of resistance gene types found using an ABRicate search with the CARD database. Each ARG was interrogated to find its presence across the whole genome sequences, then all ARGs from the same drug family were combined to give the average percentage of resistance genes per gene family/ drug class. Bars are plotted with and without efflux-related genes ("no EP") for both species.

of the *A. baumannii* and *A. lwoffii* sequences. More ARGs were found in 50% or more *A. baumannii* sequences than the *A. lwoffii* sequences, such as OXA-type β lactamases, aminoglycoside modifying enzymes and macrolide resistance genes. Notorious resistance gene NDM-1, which confers drug resistance to a range of β lactam drugs, including ampicillin and cefotaxime was detected in both species. In *A. lwoffii* NDM-1 was found in 4 sequences (11%) and in *A. baumannii* it was found in 223 sequences (5%), however due to the difference in sample sizes it is difficult to directly compare the prevalence. UKHSA data suggested that *A. lwoffii* was trending towards colistin resistance, so *A. lwoffii* was searched more closely for any peptide resistance genes, of which one, *ugd*, was highlighted in 26% (n=10) of sequences (data not shown). The *ugd* gene codes for a phosphoethanolamine transferase, which allows Gram negative bacteria to evade the action of peptide antibiotics including polymyxins; *ugd* was also found in 2% of *A. baumannii* sequences n=97 sequences.

Table 3.4: Genes found in 50% or more of *A. lwoffii* sequences using the CARD database

Gene	% of sequences
abeM	100
adeI	100
adeJ	100
adeK	100
rsmA	100
abeS	58
OXA-283	50

Table 3.5: Genes found in 50% or more of *A. baumannii* sequences using the CARD database

Gene	% of sequences
abeM	100
amvA	100
lpsB	100
adeI	100
adeJ	100
adeK	100
adeF	100
adeG	100
adeH	100
abeS	100
adeL	100
adeN	100
adeB	97
abaQ	96
adeA	96
adeS	96
adeR	95
abaF	95
*aadA*27	88
adeC	79
OXA-23	63
APH(6)-Id	60
APH(3")-Ib	59

tetB	53
msrE	53
mphE	52
OXA-66	51

When focusing on efflux genes found by the CARD database, the MATE pump *abeM* and RND system *adeIJK* were highlighted in all sequences searched of both species, Table 3.4 and Table 3.5. 58% of *A. lwoffii* also encoded SMR efflux pump *abeS*. All *A. baumannii* sequences also had the RND system *adeFGH* and RND system *adeABC* was found in a proportion of sequences, where 97% had *adeB*, 96% encoded *adeA* and only 79% of sequences had *adeC*. The associated efflux regulators for *adeABC*, *adeRS*, were found in 95-96% of sequences and *adeN*, the regulator of *adeIJK* and *adeL*, the regulator of *adeFGH* were found in all *A. baumannii* sequences tested. Of the three efflux pump regulators found in *A. lwoffii*, only one was homologous to an RND regulator (*adeR)* and this was found in 5% of sequences only. Additionally, *rsmA* (also known as *csrA*) was found in 50% or more sequences of *A. lwoffii*, *rsmA* is a regulator of virulence and RND efflux from *Pseudomonas aeruginosa*. Therefore, whilst *A. lwoffii* does not encode a regulator with homology to *adeN*, it does contain one with homology to *rsmA*, which could be acting upon *adeIJK*.

3.5.2 Finding RND efflux pump genes using the AciRND database

The CARD search included a range of efflux pump genes, but was not specific to *Acinetobacter*. Therefore, in addition to finding efflux genes using the CARD database, a manually curated *Acinetobacter* efflux database (AciRND, described in Chapter 5) was employed across all the whole genome sequences. AciRND comprises all characterised RND genes across *Acinetobacter* to date, including heavy metal efflux genes. RND transporters

were searched for because if overexpressed they can confer clinical levels of drug resistance (Yoon, Courvalin, & Grillot-Courvalin, 2013). The RND systems searched for were: *adeABC* (*A. baumannii*), *adeFGH (A. baumannii)*, *adeIJK* (*A. baumannii* and *A. lwoffii* sequences), *adeXYZ* (*A. baylyi)*, *adeDE* (*A. pittii*), *czcABCD* (*A. baumannii)*, *arpAB* (*A. baumannii)* and *acrAB* (*A. nosocomialis).* Two species' sequences were included in the database for *adeIJK* because preliminary work had shown that *adeIJK* sequence conservation can differ at the nucleotide level and *A. baumannii* and *A. lwoffii* are phylogenetically distinct, potentially having low sequence identity despite both being described as encoding *adeIJK* in the literature.

The mean number of RND genes found in *A. lwoffii* genomes was 9 and 15 in *A. baumannii* genomes. All *A. lwoffii* sequences contained the RND system *adeIJK*, the inner membrane channel *czcD* and a gene with homology to the lone RND gene *abeD* (>98% coverage, >68% nucleotide identity). Additionally, 82% of sequences also contained the metal RND pump gene *czcA* and 74% had the PAP genes *czcB* and *acrA*. 66% of sequences encoded *arpB*, 34% had *acrB*, 25% had *arpA* and lastly 8% of sequences had *adeA* and/or *adeB*.

In the *A. baumannii* sequences, 100% of sequences encoded *czcA* (RND gene), *czcB* (PAP gene), *czcD* (additional inner membrane channel gene), *adeIJK*, *abeD adeFGH* and *arpB*. Additionally, 99% of sequences contained *arpA*, 97% had *adeB*, 96% had *adeA*, 79% had *adeC*, 28% of sequences had *czcC* (OMF) and finally 0.5% had *acrA* and 0.1% had *acrB*. The data for *adeABC* and *adeFGH* are exactly the same as determined by the CARD search, Section 3.5.1.

In summary, *A. lwoffii* and *A. baumannii* have only one RND system in common: *adeIJK* and both species contain some of the genes associated with metal efflux system *czc*, but *A. baumannii* generally encodes more RND efflux systems including an additional two

tripartite complexes *adeABC* and *adeFGH*. This discrepancy in efflux complement could also explain why *A. lwoffii* is more susceptible to antibiotics than *A. baumannii*, due to its lack of efflux systems and therefore reduced capacity to export structurally diverse compounds and less transporters available to provide time to acquire drug resistance mutations. Chapter 5 further contextualises the differences in RND efflux between the two species by evaluating the complement of efflux across the *Acinetobacter* genus more broadly.

3.6 Genomic signatures of HGT

It is clear from the results thus far that *A. lwoffii* is more susceptible to antibiotics and this is likely due, in part, to a lack of antibiotic resistance genes. These genes may be acquired via horizontal gene transfer (HGT) (Domingues & Nielsen, 2017; Eijkelkamp, Stroeher, Hassan, Paulsen, *et al.*, 2014), and therefore the lack of these genes in *A. lwoffii* could be due to a lack of HGT. Given that HGT occurs by a range of mechanisms, from phage infection to the dissemination of plasmids and even the uptake of exogenous DNA, different methods were used to look for evidence of HGT in both species' genomes.

3.6.1 Searching for the presence of plasmids

The presence of plasmid DNA was confirmed in the whole genome sequences by identifying *rep* (replication) genes, found by an ABRicate search using a previously published *rep* typing scheme (Lam *et al.*, 2023). Replication genes are essential for plasmid survival, coding for Rep proteins, which initiate replication of the plasmid. The presence of *rep* genes can be used as a proxy for plasmid presence in short-read whole genome sequences (Salgado-Camargo *et al.*, 2020). Initially, plasmid *rep* genes were searched for in the laboratory strains, Table 3.6. In the laboratory strains both *A. baumannii* and *A. lwoffii* had between 0 and 5 plasmids.

Table 3.6: Replication (*rep*) genes found in laboratory strains of *A. baumannii* and *A. lwoffii* using ABRicate

	Number of rep genes
AB05	4
AB18	0
AB19	1
AB20	2
AB25	2
AB27	0
AL04	2
AL28	2
AL29	5
AL32	5
AL33	4

Across the 4,809 *A. baumannii* and 38 *A. lwoffii* whole genome sequences there was an average of 2 *rep* genes per *A. baumannii* sequence and an average of 4 per *A. lwoffii* sequence, Figure 3.3. When using a random permutation test, followed by Welch's T-test, to more accurately compare between the species there is significantly more *rep* genes in *A. lwoffii* than *A. baumannii*, p = <0.0001.

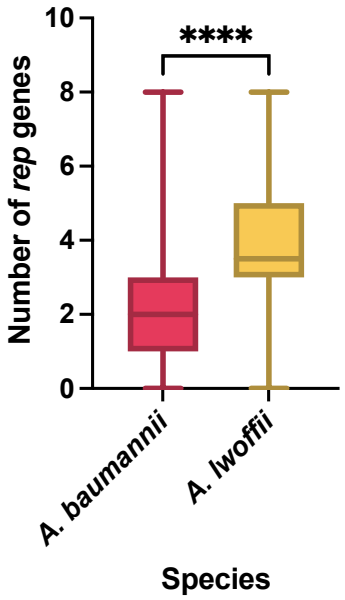


Figure 3.3: Number of *rep* genes found in *A. baumannii* and *A. lwoffii* sequences. Whiskers show the minimum and maximum number of *rep* genes found in the genomes by an ABRicate search and the line in the bar represents the mean value. *A. lwoffii* (yellow) sequences have significantly more *rep* genes than *A. baumannii* (pink), as determined by a two-tailed T-test with Welch's corrections. **** = p<0.0001.

In addition to the number of *rep* genes differing between the two species, the types of *rep* gene also differed. The presence of *rep* gene types across the whole genome sequences is shown in Table 3.7.

Table 3.7: Types of *rep* gene found in the *A. baumannii* and *A. lwoffii* genome sequences

rep type	% *A. baumannii*	% *A. lwoffii*
R1-T1	2	0
R1-T2	2	0
R1-T3	1	0
R1-T4	0	0
R1-T5	0	0
R1-T6	1	0
R2-T1	38	0
R3-T1	6	18
R3-T10	1	11
R3-T11	1	0
R3-T12	1	3
R3-T13	3	8
R3-T14	2	18
R3-T15	4	5
R3-T16	1	0
R3-T17	0	0
R3-T18	0	13
R3-T19	0	3
R3-T2	10	3
R3-T20	0	26
R3-T21	0	0
R3-T22	0	0
R3-T23	1	13

Table 3.7: Types of *rep* gene found in the *A. baumannii* and *A. lwoffii* genome sequences

rep type	% *A. baumannii*	% *A. lwoffii*
R3-T24	0	3
R3-T25	0	37
R3-T26	2	8
R3-T27	1	0
R3-T28	0	0
R3-T29	0	5
R3-T3	14	0
R3-T30	0	0
R3-T31	1	3
R3-T32	0	0
R3-T33	1	13
R3-T34	0	3
R3-T35	0	3
R3-T36	0	0
R3-T37	0	8
R3-T38	0	0
R3-T39	0	0
R3-T4	8	0
R3-T40	0	5
R3-T41	0	0
R3-T42	0	0
R3-T43	1	0
R3-T44	0	0

Table 3.7: Types of *rep* gene found in the *A. baumannii* and *A. lwoffii* genome sequences

rep type	% *A. baumannii*	% *A. lwoffii*
R3-T45	0	55
R3-T46	0	0
R3-T47	0	0
R3-T48	0	0
R3-T49	0	0
R3-T5	2	0
R3-T50	0	0
R3-T52	0	3
R3-T53	0	0
R3-T54	0	0
R3-T55	0	0
R3-T56	0	0
R3-T57	0	0
R3-T58	0	0
R3-T59	0	0
R3-T6	3	0
R3-T60	32	0
R3-T61	0	32
R3-T62	0	24
R3-T63	0	3
R3-T64	0	0
R3-T65	0	8
R3-T66	0	5

Table 3.7: Types of *rep* gene found in the *A. baumannii* and *A. lwoffii* genome sequences

rep type	% *A. baumannii*	% *A. lwoffii*
R3-T67	0	0
R3-T68	0	5
R3-T69	0	0
R3-T7	2	0
R3-T8	1	0
R3-T9	12	0
RP-T1	37	0
RP-T2	2	0
RP-T3	0	0
RP-T4	1	0
RP-T5	0	0

The most common *A. lwoffii rep* types were R3-T25/R3-T45, which were found in a total of 92% of sequences. R3-T25 and R3-T45 are 94.71% identical at the nucleotide level and therefore, although classed as different *rep* types, they are very similar and were historically known collectively as GR31. Therefore, it is possible that R3-T25/R3-T45 represents a native *A. lwoffii* plasmid, found in almost all sequences tested. Furthermore, all *A. lwoffii rep* genes highlighted in this search belonged to the R3 group.

However, the *rep* database used, like other tools, was built primarily for *A. baumannii* and therefore when cross-referencing the *rep* genes found using ABRicate and the number of plasmids known to be in complete NCBI *A. lwoffii* genomes, there were discrepancies, where ABRicate highlighted fewer *rep* genes than those in the whole genome sequences, Table 3.8. Whilst it is possible that some plasmids did not encode a *rep* gene, it is unlikely to be the

Table 3.8: Number of *rep* genes found in *A. lwoffii* complete whole genome sequences compared to the number of plasmids

	ABRicate *rep* search	NCBI plasmid number
GCA_019343495.1	3	5
GCA_009730095.1	3	4
GCA_015602705.1	0	0
GCA_019048305.1	2	5
GCA_019048525.1	5	8
GCA_019787625.1	8	17
GCA_019787645.1	3	5
GCA_002119785.3	4	10
GCA_004151415.2	6	10

case for all of the instances.

Therefore, all the *A. lwoffii rep* sequences available from complete genomes were extracted from NCBI and added into the ABRicate database of *rep* genes. Almost all *A. lwoffii rep* genes clustered in R3, including novel *rep* genes, which were not picked up using the original ABRicate search, supporting the idea that *A. lwoffii* prefers R3-type plasmids (Figure 3.4). There was one exception to this rule, which was the *rep* gene from CP080641.1 (pALWEK1.14). This *rep* gene clustered near to R1 and when it was searched against the entire NCBI database using BLASTn, it was only found in three *Acinetobacter* species (two listed as *Acinetobacter* sp. and the host *A. lwoffii*, from where it was found, >80% sequence coverage and identity). In addition to this novel *rep* gene, a further five *A. lwoffii* novel *rep* genes were found, this time all within the R3 clade - CP032104 1 (pALWEK1.11), CP080579 1 (pALWVS1.3), CP072552.1 (pH7-68), CP080580 1 (pALWVS1.4) and CP080643

1 (pALWEK1.16) and they clustered independently of known *rep* genes, Figure 3.4.

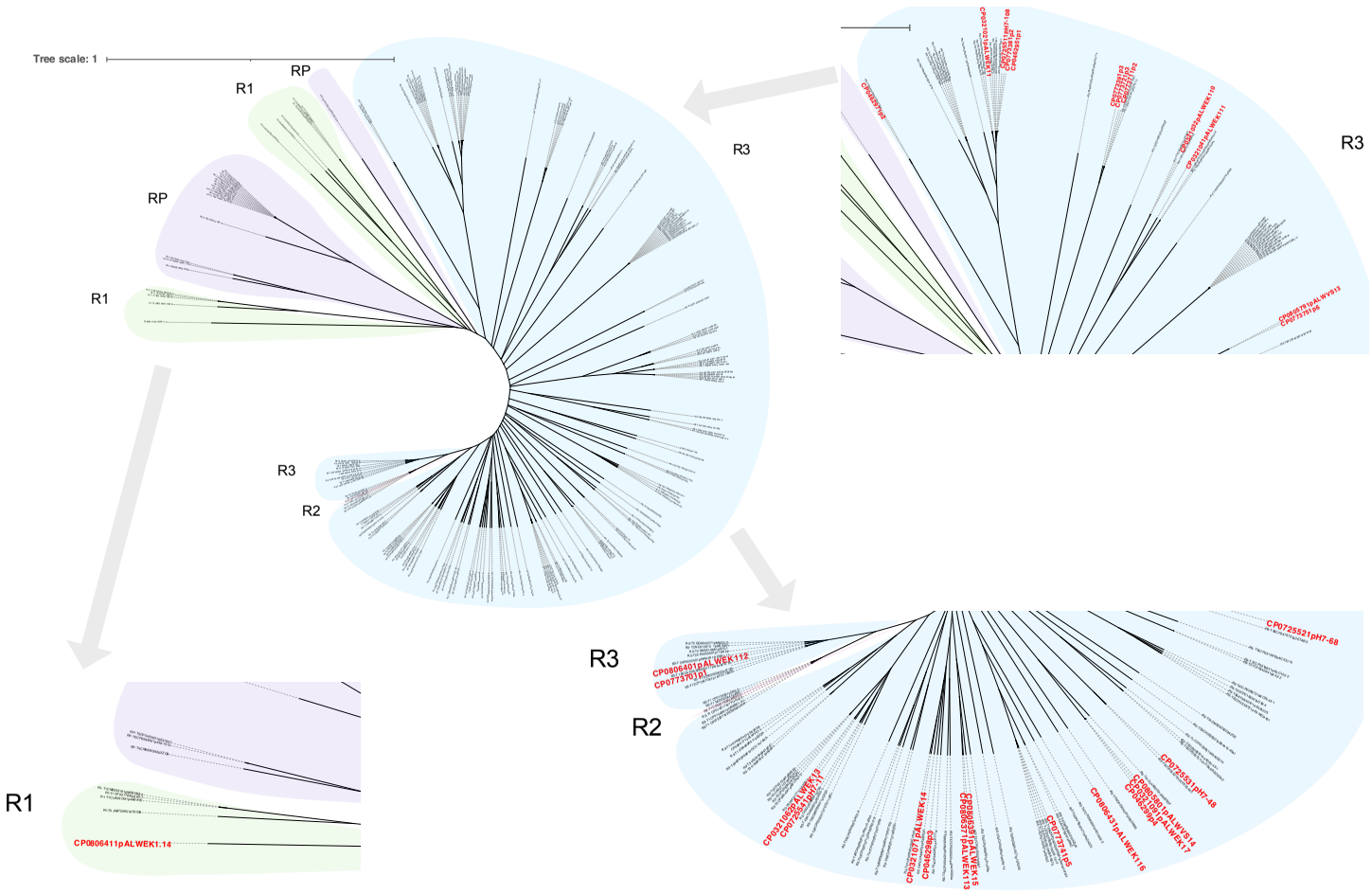


Figure 3.4: The distribution of *Acinetobacter rep* gene groups. *A. lwoffii rep* genes (red) cluster mainly in *rep* group R3 on a phylogeny created using a MAFFT alignment of the *rep* genes from (Lam *et al.*, 2023). Zoomed in areas of the tree show where additional *A. lwoffii rep* genes found in this work clustered. The tree is shaded according to *rep* type, i.e., R1 = green.

For *A. baumannii*, 38% of sequences encoded R2-T1 and 37% encoded RP-T1 *rep* types. *A. baumannii* had a total of 82 distinct rep types, including from RP, R1, R2 and R3 groups. When looking for the presence of R3-T25 and R3-T45 in *A. baumannii*, only 0.4% of sequences had these *rep* types, indicating that although highly prevalent in *A. lwoffii*, the same pattern is not seen in *A. baumannii*.

Given that both species can harbour plasmids, 42 *A. baumannii* and 42 *A. lwoffii* plasmids were looked at in more detail. The 42 *A. baumannii* plasmids represent two plasmids from each *A. baumannii* plasmid lineage as denoted in Salgado-Camargo *et al.* (2020). The 42 *A. lwoffii* plasmids included the full complement of plasmids sequenced from lab strains AL04, AL23, AL44 and AL51 and additional plasmids were downloaded at random from NCBI to make the dataset sizes equal. A full list of plasmid accession codes can be found in Table C.1, in the appendices. The plasmids were searched for the presence of antibiotic resistance (ResFinder, ABRicate) and metal resistance genes (BacMet database, BLASTp) and EggNOG was implemented to categorise open reading frames (ORF) into categories of function.

When looking most broadly at the differences between the plasmids sequenced from *A. baumannii* versus those sequenced from *A. lwoffii* there were not any clear differences in functional categories, Figure 3.5. COG categories were assigned to ORFs in each plasmid and then the percentage of genes related to i) metabolism, ii) information storage and processing, iii) cellular processing and signalling and iv) unknown were computed. The mean of each of these across either species' plasmids were determined and plotted below in Figure 3.5. The majority of genes found by EggNOG were of an unknown function and *A. lwoffii* plasmids had slightly more metabolism-related genes.

Across the 42 *A. baumannii* plasmids there was a total of 66 ARGs found by ResFinder, which were found on 21 of the plasmids. For *A. lwoffii* there were only 11

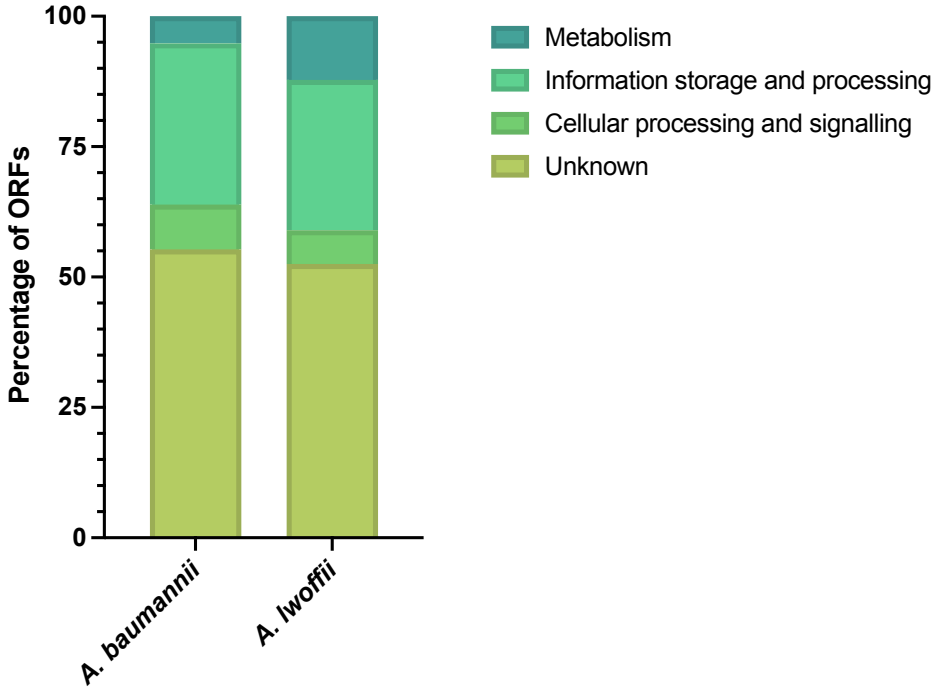


Figure 3.5: Percentage ORFs from *Acinetobacter* plasmids, divided into orthologous groups. Orthologous groups from plasmid ORFs were determined using EggNOG (Huerta-Cepas *et al.*, 2017). *A. baumannii* plasmid ORFs are shown on the left and *A. lwoffii* on the right. The ORFs in plasmids from either species were dedicated to similar physiological functions.

ARGs found, where 10 were found on a single plasmid and the remaining ARG was on a second plasmid. In summary, *A. baumannii* plasmids were more likely to encode ARGs than plasmids found in *A. lwoffii*.

A. lwoffii plasmids encoded a total of 49 metal resistance genes. The BacMet database also highlights biocide resistance genes, of which there were 55, including RND efflux pump proteins. The metal resistance genes in *A. lwoffii* provided protection against copper, cadmium, zinc, nickel, lead, arsenic, mercury, cobalt, iron, gold, tellurium, gallium and magnesium. The metal genes found in *A. baumannii* plasmids included those which provide resistance to nickel, mercury, zinc, gold, cobalt, antimony, arsenic, gallium, silver, chromium, tellurium, selenium and cadmium, this totalled 53 genes. As with *A. lwoffii* 28 biocide resistance genes were also found by the BacMet database. Of all the metals highlighted nickel, zinc, cobalt, iron, silver, copper, chromium and cadmium are biologically relevant metals. Additionally, nickel, mercury, zinc, arsenic, chromium and cadmium are found in soil environments. Furthermore, copper and zinc can be released within macrophage-derived phagosomes to poison intracellular bacteria, as well as being found in the environment (Sheldon & Skaar, 2019).

Given that *rep* types R3-T25/R3-T45 appeared in 92% of the *A. lwoffii* sequences tested, six of these *A. lwoffii* plasmids (from the plasmid sequences in appendix Table C.1) were further analysed. Panaroo was invoked to determine which genes are core to these plasmids and which are accessory. There were 63 genes core to all plasmids, of a total 434 genes found across the six plasmids. Many of the core genes encoded proteins of unknown function. In addition to the unknown genes core genes included, *srkA* (stress response kinase), *fatA* (ferric-anguibactin receptor for iron sequestering), *lldD* (L-lactate dehydrogenase) and *yqhD* (alcohol dehydrogenase). Furthermore, there were a number of IS elements which were core to this group of plasmids, for example IS5 family: IS*Aba*31 and IS*Caa*13, IS3 family: IS1236, IS982 family: IS*Acsp*2, and IS3 family: IS*Aba*2.

Accessory genes included metal genes (*czcC, czcD, mer1, copA, arsC, feoB* and *copB*), which varied in presence from 4/6 plasmids to 2/6 plasmids. Finally, the R3-T25/R3-T45 plasmids did not contain any antibiotic resistance genes.

Occasionally, the presence of RND efflux pumps has been seen on plasmids, for example in Dong *et al.,* (2022). Since RND efflux pumps can export a wide range of structurally diverse compounds, including antibiotics, out of the cell (Colclough et al., 2020) the evidence of them on plasmids is potentially alarming for the development of antibiotic resistance. Indeed, the presence of RND components on plasmids has been seen before for *A. baumannii*, for example pDETAB2 (Liu, Moran, *et al.*, 2021). As discussed earlier, the presence of ARGs, in general, on *A. lwoffii* plasmids is rare, compared with *A. baumannii*. When specifically looking at RND pumps, only one study shows *A. lwoffii* (AL_065) encoding a plasmid with an RND transporter and PAP gene on it (Naidu *et al.*, 2023). *A. lwoffii* AL_065 was isolated from a hospital bed rail in Pakistan. This plasmid (GCA_004151415.2) is also found in *A. baumannii* and when looking at it more closely it is an R3-T25 plasmid and therefore may have the potential to disseminate efflux genes across *A. lwoffii* more broadly. The RND pump is closest in homology to AdeB (Naidu *et al.*, 2023) and may therefore represent the acquisition of an additional RND system, which could be adaptive (like chromosomal AdeB) to environmental stressors, reducing the susceptibility of this strain to structurally different substrates than those exported by its native RND system: AdeIJK (Darby, Bavro, *et al.*, 2023). Furthermore, RND pumps also contribute to infection, so another potential result of a horizontally acquired RND pump could be increased virulence (Fernando & Kumar, 2013).

3.6.2 Prophage DNA within the genomes

In addition to the transformation and conjugation of plasmids, DNA may also be introduced into a bacterial cell via a bacteriophage infection. Once a phage has inserted into the genome, it is known as a prophage. Therefore, the whole genome sequences of a random 10 *A. baumannii* and a random 10 *A. lwoffii* were searched for the presence of prophage DNA using PHASTER (Arndt *et al.*, 2016). Ten sequences from each species were used because PHASTER is a web-based program and was not available on the command line (Arndt *et al.*, 2016). The hypothesis was that *A. lwoffii* might express a system to reduce or stop phage infection, which in turn could explain why it encodes fewer antibiotic resistance genes. ARGs, and bacterial DNA in general, are often mispackaged with phage DNA before a lytic phage goes on to infect another host.

The *A. lwoffii* results are shown in Table 3.9, where every sequence tested contained intact prophage DNA, indicating that the *A. lwoffii* sequences tested had been infected previously by phages.

Table 3.9: Presence of prophage DNA in *A. lwoffii* sequences

Sequence ID	Number of intact phages found	Types of phage
GCA_900699155.1	3	PHAGE_Paenib_Tripp_NC_028930(3), PHAGE_Acinet_Bphi_B1251_NC_019541(4), PHAGE_Acinet_vB_AbaS_TRS1_NC_031098(13)
GCA_000248355.2	2	PHAGE_Acinet_vB_AbaS_TRS1_NC_031098(12), PHAGE_Acinet_YMC11/11/R3177_NC_041866(4)
GCA_002119785.3	5	PHAGE_Entero_mEp235_NC_019708(6), PHAGE_Psychr_Psymv2_NC_023734(11), PHAGE_Stx2_c_1717_NC_011357(3), PHAGE_Escher_PA28_NC_041935(3), PHAGE_Stx2_c_1717_NC_011357(4)
GCA_013530545.1	2	PHAGE_Acinet_YMC11/11/R3177_NC_041866(13), PHAGE_Pseudo_phiCTX_NC_003278(14)
GCA_006538585.1	2	PHAGE_Acinet_YMC11/11/R3177_NC_041866(4), PHAGE_Acinet_vB_AbaS_TRS1_NC_031098(12)
GCA_009645785.1	1	PHAGE_Pelagi_HTVC010P_NC_020481(5)

		PHAGE_Acinet_vB_AbaS_TRS1_NC_031098(2),
GCA_009730095.1	3	PHAGE_Stx2_c_Stx2a_F451_NC_049924(3),
		PHAGE_Stx2_II_NC_004914(2)
		PHAGE_Pseudo_Dobby_NC_048109(14),
GCA_000301755.1	2	PHAGE_Entero_HK022_NC_002166(6)
GCA_011058205.1	1	PHAGE_Acinet_YMC11/11/R3177_NC_041866(19)
		PHAGE_Acinet_Bphi_B1251_NC_019541(4),
GCA_000836095.1	2	PHAGE_Acinet_vB_AbaS_TRS1_NC_031098(14)

10 *A. baumannii* sequences were also searched using PHASTER, Table 3.10. Unlike the *A. lwoffii* sequences tested, not all of the *A. baumannii* sequences contained the presence of intact propage DNA. However, every sequence did contain some partial phage DNA hits, data not shown. Furthermore, in *A. baumannii* the sequence with the most intact phage DNA had two prophages, whereas for *A. lwoffii* one sequence (GCA_002119785.3) had a total of 5 intact phages.

For both species, mainly phage characterised in *Acinetobacter* "Acinet" were found in the sequences searched. In addition to phages characterised from *Acinetobacter*, phage associated with other Gram negative species were also found, for example "Escher" *Escherichia*, "Pseudo" *Pseudomonas* and "Salmon" *Salmonella*. The PHASTER data indicates that both species are susceptible to phage infection and have had previous phage infection and therefore *A. lwoffii* is not particularly immune to this mechanism of HGT.

Table 3.10: Presence of prophage DNA in *A. baumannii* sequences

Sequence ID	Number of intact phages found	Types of phage
GCA_001862775.1	0	
GCA_018069995.1	0	
GCA_021173655.1	1	PHAGE_Acinet_vB_AbaS_TRS1_NC_031098(6)
GCA_009736385.1	2	PHAGE_Acinet_YMC11/11/R3177_NC_041866(26) PHAGE_Mannhe_vB_MhM_3927AP2_NC_028766(13)
GCA_001704685.1	0	
GCA_020251365.1	1	PHAGE_Mannhe_vB_MhM_3927AP2_NC_028766(11)
GCA_000581875.1	1	PHAGE_Salmon_SSU5_NC_018843(27)
GCA_904890255.1	0	
GCA_001673825.1	1	PHAGE_Acinet_vB_AbaS_TRS1_NC_031098(7)
GCA_019920745.1	0	

3.6.3 Natural transformation and twitching

Thus far *A. lwoffii* seems as capable as *A. baumannii* at acquiring plasmids and being susceptible to phage infection. Another mechanism for the horizontal acquisition of DNA is the ability to naturally transform DNA, which like the motility phenotype twitching, uses the type IV pilus in *Acinetobacter* (Leong *et al.*, 2017). Therefore, to first investigate this, the twitching ability of strains from both species was tested in the laboratory.

3.6.3.1 Both species display twitching motility under laboratory conditions

To determine if the 10 *Acinetobacter* strains could twitch and to what degree, a subsurface twitching motility assay was set up. Bacterial culture was inoculated into a well within an agar plate, incubated overnight at 37°C and the twitching halo was stained using crystal violet and measured, Figure 3.6A. There was large variation in the twitching capacity between strains. For example, AL29 twitched the least even when compared to other *A. lwoffii*. AL29 is an *A. lwoffii* isolated from urine in the 1960s (ATCC, 2023). Additionally, AB25 (*A. baumannii*) twitched less than other *A. baumannii* strains tested.

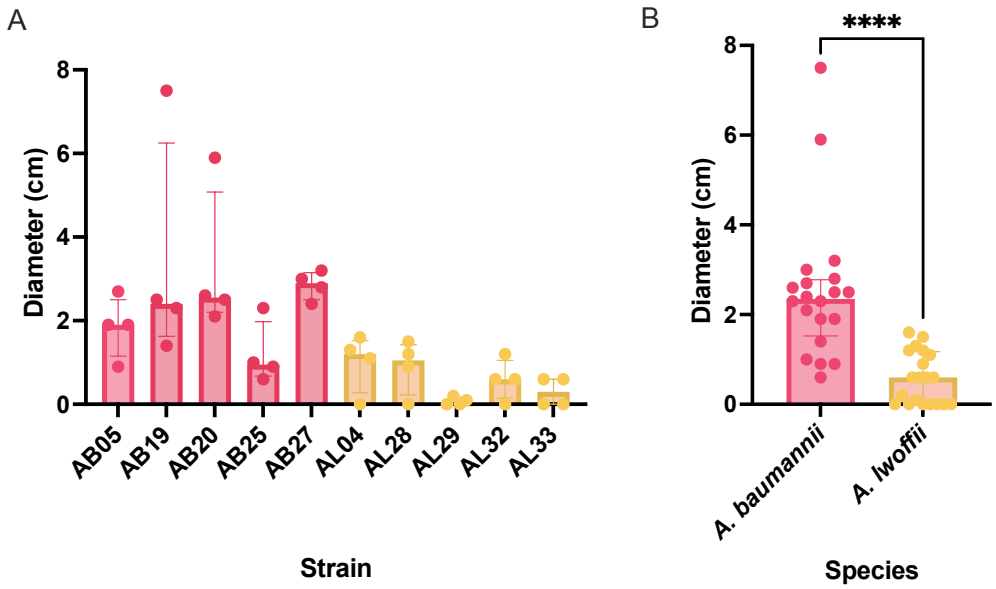


Figure 3.6: Twitching motility of *A. baumannii* (AB, pink) and *A. lwoffii* (AL, yellow). A: The average sub-surface twitching halo (cm) was stained using crystal violet and measured. Median and interquartile range are plotted. The experiment was independently repeated four times, using strains available in the laboratory. B: Data from panel A plotted by species. Median and interquartile ranges are shown and Welch's unpaired T-test was used determine if the differences were statistically significant between the two groups (**** p=<0.0001).

When comparing between the two species, *A. lwoffii* twitched significantly less than *A. baumannii* under laboratory conditions at 37°C (p=<0.0001), Figure 3.6B. Natural competence is a well characterised method of gaining exogenous DNA and is employed by other bacteria such as *Neisseria gonorrhoeae* (Hamilton & Dillard, 2006) and also *Acinetobacter baylyi* (Vaneechoutte *et al.*, 2006). Therefore, if the strains that twitched well were also more adapted at transforming foreign DNA it could be an advantage for the acquisition of ARGs and begin to explain why *A. baumannii* displays more drug resistance than *A. lwoffii*.

3.6.3.2 Both species encode genes related to type IV pili and natural competence

To determine if there was a difference in the complement of genes associated with natural transformation, twitching and type IV pili, the *A. baumannii* and *A. lwoffii* whole genome sequences were searched, using a custom ABRicate database with gene information from Leong *et al.* (2017).

Initially, the laboratory genomes were searched. In *A. lwoffii*, AL29 twitched the least and AL04 twitched the most, Figure 3.6A. Both of these strains had the same complement of type IV genes, however AL04 had two copies of *pilH*. When focusing on *A. baumannii*, AB19 twitched the most and AB25 the least. Again, both strains had the same complement of type IV genes but there was a 5% discrepancy in the coverage of the *comL* gene, where AB25 had a shorter region of homology, compared to the reference sequence (*A. baylyi* ADP1).

In summary, there was no clear genotype to phenotype link for the type IV genes and twitching. It is therefore possible that both species can be naturally competent but perhaps not under the conditions tested in this study.

When looking across all of the whole genome sequences, including those from NCBI, similar numbers of twitching genes were found in both species; there was an average of 15 twitching-related genes in *A. lwoffii* and 16 in *A. baumannii* whole genome sequences, however this was significantly higher in *A. baumannii* than *A. lwoffii* when comparing the number of twitching genes across all the sequences using an unpaired T-test with Welch's corrections ($p=<0.0001$), Figure 3.7. Again, because of the dataset size disparity, a random permutation and Welch's T test was done to confirm the statistically significant difference between the two species ($p = <0.0001$).

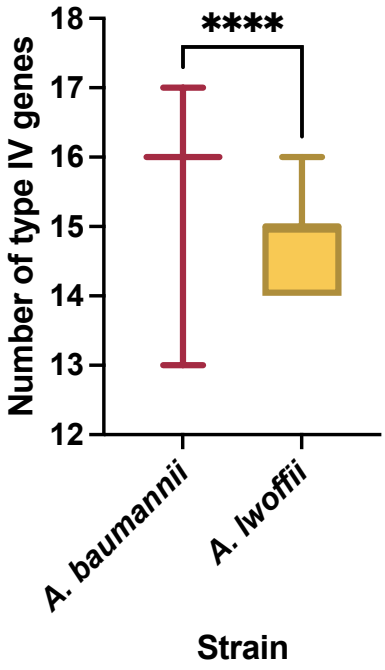


Figure 3.7: Number of type IV genes in *A. baumannii* and *A. lwoffii* genomes. Box plots show the average (darker line in the box) number of type IV associated genes in either *A. baumannii* (pink) or *A. lwoffii* (yellow) genomes, as determined by ABRicate. Whiskers denote the minimum and maximum number of type IV genes found. To compare the groups, an unpaired T-test with Welch's corrections was used (**** p=<0.0001).

A breakdown of the types of twitching genes and their presence in the *A. baumannii* and *A. lwoffii* sequences can be seen below in Table 3.11. Again, the two species had very similar types of genes associated with type IV pili and natural transformation, indicating that both species may be able to transform exogenous DNA (and by extension ARGs) from the environment under certain conditions.

Table 3.11: Presence of genes involved in type IV pili, natural transformation, twitching and competence as determined by ABRicate

Twitching gene	Product	% *A. lwoffii*	% *A. baumannii*
comL	Pilus assembly complex	100	100
comM		100	100
comN		100	100
comO		100	100
comQ	Secretin	100	100
pilB	Pilin assembly ATPase	100	100
pilC	Assembly platform	100	100
pilD	Prepilin peptidase	100	100
pilF	Secretin stability	100	100
pilG	Twitching signal transduction	100	100
pilH		100	100
pilR		100	100
pilT	Retraction ATPase	100	100
pilU		100	100
pilS	Twitching signal transduction	61	100
pilI	Prepilin peptidase	21	100
comA	DNA transport protein	0	5
comE	Pilin	3	0

3.6.4 Homologous recombination within the genomes

When foreign DNA enters a bacterial cell in order for it to be incorporated to the genome it must have some similarity (homology) to existing DNA within the bacterial cell, following this the DNA can be recombined to include host and foreign DNA. It is important to note that homologous recombination is effective for HGT but also has roles in general DNA repair and the life of the cell (Vos, 2009). Since homologous recombination is needed for HGT to be complete, i.e. the DNA must be transferred *and* incorporated into the host, the next section focuses on this.

To quantify if the overall amount of homologous recombination differs between the two species, a random sample of 38 *A. baumannii* and all 38 *A. lwoffii* genomes were analysed by Gubbins (Croucher *et al.*, 2015). Homologous recombination was used as a proxy for HGT, where the higher the recombination the more HGT occured across the genomes. Gubbins outputs two values for recombination a rho/theta and r/m ratio. Rho/theta is the ration of rates at which rho (recombination) and theta (point mutation) occurred and describes the relative number of recombination events compared to normal genetic drift point mutations. Furthermore, r/m is the ration of probabilities that a given position within a genome was altered by either recombination (r) or mutation (m) and how important recombination, relative to mutation, was in the diversification of the site (González-Torres *et al.*, 2019).

A. baumannii had a median rho/theta of 0.006877 and a median r/m of 0.114434. *A. lwoffii* had a median rho/theta of 0.0099935 and a median r/m or 0.233044. Therefore, *A. lwoffii* genomes contained more recombination relative to the occurrence of point mutations. Gubbins works by comparing the test sequences to a reference and then to each other to determine what is a mutation and what is recombination. When comparing the rho/theta values of either dataset using an unpaired two-tailed T-test, there is no significant difference between *A. baumannii* and *A. lwoffii*, therefore they have similar

levels of recombination and therefore likely similar levels of HGT. Finally, both species had positive rho/theta values, indicating recombination occurred more than point mutations.

Both the rho/theta and r/m values display strain-level differences. For example across the *A. baumannii* sequences there were rho/theta values between 0 and 0.4 and r/m values ranging from 0 to 135.8. A similarly varied picture was seen in *A. lwoffii* where rho/theta values also ranged from 0 to 0.4 and r/m values ranged from 0 to 77.6. Therefore, it is too simplistic to broadly compare recombination and HGT between the two species and when more sequences are available for *A. lwoffii*, it would be useful to redo these analyses based upon MLST or clonal groups of strains instead.

3.7 DNA defense mechanisms

3.7.1 Defense systems within *A. lwoffii* and *A. baumannii* genomes

Previous work in this chapter has highlighted that *A. lwoffii* encodes fewer ARGs than *A. baumannii* and this is independent of the ability to accept foreign DNA via HGT. Furthermore, *A. lwoffii* harbours distinct plasmid types to *A. baumannii* and often those plasmids do not encode ARGs. It is unclear thus far why *A. lwoffii* does not often harbour plasmids outside of *rep* group R3, but a potential explanation for this could be the expression of specific DNA defence systems. DNA defence systems include restriction modification systems, which cause double strand breaks in certain sequences of unmethylated DNA, toxin/antitoxin systems where a stable toxin can kill the host if the antitoxin-encoding DNA is lost and anti-phage mechanisms such as abortive infection, where cells lyse if infected by certain phage DNA before the phage can complete its replication cycle.

Consequently, DNA defence systems within *A. lwoffii* and *A. baumannii* were searched for using DefenseFinder (Tesson *et al.*, 2022). All *A. baumannii* (4,809) and *A. lwoffii* (38) sequences were searched. For *A. lwoffii* between 3 and 24 defence systems were highlighted per sequence, giving a median defense system number of 7 per genome. The most common types highlighted were Type I and Type IV restriction modification systems, toxin/antitoxin systems (PsyrTA) and antiphage systems (such as Abi2, abortive infection systems). In *A. baumannii* between 1 and 14 defence systems were found in any given genome with a median of 3 defense systems per genome. In *A. baumannii* the most common types were toxin/antitoxin systems (PsyrTA), Antiphage DNA phosphorothioate-based (SspBCDE), enzymatic anti-phage (Gao_Qat) and Type I restriction modification systems.

In order to determine if there were any significant differences in the number of DNA defence systems between either species, the random permutation script from Section 3.5.1 was invoked. Again, the *A. baumannii* dataset was sampled 38 times with 100 iterations and compared to the 38 *A. lwoffii* results. By using an unpaired two-tailed T test with Welch's corrections it was clear that *A. lwoffii* sequences have significantly more DNA defense systems in total than *A. baumannii* ($p=<0.0001$). Furthermore, there seems to be differences in the types of defense systems found in either species. This means that perhaps the difference in the amount of ARGs and the types of plasmids that are seen in *A. lwoffii* genome sequences is due to more DNA defence systems and therefore more stringent control over the integration of foreign DNA.

3.7.2 CRISPR-Cas systems

CRISPR-Cas systems are another method of DNA defence. CRISPR-Cas works as an immune system memory of previous foreign DNA it has encountered, for example phage DNA, and stores this information in spacers. Once the bacterium is reintroduced to this

DNA, proteins destroy it, protecting the cell. Since this memory is encoded within the bacterial genomes, it can be vertically passed down to daughter cells.

Although DefenseFinder highlighted some CRISPR-Cas systems, they were not the most abundant type of DNA defense system. To probe more closely at the CRISPR sequences within either species, the same genomes were subjected to a search using CCtyper (Russel *et al.*, 2020). When looking at CRISPR-Cas systems, *A. lwoffii* genomes had between 0 and 1 system per sequence with a total of 2x I-F systems found in the 38 genomes. In *A. baumannii*, however, an average of 0 to 2 CRISPR Cas systems were found per sequence with a total of 721 I-F systems found in the 4,809 genomes. An I-F system is a transposon encoded Class I CRISPR system, first discovered in *Yersinia pestis* (Chen, Liu, *et al.*, 2020; Cady *et al.*, 2011; Haft *et al.*, 2005). The *A. baumannii* sequences also encoded 1 type IV-A3 system, often associated with IncH1B/IncFIB plasmids in Enterobacterales and it is thought to be involved in plasmid-plasmid competition (Newire *et al.*, 2020).

To determine if there were any statistically significant differences between the number of CRISPR-Cas systems highlighted by CCtyper in either species, a random permutation test was again conducted, see Section 2.3.2 for further details. By using a Welch's two tailed T-test there was a p value of <0.0001, indicating that there is significantly more CRISPR-Cas systems found in *A. baumannii* sequences using CCtyper than in *A. lwoffii* genomes, furthermore the mean number of CRISPR-Cas systems per sequence was 0.053 for *A. lwoffii* and 0.152 for *A. baumannii*.

In summary, *A. lwoffii* may encode more DNA defence systems in general, but when specifically searching for adaptive immunity through CRISPR-Cas, *A. baumannii* encodes more systems from the CCtyper database. CRISPR systems are still regularly being discovered and therefore it is possible that some arrays were missed in both species' genomes but it is more likely that that *A. lwoffii* would be underrepresented due to the

lack of available sequences for this species and relative lack of published research.

3.8 Selection for antibiotic resistant mutants

Work so far has shown that *A. baumannii* is frequently resistant to clinically used antibiotics while *A. lwoffii* is not and this is likely due in part to the lack of efflux and antibiotic resistance genes in *A. lwoffii*. However, acquiring foreign DNA is not the only mechanism to decrease susceptibility to antibiotics, bacteria may select for antibiotic resistance mutations within their genomes. To determine if *A. lwoffii* and *A. baumannii* could evolve drug resistance mutations, mutant evolution experiments were carried out, where strains were grown in increasing concentrations of a clinically-relevant antibiotic and whole genome sequenced to identify mutations that were selected for over the experiment. The antibiotics chosen were those with defined EUCAST breakpoints that are used clinically to treat *Acinetobacter* infections. The same ancestral strains (AB18 and AL28) were used throughout each experiment due to them both being clinically susceptible to the antibiotics, at the start of each experiment, as defined by EUCAST.

3.8.1 Strains evolved to grow in the presence of meropenem

Meropenem is an antibiotic used to treat *Acinetobacter* infections caused by carbapenem-sensitive *Acinetobacter*. Meropenem targets peptidoglycan cross-linking by inhibiting penicillin binding proteins. The EUCAST clinical breakpoint for meropenem resistance in *Acinetobacter* is >8 and the ancestral *A. baumannii* AB18 has an MIC of 1 and *A. lwoffii* AL28 has an MIC of 0.015 μg/mL. Briefly, single colonies were grown over a period of seven days in increasing concentrations of meropenem (1/4 MIC, 1/2 MIC, MIC and a fold above MIC) in nutrient broth. Cells were passaged over time using a 1%

suspension to avoid population bottlenecks. After 7 days, single colonies from each biological replicate (n=5) were chosen, at random, and sent for whole genome sequencing using Illumina platforms (MicrobesNG). The short reads were subsequently assembled and searched for the presence of mutations by comparing to the sequences of the ancestral strains. Additionally, cultures without meropenem and just nutrient broth were set up as controls for natural genetic drift.

The results of the meropenem-evolved strains can be seen below in Figure 3.8, where SNPs are shown as black squares and the coloured dots correspond to MIC changes compared to the ancestral strain for a range of compounds.

Despite *A. lwoffii* being able to grow at 2x/1-fold above MIC (the final concentration of meropenem used in the evolution experiment) no genetic changes were seen. In fact, the selected strains had fewer SNPs than the strains just passaged in nutrient broth alone. The level of sensitivity in *A. lwoffii* strains stayed the same as the strains grown in nutrient broth alone (0.06 μg/mL), which was within a fold of the ancestor (0.015 μg/mL). All strains were still susceptible to meropenem using the EUCAST resistance breakpoint. However, for *A. baumannii* the mutant strains of AB18 (M1-M5) consistently had SNPs within the *adeJ* gene, encoding the RND transporter for efflux pump AdeIJK, and in the gene, which encodes the repressor protein for this system - *adeN*. Additionally, AB18 mutant 5 had target-site mutations in genes encoding penicillin binding proteins 2 and 3 (where meropenem binds). The mutations seen in the AB18 mutants caused significant MIC increases in ampicillin, meropenem, ciprofloxacin and tetracycline with some mutants also being less susceptible to moxifloxacin and erythromycin as well. The AB18 ancestor had a meropenem MIC of 1 μg/mL, the strains in nutrient broth had MICs of 0.5 μg/mL and the mutants displayed a reduced susceptibility, where their MICs were between 2 and 4 μg/mL.

In summary, only the *A. baumannii* (AB18) derived strains, and not the *A. lwoffii*

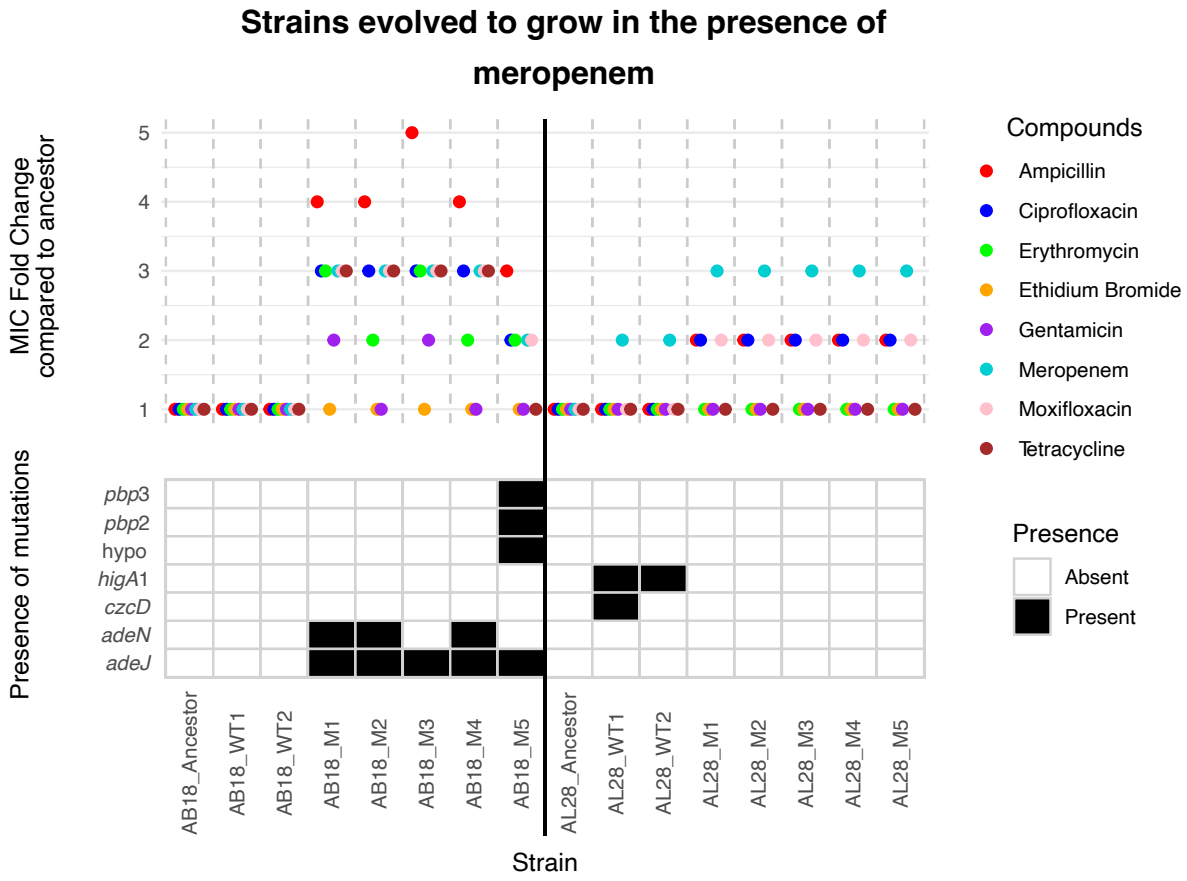


Figure 3.8: Mutations in, and the susceptibility of, strains grown in the presence of meropenem. Susceptibility to a range of compounds is shown as coloured dots corresponding to MIC fold change compared to the ancestral strain and the presence of SNPs is shown as black squares. AB18 is an *A. baumannii* strain (left-hand side) and AL28 is an *A. lwoffii* strain (right-hand side). Ancestral strains were not part of the evolution experiment and included for context. The WT strains were passaged in nutrient broth only as genetic drift controls. hypo= gene encoding for a hypothetical protein.

strains, were able to display clinical levels of resistance to meropenem and they did this by target site and efflux mutations.

3.8.2 Strains evolved to grow in the presence of ciprofloxacin

To determine whether *A. lwoffii* strain AL28 is less able to evolve resistance across multiple classes of drugs, the evolution experiment was repeated. This time a drug from a different class, with a different mechanism of action, was used - ciprofloxacin. Ciprofloxacin works by inhibiting DNA replication by DNA gyrase and topoisomerase IV and often resistance to this drug arises from mutations in these bacterial targets (Willmott & Maxwell, 1993). The EUCAST breakpoint for resistance to ciprofloxacin in *Acinetobacter* is >1 μg/mL. Again the experiment incrementally increased ciprofloxacin concentration over a week until cells were growing in 2x MIC.

The results for the ciprofloxacin evolution experiment are in Figure 3.9, below. For ciprofloxacin a different pattern was seen, where mutants were able to be captured from both *A. baumannii* (AB18) and *A. lwoffii* (AL28) and these strains had genetic changes in ciprofloxacin's targets as well as efflux-related genes, conferring the increases in MIC. For *A. baumannii* the ancestral strain and cells grown only in nutrient broth had ciprofloxacin MIC values of 0.25 μg/mL, whereas colonies passaged in ciprofloxacin had MIC values ranging from 16 to 128 μg/mL. For *A. lwoffii* the ancestor and cells grown in nutrient broth had MIC values of <0.03 μg/mL whereas colonies from the antibiotic conditions had MIC values of between 1 and 2 μg/mL. Therefore, both species were able to select for clinical levels of resistance in the presence of ciprofloxacin, although there was a difference in the final level of resistance observed between the species.

For *A. baumannii*, each mutant strain had a different complement of SNPs including

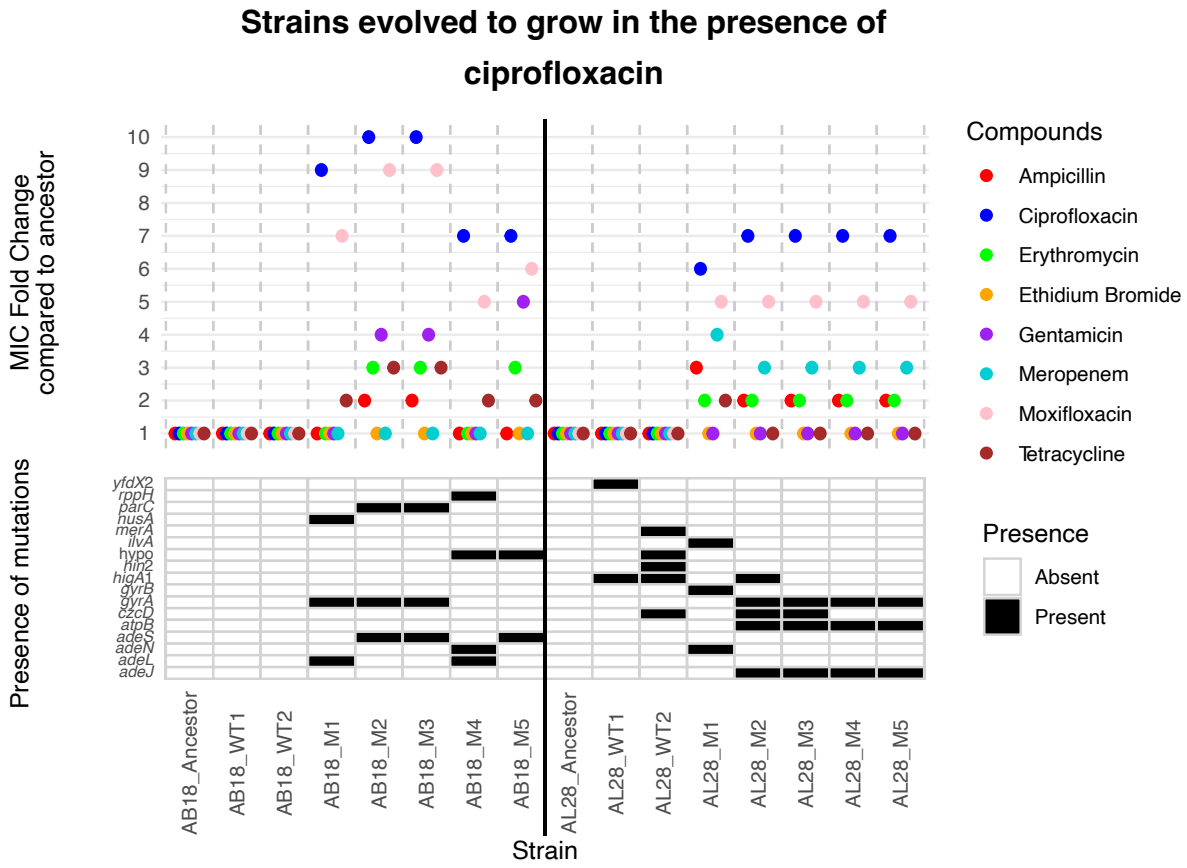


Figure 3.9: Mutations in, and the susceptibility of, strains grown in the presence of ciprofloxacin. Susceptibility to a range of compounds is shown as coloured dots corresponding to MIC fold change compared to the ancestral strain and the presence of SNPs is shown as black squares. AB18 is an *A. baumannii* strain (left-hand side) and AL28 is an *A. lwoffii* strain (right-hand side). Ancestral strains were not part of the evolution experiment and included for context. The WT strains were passaged in nutrient broth only as genetic drift controls. hypo= gene encoding for a hypothetical protein.

in the targets of ciprofloxacin (*gyrA* and *parC*) as well as in genes encoding for RND efflux regulation proteins (*adeN*, *adeS* and *adeL*). AdeN is the repressor of AdeIJK and mutations were seen near to the distal binding pocket, where efflux substrates such as β lactams can bind (Darby, Bavro, *et al.*, 2023). AdeS is part of a two-component system activator of AdeABC and mutations were seen in the periplasmic loop of this regualtory protein. Finally, AdeL, the repressor of AdeFGH, had mutations in areas outside of where DNA binds. In addition SNPs were seen in other genes - *rppH* (a regulator of 5'-end-dependent mRNA decay), *nusA* (a protein involved in transcription termination) and genes encoding for hypothetical proteins. In AB18 large MIC changes, up to 10-fold higher than the ancestral strain, were seen for both ciprofloxacin and moxifloxacin, where strains had a combination of target site and efflux-related mutations. Furthermore cross-resistance was gained for gentamicin and erythromycin in some mutants (AB18 M2, M3, M5). The tetracycline MIC was also elevated in AB18 M2 and M3. Cross-resistance is likely due to efflux mutations where AdeB/AdeG/AdeK can export β lactams, aminoglycosides, erythromycin, tetracycline and fluoroquinolones (Kornelsen & Kumar, 2021). Of the *A. baumannii* SNPs identified in this study, those in *adeL, gyrB* and *parC* are novel.

For *A. lwoffii*, in contrast to the results seen with meropenem, mutations in genes coding for target sites and efflux pumps were seen in the AL28 selected mutants. It is also worth noting the AL28 WT strains also harboured SNPs, despite being passaged in only nutrient broth. AL28 WT1 had SNPs in *higA*1 and *yfdX*2 genes. HigA is an antitoxin protein and YfdX is a heat-resistance protein. For AL28 WT2 additional SNPs were also seen in *czcD*, *hin*2, *merA* and genes encoding for hypothetical proteins. CzcD is the second inner membrane channel of the Czc RND efflux transporter system that extrudes metals. Hin is a DNA invertase and MerA is a mercuric reductase protein. Since these SNPs occurred in the nutrient broth conditions, they are excluded from the discussion below on AL28 mutant SNPs.

A. lwoffii (AL28) mutants had currently undescribed polymorphisms in *adeJ*, near to the distal binding pocket according to predicted pocket locations in Darby *et al.,* (2023). Novel mutations were also seen in *adeN* (outside of the DNA binding region), *atpB*, *gyrA* (within the quinolone resistance determining region) and *gyrB* (proximal to the quinolone resistance determining region). Locations of polymorphisms were informed by matching the data generated from whole genome sequences to published literature describing important regions relating to the protein's functions (Rosenfeld *et al.*, 2012; Attia & Elbaradei, 2020). GyrA and GyrB are the targets of ciprofloxacin, additionally AdeJ is the RND transporter from efflux pump AdeIJK, which can export fluoroquinolones, and AdeN is the repressor of this system. Finally, AtpB is an ATP-synthase. Presumably, the combination of mutations in efflux-related genes and target-site genes contributed to the reduced susceptibility of the mutants to ciprofloxacin, moxifloxacin and efflux mutations also allowed for cross-resistance to meropenem, where the combination of mutations in *adeJ* and *gyrA* and *gyrB* provided the highest levels of resistance.

In summary, both species naturally evolved mutations in the presence of ciprofloxacin, which enabled them to survive higher in concentrations of it. For some *A. baumannii* AB18 mutants, the MIC fold change compared to wild type was large (10 fold), 0.25 - 128 μg/mL and larger than the best-performing *A. lwoffii* AL28 strains (7 fold), 0.03 - 2 μg/mL and overall the *A. baumannii* strains are less susceptible than the *A. lwoffii* strains to ciprofloxacin. In this experiment both *A. baumannii* AB18 and *A. lwoffii* AL28 managed to evolve resistance from clinical susceptibility to clinical drug resistance, apart from AL28 M1.

3.8.3 Strains evolved to grow in the presence of gentamicin

Since *A. lwoffii* seemed to be capable of evolving mutations conferring resistance to ciprofloxacin but not meropenem, a third antibiotic evolution experiment was conducted. The last antibiotic chosen, gentamicin, is again used clinically to treat *Acinetobacter* infections, belonging to the aminolgycoside class of antibiotics. Aminoglycosides work by interacting with the 30S ribosomal subunit causing misreading and truncated proteins during protein translation and cell death. The experimental conditions were kept the same as before with AB18 and AL28 ancestral strains used.

The results of the mutants evolved to grow in the presence of gentamicin can be seen in Figure 3.10. In the gentamicin conditions more mutations in different genes were seen, mainly mutations captured in *A. lwoffii* (AL28) derived strains. Again, as with meropenem, AL28 derived strains did not evolve drug target mutations to gentamicin and cross-resistance was not observed. However, for *A. baumannii* AB18 large MIC increases were seen for gentamicin (up to 9 fold) and cross resistance was also seen (albeit to a lesser level) to ciprofloxacin, moxifloxacin, erythromycin and in one instance (AB18 M1) tetracycline.

All *A. baumannii* AB18 mutants had elevated MICs to gentamicin (8 or 9 fold above ancestral strain MIC), taking them from clinically susceptible to resistant ($>$ 4 μg/mL). The ancestral strain and cells grown in nutrient broth had MIC values of 0.25 μg/mL and cultures grown in the presence of gentamicin had MIC values of between 32 and 64 μg/mL. These mutants also displayed a reduced susceptibility to ciprofloxacin and moxifloxacin and some of the AB18 mutants (1, 2 and 4) also had reduced susceptibility to erythromycin and tetracycline too. The wild-type strains grown in broth did not have any mutations, whereas the mutant strains, selected following growth in the presence of gentamicin, had mutations in *adeB* (not within the predicted binding pockets), *adeR* (in the DNA binding region), *adeS* (in the transmembrane part of the protein), *fusA*, *ptsP* and

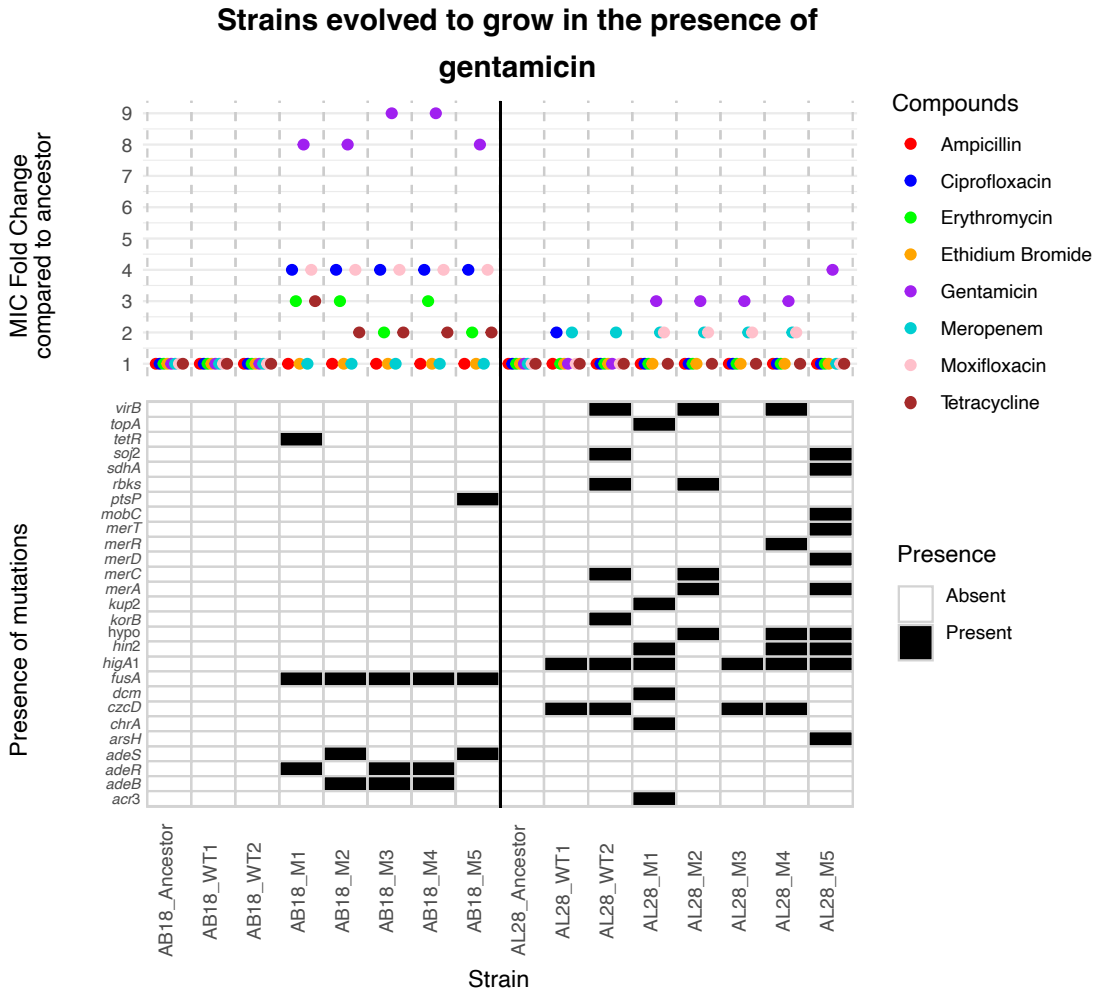


Figure 3.10: Mutations in, and the susceptibility of, strains grown in the presence of gentamicin. Susceptibility to a range of compounds is shown as coloured dots corresponding to MIC fold change compared to the ancestral strain and the presence of SNPs is shown as black squares. AB18 is an *A. baumannii* strain (left-hand side) and AL28 is an *A. lwoffii* strain (right-hand side). Ancestral strains were not part of the evolution experiment and included for context. The WT strains were passaged in nutrient broth only as genetic drift controls. hypo= gene encoding for a hypothetical protein.

tetR (outside of the DNA binding region). As mentioned previously, AdeB is the inner membrane pump of RND system AdeABC and AdeRS is the two-component system that activates the expression of it. Mutations within efflux related genes can lead to the overexpression of efflux pumps and increased export capacity of a bacterium, to protect it from toxic compounds in the environment. The gene *fusA* encodes for elongation factor B, which is involved in the translation of proteins, *ptsP* encodes for phosphoenolpyruvate protein phosphotransferase which is important in sugar transport and *tetR* encodes for a TetR-family repressor protein - these proteins often repress efflux expression and an example of one is AdeN. The *tetR* gene here, had no homology to characterised TetR proteins, such as AdeN, and potentially represents a novel regulator.

As with meropenem, the *A. lwoffii* (AL28) strain tested did not evolve clinical levels of resistance to gentamicin (MIC $\leq$ 4 μg/mL) or other drugs tested, the ancestral AL28 strain and cells grown in nutrient broth only had MIC values of 0.06 μg/mL and the cells grown in the presence of gentamicin had MIC values of between 0.25 and 0.5 μg/mL. However, during this experiment many SNPs were selected for in both the nutrient broth only conditions (WT1 and WT2) and gentamicin conditions (M1-5). The mutations seen in the WT strains were in *czcD*, *higA*1, *korB*, *merC*, *rbks*, *soj*2 and *virB*. These genes encode a metal RND efflux protein, an antitoxin protein, a plasmid copy number control element, mercuric reductase, a ribokinase, a sporulation initiator inhibitor protein and finally a virulence transcriptional regulator protein, respectively. These genes are not discussed below when looking at the mutant conditions, since they also appeared in broth only. The AL28 cells grown in gentamicin additionally had mutations in *acr*3 and *arsH* (arsenic resistance), *chrA* (chromate resistance), *dcm* (DNA-cytosine methyltransferase), *hin*2 (DNA invertase), *kup*2 (potassium transport), *topA* (DNA topoisomerase I), hypothetical proteins, *merA*, *merD* *merR* and *merT* (mercuric transport proteins), and *sdhA* (succinate dehydrogenase). Therefore these cells demonstrate unexpected genomic changes,

particularly in metal-tolerance genes, as opposed to canonical drug target and efflux pump mutations often seen in drug resistance.

In conclusion, *A. baumannii* AB18 was able to rapidly evolve resistance to three clinically relevant antibiotics, which provided not only elevated MICs to that antibiotic but cross-resistance to drugs from other classes. Furthermore AB18 went from clinically susceptible to resistant, as defined by EUCAST breakpoints, in each instance. However, for *A. lwoffii*, resistance was only seen for ciprofloxacin. In this work, which included with one strain of each species, these results suggest that *A. lwoffii* has a more limited capacity to evolve resistance to clinically-used antibiotics and due to the diversity of efflux-related mutations in *A. baumannii* this may be due to the lack of RND systems in *A. lwoffii*. A comprehensive list of all mutations found and MIC values for each strain can be found in Appendix D.1.

3.9 Discussion

This chapter focused on understanding the differences in susceptibility between two *Acinetobacter* species, which cause bacteraemia in the UK: *A. baumannii* and *A. lwoffii*. Despite *A. lwoffii* causing more bloodstream infections than *A. baumannii* (United Kingdom Health Security Agency, 2021), comparatively little is known about this species. Additionally, surveillance data in the UK, suggested *A. lwoffii* is generally drug sensitive, which is in contrast to *A. baumannii*, which is often multidrug resistant (United Kingdom Health Security Agency, 2021). Therefore, to understand each species response to antibiotics, a series of experiments were conducted.

Firstly, the study focused on determining the differences in susceptibility to antibiotics. As shown to be the case in the UKHSA surveillance report, all *A. lwoffii*

strains tested were more susceptible to antibiotics than the *A. baumannii* strains. There are EUCAST breakpoint values for *Acinetobacter* for meropenem (>2 μg/ml), ciprofloxacin (>1 μg/ml) and gentamicin (>4 μg/ml) (EUCAST, 2023). All the *A. lwoffii* strains were clinically susceptible to these antibiotics. However for *A. baumannii* four strains were resistant to meropenem, 11 strains were resistant to ciprofloxacin and 10 strains were resistant to gentamicin. Carbapenem, such as meropenem, resistance in *A. baumannii* is very common (Bou *et al.*, 2000). From the UKHSA data on bloodstream isolates of *A. baumannii*, 9% of strains are resistant to meropenem, 10% are resistant to gentamicin and 15% to ciprofloxacin (United Kingdom Health Security Agency, 2021). The data in this work agree with the literature where generally *A. lwoffii* can be treated with relative ease compared to *A. baumannii* infections (Regalado, Martin, & Antony, 2009; Dijkshoorn, Nemec, & Seifert, 2007). Furthermore, the antibiotics tested belong to a range of drug classes with differing modes of action, showing the mechanisms of resistance are diverse in *A. baumannii*.

By using the CARD database to search assembled genomes of both species, it became clear that *A. lwoffii* encodes far fewer antibiotic resistance genes than *A. baumannii*. This explains the susceptibility data and that from the UKHSA, showing *A. lwoffii* does not display MDR (United Kingdom Health Security Agency, 2021). Furthermore, compared to *A. baumannii*, there are very few reports of drug-resistant *A. lwoffii* infections (Tega *et al.*, 2007; Ahmadi *et al.*, 2009; Mittal *et al.*, 2015). The most predominant resistance genes found in both species conferred resistance to β lactam and aminoglycoside drugs. *Acinetobacter* bacteraemia treatment often includes β lactams, including carbapenems, as well as aminoglycosides and fluoroquinolones. If the infection is not clearing, tetracyclines and polymyxins may also be prescribed (Kanafani & Kanj, 2022). Therefore, it is logical that the drugs used to treat *Acinetobacter* infections are often the classes which resistance genes were found to protect against. *A. lwoffii* had few

resistance genes, but ten genomes did contain the presence of a peptide resistance gene, *ugd*. Given the small trend towards increasing colistin resistance in *A. lwoffii*, documented by the UKHSA, it is possible that peptide resistance genes such as *ugd* may reduce susceptibility to polymyxins. Whilst polymyxin resistance is common in other species, such as *A. baumannii*, it is puzzling to see the presence of a resistance gene to peptides in *A. lwoffii*, given that it is often clinically susceptible so would be treated using a different drug class in the first instance. In summary, *A. lwoffii* is likely more drug sensitive than *A. baumannii* due to a reduced number of antibiotic resistance in their genomes.

In addition to antibiotic resistance genes, the presence of RND efflux pumps was also determined across either species. Firstly, efflux in general was searched for using the CARD database, then a second, custom, *Acinetobacter*-specific RND database (AciRND) was used to pull out known RND systems. In general, there were more efflux-related genes found in the *A. baumannii* sequences than in the *A. lwoffii* sequences. When focusing on RND systems, again, *A. lwoffii* sequences had fewer than *A. baumannii*, but both species shared one RND system in common: *adeIJK*. AdeIJK is tightly controlled and important to the species that express it - it modulates lipids contributing to the maintenance of the cell wall and provides intrinsic resistance to some antibiotics, including β lactams (Damier-Piolle *et al.*, 2008; Rosenfeld *et al.*, 2012). AdeIJK is also known to be toxic when expressed at high levels (Rosenfeld *et al.*, 2012). The fact that *A. lwoffii* only encodes one complete tripartite efflux pump system (*adeIJK*) means that it has a more limited efflux capacity than *A. baumannii*. Furthermore, it has been shown that efflux not only directly reduces susceptibility to antimicrobials via extrusion, it also allows cells evolutionary time to gain drug resistance mutations, for example in the drug's target site, by extruding enough toxic compounds to keep the cell alive (Papkou *et al.*, 2020; Yoon, Courvalin, & Grillot-Courvalin, 2013). Additionally, the number of RND pumps does not always correlate with a species' ability to cause human infection, for example in *Neisseria gonorrhoeae* (Colclough, Alav,

et al., 2020). However, more RND pumps does allow a species to survive in more diverse environments. *N. gonorrhoeae* is an obligate pathogen, but *E. coli*, which has 6 RND pumps, can survive in a variety of niches causing different types of infections (Anes *et al.*, 2015). So whilst *A. lwoffii* has fewer RND transporters than *A. baumannii* it may still be able to cause bloodstream infections, which is what is reflected in the UKHSA surveillance report (United Kingdom Health Security Agency, 2021), but it may be less well adapted at surviving in different environments.

Given that *A. lwoffii* has fewer antibiotic resistance genes, but is regularly treated with antibiotics when it causes infection, it seemed paradoxical that it did not encode more ARGs. A potential explanation for the lack of ARGs is less horizontal gene transfer, or at least, a reduced capacity to recombine and maintain foreign DNA. To investigate this, the presence of plasmids, twitching genes (as a proxy for natural transformation) prophage DNA and homologous recombination regions were searched for. Additionally, DNA defense systems were also highlighted to determine if *A. lwoffii* is more selective about the DNA it encodes. A species might limit the DNA horizontally acquired because not all DNA will be advantageous and some may cause a fitness decrease (Thomas & Nielsen, 2005).

Both *A. baumannii* and *A. lwoffii* can maintain plasmids, but the most common *rep* type differed between the two species. 92% of *A. lwoffii* sequences tested encoded a R3-T25/R3-T45 plasmid, whereas the most common type in *A. baumannii* (37%) was RP-T1. The naming system for *rep* genes has recently changed (Lam *et al.*, 2023). For example, originally *rep* genes were classified based upon PCR but this was not sensitive enough to distinguish between some plasmids (Lam *et al.*, 2023). The scheme used in this thesis categorises *rep* types first by family (Rep1, Rep3 or RepPriCT) and secondly by homology (>95% nucleotide identity) to a *rep* type (Lam *et al.*, 2023). It is then easy to see the types of *rep* genes found, for example it became clear than generally *A. lwoffii* only harbours R3-type plasmids, whereas *A. baumannii* can maintain many different types. A possible explanation

for why *A. lwoffii* maintains R3 plasmids is due to a lack of a conserved backbone. The Panaroo results showed few core genes across R3-T25/R3-T45, which represent two closely related R3 types. Therefore R3 plasmids are very plastic and it is possible that it is harder for a defence system to target R3 plasmids, as opposed to plasmids with well defined backbones.

Little is known about the R3-T25/R3-T45 *rep* type plasmids and what their function and mode of replication may be. These plasmids from *A. lwoffii* contained many IS elements, again indicating the plasmids are plastic. This is common in *Acinetobacter* plasmids, where metal and/or resistance genes can be flanked by insertion sequences and transposons (Zong *et al.*, 2020; Nigro & Hall, 2016; Maslova *et al.*, 2022; Mindlin, Maslova, *et al.*, 2021). Additionally, *Acinetobacter* plasmids can contain *dif* modules (p*dif*), which can easily mobilise genes such as OXA-type β lactamases (D'Andrea *et al.*, 2009; Mindlin, Beletsky, *et al.*, 2019). However, the plasmids did not encode antibiotic resistance genes so they do not contribute to MDR. Some accessory genes were related to metal tolerance, but only the iron sequestering gene *fatA* is part of the core genes. FatA has been shown to aid iron sequestering in *A. baumannii* clinical isolates but the types of siderophore expressed differs among different *A. baumannii* isolates (Dorsey, Beglin, & Actis, 2003).

When looking more closely at *Acinetobacter* plasmids between the two species, the amount of genes per functional orthologous group was similar, the only discrepancy was *A. lwoffii* plasmids encoded a higher percentage of genes relating to metabolism. Additionally, many more more ARGs were found on *A. baumannii* plasmids than those from *A. lwoffii*. This agrees with the data on whole genome sequences discussed above. Previous work has shown that clinically-derived *A. lwoffii* plasmids can contain ARGs and that it is environmental or ancient strains that lack them (Maslova *et al.*, 2022; Mindlin, Maslova, *et al.*, 2021). In contrast, in this work the *A. lwoffii* laboratory strains that were sequenced (which comprised both clinical and environmental strains) had no clear difference in the number of total resistance genes, although because these were short-read

sequenced it was impossible to assign which were plasmid- or chromosome-derived.

Often antibiotic resistance genes can occur with metal resistance genes on plasmids (Baker-Austin *et al.*, 2006), so the presence of metal genes was also determined. Both species contained similar levels of metal resistance genes, but those found in *A. lwoffii*, unlike *A. baumannii*, did not co-occur with ARGs. The metals that the genes often conferred resistance to, were biologically relevant metals, indicating these genes help both species to survive in the host, for example the presence of copper and zinc resistance genes, which are used to kill bacteria in phagosomes (Sheldon & Skaar, 2019).

After searching for plasmids to determine why *A. lwoffii* does not encode ARGs, the search continued to find out if *A. lwoffii* is naturally competent and can transform free DNA in the environment as a potential mechanism to acquire foreign DNA and ARGs. As a proxy for natural competence and therefore the uptake of foreign DNA including AMR genes, twitching motility was measured because both mechanisms use the same molecular machinery (Leong *et al.*, 2017). In general, the *A. lwoffii* strains twitched less. Twitching motility has been shown to be strain dependent and can also depend on the environment in which it is measured (Eijkelkamp, Stroeher, Hassan, Papadimitrious, *et al.*, 2011). Additionally, the rate of transformation in *A. baylyi* is dependent on the environment, where the presence of bromoacetic acid, a regulated drinking water disinfection byproduct, can increase it (Mantilla-Calderon *et al.*, 2019). Therefore, whilst it seems that *A. lwoffii* is less able to twitch and as such less able to naturally transform DNA, including resistance genes from the environment, it could be that the conditions of this experiment were not optimal to encourage that phenotype.

To relate the twitching phenotypes to genetic determinants of competence, the presence of type IV pilus and twitching genes was established across the whole genome sequences. Both species had a similar complement of genes. The database was curated

using genes characterised in *Acinetobacter baylyi* but using the *A. baumannii* sequence, so it is possible this database may be missing genes important for competence in both *A. lwoffii* and *A. baumannii*. Previous work has shown that interruption of *pilA, pilD* or *pilT* leads to *A. baumannii* no longer being able to form functional type IV pili and no longer being naturally transformable (Harding, Tracy, *et al.*, 2013). Pilin genes *pilT* and *pilD* were found in all sequences tested in both species, however *pilA* was not included because of its absence in *A. baylyi* (Leong *et al.*, 2017). The structure of PilA from two distinct *A. baumannii* strains has recently been compared, which showed that despite similar structures, PilA can modulate biofilm formation and twitching motility depending on its sequence, suggesting changes in the protein charges can lead to changes in function (Ronish *et al.*, 2019). When trying to relate genes found in the laboratory strains to measured twitching phenotypes there was no clear pattern, suggesting that simply the presence of these genes alone does not dictate twitching capacity. It is likely that the regulation of expression of these genes is also important. Twitching is a temporal phenotype that can be turned on and off rapidly due to regulators such as cyclic diguanosine monophosphate (Opoku-Temeng *et al.*, 2016), two component systems (Kilmury & Burrows, 2018) and quorum sensing signals (Mayer *et al.*, 2020) and therefore these systems may be more important than the presence of type IV genes, when trying to uncover the mechanisms underpinning the differential twitching capacities across the strains tested. Additionally, it has been shown that transformation efficiency is affected by the methylome pattern that these epigenetic marks differ substantially across different *A. baumannii* strains and therefore the ability to transform DNA is likely to not be universal across a whole species but differ at the strain level (Vesel *et al.*, 2023).

Both *A. baumannii* and *A. lwoffii* contained intact prophage DNA, proving that both species have previously been infected by bacteriophages. Fewer regions of phage DNA were found across *A. baumannii*, but the sample sizes (n=10) were very small for both species, so

it is difficult to extrapolate these data. Phages can inject antibiotic resistance genes from bacterium to bacterium (Arndt *et al.*, 2016), but this is unlikely to be the case in the *A. lwoffii* sequences tested because they do not encode many ARGs despite having many regions of intact prophage DNA. Another function of phages is medicinal, where phage cocktails may be a way of combatting *Acinetobacter* infections in the clinic (Wang, Kuo, *et al.*, 2018; LaVergne *et al.*, 2018). Therefore, by understanding how susceptible either species is to certain types of phages, infections may be treated avoiding the use of antibiotics.

Homologous recombination within the genomes of both species was analysed using Gubbins to infer how much horizontal gene transfer is occurring. Both had similar levels of recombination, indicating both species actively undergo HGT, but the type of DNA maintained by *A. lwoffii* differs to that of *A. baumannii*. Large differences in r/m values were obtained across both *A. lwoffii* and *A. baumannii* sequences by Gubbins, when estimating homologous recombination. A paper by Vos and Didelot (2009) showed example r/m values for well characterised species, including *E. coli* and *S. aureus*, however they only used one representative sequence per species. In that paper r/m values between 0.02 and 63.6 were found across archaea and bacteria, with *C. jejuni*, *E. coli*, *K. pneumoniae* and *S. aureus* harbouring r/m values of 2.2, 0.7, 0.3 and 0.1 respectively (Vos & Didelot, 2009). The average values for *A. baumannii* and *A. lwoffii* puts both species between *K. pneumoniae* and *S. aureus* for recombination levels relative to point mutations. A more recent study was conducted looking at recombination across *Mycobacterium bovis*, where the authors found r/m average values between 0.025 and 0.037 (Reis & Cunha, 2021). The authors suggest that r/m value differences are consistent with the idea that recombination rates vary widely among clonal lineages of the same species and this could also explain the differences seen in r/m values in this work (Reis & Cunha, 2021).

Given that *A. lwoffii* and *A. baumannii* both encode plasmids, can be infected by phage and have similar levels of HGT, the hypothesis changed to that *A. lwoffii* may

encode DNA defence systems that stop the integration of certain foreign DNA (such as ARGs). To test this, the genomes were searched for DNA defence systems. When looking at many different DNA defence systems, *A. lwoffii* genomes encoded statistically more than *A. baumannii*, particularly restriction modification systems, which provide intrinsic resistance to foreign DNA. However, when looking at CRISPR-Cas systems only, *A. baumannii* encoded more. It has been shown in *Enterococcus faecalis* that both CRISPR-Cas and restriction modification systems act as immunity reducing the capacity of the bacterium to accept conjugative plasmids (Price, Huo, *et al.*, 2016). A pangenome analysis of 2,500 *A. baumannii* strains separated the species into two groups, where one group rarely harbours plasmids but has CRISPR-Cas and restriction modification genes and the other does not but harbours plasmids (Mangas *et al.*, 2019). It is therefore possible that it is too broad to generalise "*A. baumannii*" and "*A. lwoffii*" and it is better to take into account strain-to-strain variation. CRISPR-Cas systems provide adaptive immunity to previous invading genetic elements, and act synergystically with other DNA defense systems. The increased presence of CRISPR-Cas systems in *A. baumannii* may indicate a historical increase in a certain type of infection, e.g. a type of phage.

In addition to acquiring resistance from foreign DNA, bacteria may also select for mutations leading to reduced susceptibility, for example in the gene encoding for the target protein of an antibiotic (Darby, Trampari, *et al.*, 2023). When trying to experimentally mimic the selection for drug resistant clones, *A. lwoffii* was only able to select for genetically resistant mutations to one of the antibiotics tested: ciprofloxacin. It is well characterised that ciprofloxacin resistance arises rapidly, in a number of Gram negative species, via mutations in the genes encoding for the drug target - GyrA, GyrB, ParC and ParE (Lindbäck *et al.*, 2002; Lee, Lee, *et al.*, 2005; Mouneimné *et al.*, 1999; Park *et al.*, 2011). Furthermore, previous work has shown that fitness costs associated with high-level fluoroquinolone resistance can vary (Tóth *et al.*, 2014) and in some cases fitness can be gained with GyrA mutations

(Marcusson, Frimodt-Møller, & Hughes, 2009). Therefore, the fitness of *A. lwoffii* in the presence of ciprofloxacin may not have been as negatively impacted as in the meropenem and gentamicin experiments. Additionally, the mutation rate for *A. lwoffii* may be lower than *A. baumannii* (not tested in this work) and therefore single-step SNPs, which can lead to decreased susceptibility to fluoroquinolones with a relatively low fitness cost compared to mutations in other targets, may be more likely to arise in *A. lwoffii*.

Ciprofloxacin drug resistance mutations are often found in the quinolone resistance determining region (QRDR) of GyrA (amino acids 65-104 in *A. baumannii* and *A. lwoffii*), where a point mutation here can lead to fluoroquinolone resistance because the drug has a reduced affinity to bind to its target (Attia & Elbaradei, 2020; Price, Vogler, *et al.*, 2003). QRDR GyrA mutations were also seen in this study (Gly79Cys and Ser81Leu in *A. baumannii* and Ser81Tyr in *A. lwoffii*). There are also QRDRs in GyrB and ParC, but the mutations that arose during the ciprofloxacin evolution experiments in both species did not fall within these regions. The *A. lwoffii* GyrB SNP was at amino acid position 452, where the QRDR sits at positions 410 to 431 (Attia & Elbaradei, 2020). Additionally, the QRDR in ParC is at positions 59 to 98 and the *A. baumannii* SNPs were at position 332. This is not unique to this experiment and other studies have found fluoroquinolone resistance mutations outside of the QRDR (El-Badawy *et al.*, 2022), however these exact mutations in ParC and GyrB have not been characterised in *A. baumannii* and *A. lwoffii* before.

In addition to drug target mutations, efflux-related mutations were also selected for in the ciprofloxacin evolution experiments. Two *A. baumannii* mutants had missense SNPs in *adeL* (Phe295Ile in a beta sheet and Arg278Leu in an alpha helix). Given that AdeL decreases AdeFGH expression in *A. baumannii* and mutations within this repressor can lead to an increased expression of AdeFGH (Yoon, Courvalin, & Grillot-Courvalin, 2013), the mutations seen in this work could also lead to de-repression and thus the overexpression of AdeFGH. AdeFGH exports fluoroquinolones (Kornelsen & Kumar, 2021)

and therefore more AdeFGH activity could mean more export of ciprofloxacin and a reduced cellular toxicity. All four *A. lwoffii* AdeJ SNPs were at position Arg128Gln, which is situated between the distal binding pocket and Q124 gate. Fluoroquinones bind to the distal binding pocket of AdeJ (Darby, Bavro, *et al.*, 2023) so this mutation could increase the binding of ciprofloxacin to AdeJ and therefore its export out of the cell, although this SNP has not been characterised before so more work would need to be done to determine this. Similarly, an *A. lwoffii* mutant had a frameshift in AdeN due to the deletion of nucleic acids 497 to 506 (CATCTCCTGA), this frameshift could affect subsequent protein folding and trafficking, reducing the function of AdeN, de-repressing and therefore increasing the expression of AdeIJK, which as mentioned previously exports ciprofloxacin. Finally, mutations in AdeS were also detected in the ciprofloxacin mutants. The SNP Gly30Arg was seen in two mutants and it has been shown previously that clinical, ciprofloxacin resistant, *A. baumannii* often encode a Gly30Asp SNP located in the periplasmic loop, which leads to overexpression of AdeB (Lari, Ardebili, & Hashemi, 2018; Coyne, Guigon, *et al.*, 2010). Additionally increased AdeABC expression with ParC/GyrA mutations decreases susceptibility to fluoroquinolones (Higgins, Wisplinghoff, *et al.*, 2004). Therefore, in this evolution experiment, mutants were selected for which had both drug target mutations and mutations that can lead to the overexpression of AdeABC, AdeFGH and AdeIJK efflux pumps. Due to the polyspecificity of the RND pumps, it is not surprising that cross-resistance also occurred in these mutants to other quinolones (moxifloxacin), aminoglycosides (gentamicin), macrolides (erythromycin) and β lactams (meropenem), which are all exported by at least one of these systems.

Evolution experiments were also conducted in the presence of meropenem and gentamicin, where only *A. baumannii* selected for mutations that lead to significant changes in susceptibility. For meropenem, both drug target (*pbp*2 and *pbp*3) and efflux (*adeN* and *adeJ*) mutations were selected for. AdeIJK is the only RND pump in

A. baumannii that exports β lactams (Kornelsen & Kumar, 2021), therefore the mutations in the transporter protein (AdeJ) and repressor protein (AdeN), likely increased the efflux of meropenem out of the mutants. Previous work has shown that a $\Delta adeIJK$ mutant was more than 1,000-fold more susceptible to meropenem (Islam *et al.*, 2022). Additionally, of the AdeJ mutations in this work, three were in the distal binding pocket, where meropenem binds (Darby, Bavro, *et al.*, 2023). Furthermore, it has been shown in *E. coli* that meropenem binds to penicillin binding protein 2 and 3 (Sumita & Fakasawa, 1995) and therefore the mutations ($pbp2/3$) seen in this experiment fit with the hypothesis that the mutants have less meropenem binding to its target and export it out of the cell in order to survive. Mutations that decreased susceptibility to meropenem also reduced susceptibility to ampicillin, moxifloxacin, ciprofloxacin and erythromycin, which are all exported by AdeIJK, therefore it is even more likely that this pump is being overexpressed due to the cross-resistance observed.

Finally, in the gentamicin evolution experiments, the *A. baumannii* mutants selected for mutations within efflux genes encoding for AdeB and the two component system activator of it AdeRS, as well as in FusA, which is involved in translation (the target of gentamicin). Mutations in AdeB were at amino acid positions 97 and 136. Gentamicin can bind to both the proximal and distal binding pockets and residues Y77, T91, and S134 are thought to be essential for gentamicin to bind to the proximal pocket of AdeB (Su, Morgan, *et al.*, 2019). Given the close proximity of the mutations in this study to residues reported to be essential for binding in the literature (Su, Morgan, *et al.*, 2019), it is likely that these mutations led to increased gentamicin export via AdeB. Mutations in AdeR and AdeS have been reported to increase AdeABC expression, for example A91V, A136V and L192R in AdeR and A94V, G103D and G186V in AdeS (Lari, Ardebili, & Hashemi, 2018). This study also captured an A91V SNP in AdeR, which sits in the signal receiver domain, as well as mutations at positions 93 in AdeR and 60 and 148 in AdeS, indicating that AdeRS may be being modulated to

increase AdeABC expression and therefore increase the export of aminoglycosides. As with other experiments, cross-resistance was seen to fluoroquinolones and tetracycline (exported by AdeABC) and macrolides (exported by AdeIJK), whilst the mechanism for macrolides is unclear, it is likely that more AdeABC expression means an increased efflux capacity to all of its substrates, and therefore cross-resistance.

The efflux mutations selected for in the *A. baumannii* evolution experiments were diverse, observed across three RND systems. Therefore, it is possible that because *A. lwoffii* only encodes AdeIJK (and not AdeABC and AdeFGH) it has a reduced capacity to evolve antibiotic resistance mutations, explaining its ability to only evolve resistance to ciprofloxacin. These data suggest that efflux is important for the development for antibiotic resistance. Additionally, it is common for Gram negative bacteria to have multiple RND systems, especially species that cause different types of infection such as *E. coli*, and therefore *A. lwoffii* is unusual in that respect, only encoding AdeIJK.

3.10 Conclusions

In conclusion this work has shown that *A. lwoffii* is more susceptible than *A. baumannii* to a range of antibiotics and fewer antibiotic resistance mutations are selected for across three clinically relevant drugs with different mechanisms of action. Additionally, *A. lwoffii* encoded fewer ARGs and had more DNA defence systems than *A. baumannii*. This begins to explain why *A. lwoffii* is easier to treat clinically than *A. baumannii* and does not typically cause drug resistant infections in patients.

However, while we now understand what mediates the greater sensitivity of *A. lwoffii* to antimicrobials compared to *A. baumannii*, this leaves open the question of why *A. lwoffii* has not evolved towards resistance despite the likely selective pressure placed upon it in by

antimicrobial use in the clinical environment.

3.11 Further Work

One of the biggest limitations of this chapter (and Chapter 4) was the limited number of *A. lwoffii* whole genome sequences available. To make this work more robust, redoing the analyses with a larger dataset would help to minimise the risk of skewing the data due to unusual sequences.

Further work in this chapter would include looking not only at the presence of antibiotic resistance genes but also looking for the presence of SNPs in genes that lead to AMR, for example *gyrA* SNPs causing a decreased susceptibility to fluoroquinolones. This would provide a more comprehensive view of antibiotic resistance in either species.

With respect to characterising DNA defense systems and horizontal gene transfer, the presence of IS elements and other mobile genetic elements could be analysed. IS elements can both mobilise antibiotic resistance genes (Poirel, Figueiredo, *et al.*, 2008) and increase their expression (Segal, Garny, & Elisha, 2005). IS elements, such as IS*Aba*1 contribute to the spread of carbapenemase ARGs in *Acinetobacter*, such as by the mobilisation of ARGs from *A. radioresistens* to *A. baumannii* (Pagano, Martins, & Barth, 2016). It would be beneficial to determine if this could also occur between *Acinetobacter* species and in *A. lwoffii*.

Additionally, some of the bioinformatics conclusions could be supported by additional laboratory experiments. For example, one hypothesis is that *A. lwoffii* and *A. baumannii* harbour distinct types of plasmids and that *A. lwoffii* does not generally carry MDR plasmids. To investigate this a variety of *Acinetobacter* plasmids could be introduced by conjugation experiments to see if *A. lwoffii* can accept and maintain the

plasmids. Similarly, competence could also be tested to see if any of the lab strains are naturally competent. It is well documented that *A. baylyi* ADP1 is naturally competent and could act as a positive control (Jiang, Palazzotto, *et al.*, 2020).

Furthermore, the experiments used a limited number of strains to try to extrapolate how a species behaves, and whilst not in the scope of this thesis, it would be really interesting to increase the number and diversity of strains used in order to pull apart any correlations in phenotype.

To build upon some of the findings from the antibiotic evolution experiments it would be beneficial to a) determine the mutation frequency between either species, b) measure relative fitness of the mutants compared to ancestor using a growth kinetics assay and c) measure any changes in efflux pump transcription using qPCR to prove that the mutations seen did in fact cause upregulation of efflux.

Finally, bacteria exist in communities. The community environment is extremely complex, where not all bacteria are working synergistically and competition can occur between different species. This competition actually reduces the selection for antibiotic resistance, because often resistant strains are not as fit as susceptible ones (Fang, Elena, *et al.*, 2023). Therefore, in order to understand how bacteria become resistant, like *A. baumannii*, a more holistic approach is required where experiments are designed to look at multiple species and their interactions in more natural environments. Mutant evolution experiments exert one selection pressure: the antibiotic, whereas in reality there are many other selection pressures in nature, including the presence of other microorganisms and scarcity of nutrients.

3.12 Key findings

- *A. lwoffii* is more susceptible to antibiotics than *A. baumannii*
- *A. lwoffii* has fewer ARGs and efflux pump associated genes than *A. baumannii*
- *A. lwoffii* shows evidence of HGT within its genomes, similar to *A. baumannii*
- *A. lwoffii* harbours different plasmid types, based upon *rep* gene, compared to *A. baumannii*
- *A. baumannii* AB18 can evolve resistance mutations in the presence of meropenem, ciprofloxacin and gentamicin. *A. lwoffii* AL28 can only evolve resistance in the presence of ciprofloxacin.

Chapter Four

Comparing the biology of *A. lwoffii* and *A. baumannii.*

4.1 Background

Work in Chapter 3 showed that *A. lwoffii* is more sensitive to antibiotics than *A. baumannii* and this is likely due to the acquisition of fewer ARGs and a reduced ability to evolve drug resistance mutations. Despite this, *A. lwoffii* causes more bloodstream infections, in England and Wales, than *A. baumannii* (United Kingdom Health Security Agency, 2021). To date, there is no data about whether *A. lwoffii* is better adapted to surviving in the blood than *A. baumannii*, but due to its ability to cause more bacteraemia than *A. baumannii*, it is plausible this may be the case. Therefore, in this Chapter work focused on understanding any differences between the two species with respect to both their biology and ability to cause infection.

4.2 Aims and hypotheses

The aim of this chapter is to compare the biology and ability to cause infection of the two species. As with Chapter 3, a combination of laboratory experiments and searching the whole genome sequences of *A. baumannii* (n=4,809) and *A. lwoffii* (n=38) will be done. Based upon previously published literature, the hypothesis is that both species can cause infections but *A. lwoffii* may be better at surviving in conditions that mimic bloodstream infections.

4.3 *A. lwoffii* grows less well in LB than *A. baumannii*

Initially, since little information has been published about *A. lwoffii*, the growth of five strains were compared to five strains of *A. baumannii*. The optical density (OD) of these strains in LB was measured for 12 hours at a wavelength of 600_{nm} in a plate reader. Growth was measured at 25°C, 30°C and 37°C. Figure 4.1 shows the growth curves at each temperature for each strain.

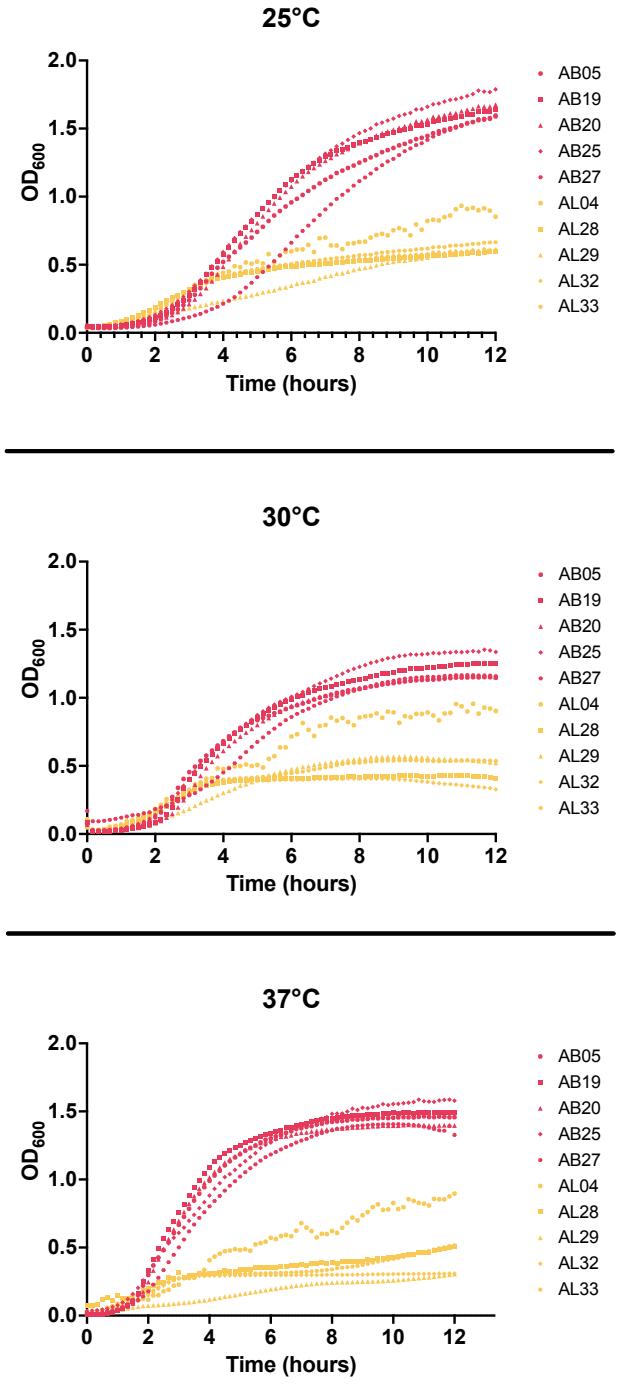


Figure 4.1: Growth of wild-type *A. baumannii* (AB, pink) and *A. lwoffii* (AL, yellow) at 25°C, 30°C and 37°C in LB. Strains chosen were those available in the laboratory at the time of the experiment. The absorbance was measured every 20 minutes for 12 hours in a plate reader at 600_{nm}. Experiments were independently repeated 4 times and mean optical density (OD) values are plotted.

A. baumannii grew to a higher final OD_{600} at all three temperatures than *A. lwoffii*. At 25°C the mean final OD for *A. lwoffii* was 0.67 whereas it was 1.63 for *A. baumannii*. Similarly, at 30°C the mean final OD for *A. lwoffii* was 0.54 and it was 1.21 for *A. baumannii*. Lastly, at 37°C, the final OD for *A. lwoffii* was 0.51 and 1.45 for *A. baumannii*. *A. lwoffii* grew to a higher OD at lower temperatures and *A. baumannii* also grew to the highest final OD at 25°C. The growth data indicates that in LB, in a microtitre plate, *A. baumannii* can grow to a higher final OD. This may impact phenotypic experiments where the cell densities are disparate in the two species, for example in biofilm experiments in Section 4.5, where *A. lwoffii* has fewer cells but may still be forming similar levels of biofilm relative to the number of cells present.

The mean generation times for each species was also determined Table 4.1. When combining all *A. baumannii* and all *A. lwoffii* strains and looking at average generation times at 37°C and 25°C there were no significant differences, despite clear differences in final OD. At 30°C when comparing the average generation time of all *A. baumannii* strains and all *A. lwoffii* strains, using an unpaired T test, there was a statistically significant difference in growth rate (p=0.046), where *A. lwoffii* grew faster, despite having a lower final OD, but at other temperatures there were no significant differences in generation time.

Table 4.1: Mean generation times (hours) for strains grown in LB at different temperatures for twelve hours

Strain	37°C – Mean generation time ± standard error	30°C - Mean generation time ± standard error	25°C - Mean generation time ± standard error
AB05	0.650 ± 0.003	0.758 ± 0.070	1.002 ± 0.016
AB19	0.595 ± 0.005	0.758 ± 0.067	0.813 ± 0.032
AB20	0.563 ± 0.017	0.703 ± 0.044	0.839 ± 0.019

AB25	0.861 ± 0.014	0.880 ± 0.063	0.972 ± 0.012
AB27	0.701 ± 0.006	0.740 ± 0.058	0.918 ± 0.045
AL04	1.070 ± 0.165	0.515 ± 0.058	0.960 ± 0.170
AL28	0.797 ± 0.406	0.406 ± 0.032	0.818 ± 0.195
AL29	1.689 ± 0.448	0.832 ± 0.189	1.362 ± 0.086
AL32	0.348 ± 0.033	0.337 ± 0.076	0.637 ± 0.135
AL33	0.434 ± 0.117	0.648 ± 0.183	0.784 ± 0.156

4.4 Scanning electron microscopy of *A. lwoffii* and *A. baumannii*

To visualise any differences in cell morphology between the two species, scanning electron micrographs were taken of two *A. baumannii* (AB05, AB18) and two *A. lwoffii* (AL04, AL28) strains. These strains were chosen as there was a mix of clinical (AL04, AB05) and non-clinical strains (AB18 and AL28), Figure 4.2. Generally all strains tested showed a typical *Acinetobacter* cell morphology of coccobacillary rods. For AL28, longer rods could be seen, where division was not occurring as expected, potentially indicating a stress response. In addition, pili can be seen such as in AB05 at 25,000 x magnification as little strands connecting the cells, agreeing with data from Section 3.6.3 that *A. baumannii* twitches more than *A. lwoffii*.

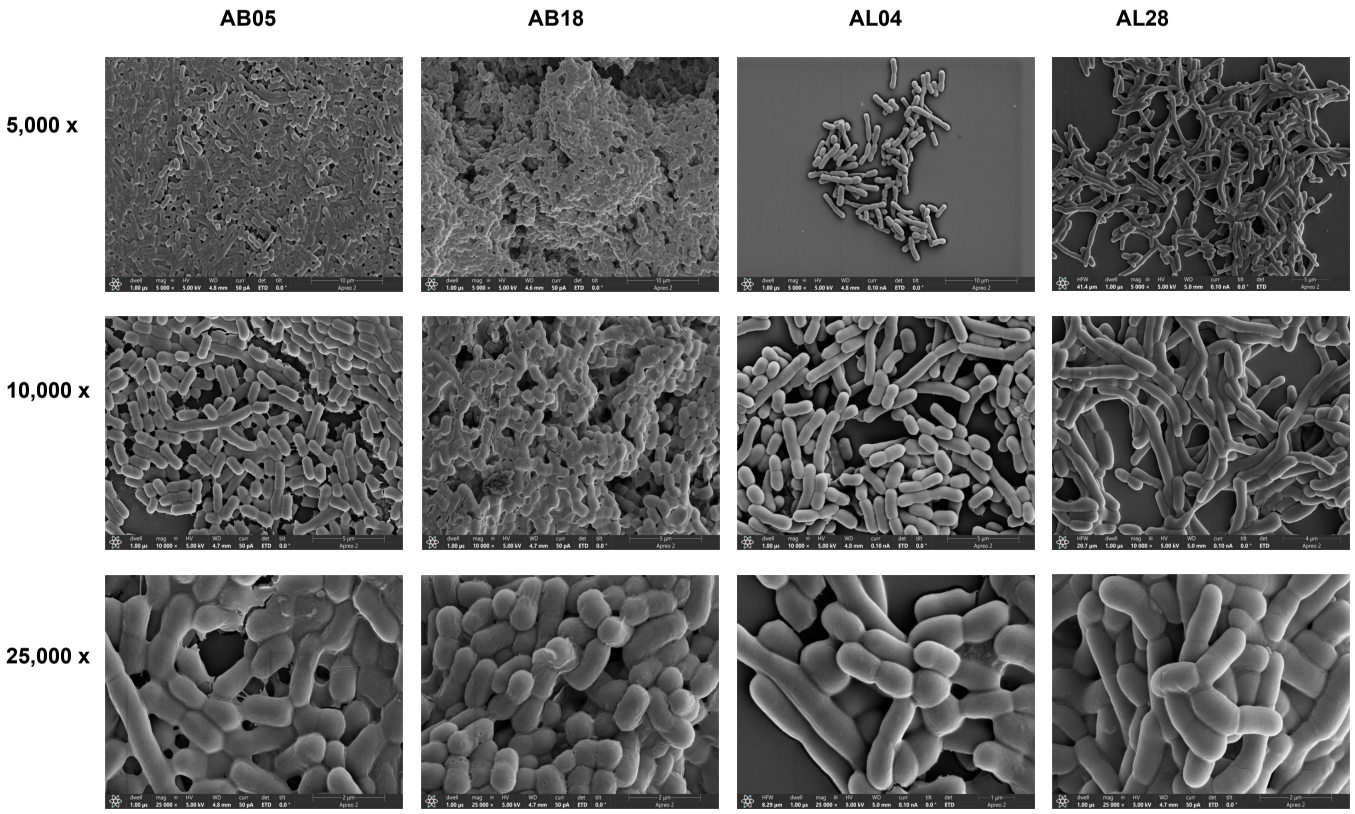


Figure 4.2: Scanning electron micrographs (Apreo 2, Thermo Fisher) of *A. baumannii* (AB) and *A. lwoffii* (AL). Clinical (AL04, AB05) and non-clinical (AB18, AL28) strains were used. Bacterial cells were fixed with 2.5% glutaraldehyde and images were taken at 25,000, 10,000 and 5,000x magnification at random. Pili can be seen connecting bacterial cells in AB. Scale bars are shown on the bottom right of each image. Images taken by Theresa Morris at the Centre for Electron Microscopy, Birmingham.

To quantify if there was any significant differences between the lengths of the *Acinetobacter* cells from each strain, the micrographs were analysed in ImageJ, where 100 cells per strain were measured. *A. baumannii* had significantly shorter cell morphology than *A. lwoffii* (p=<0.0001, one-way ANOVA), and there was no significant difference in cell length within different strains of the same species, Figure 4.3.

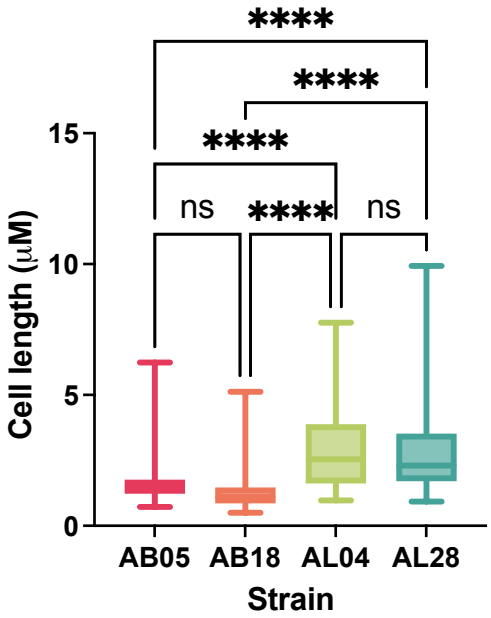


Figure 4.3: Average cell length (μM). Box and whisker plot showing average cell length with minimum and maximum cell length (whiskers), plotted for *A. baumannii* (AB05, AB18) and *A. lwoffii* (AL04, AL28). A one-way ANOVA was performed with Tukey's multiple comparisons, ****=p<0.0001. n=100 cells measured per strain.

4.5 Both *A. lwoffii* and *A. baumanii* can form biofilm

Antibiotic resistance is often be linked to a biofilm lifestyle, where bacteria in a biofilm may be less susceptible to antibiotics than those in planktonic culture. In *Acinetobacter*, biofilms allow cells to survive longer on surfaces (Espinal, Martí, & Vila, 2012) and form

persistent infections, for example on implanted medical devices (Gedefie *et al.*, 2021). Biofilm-forming strains of *A. baumannii* can often lead to catheter-related and bloodstream infections (Rodriguez-Bano *et al.*, 2008).

4.5.1 Static biofilm formation

Biofilm formation during static incubation at 37°C was measured using crystal violet dye for the same ten *Acinetobacter* strains (Figure 4.4A). Crystal violet binds to DNA and proteins in and around the bacterial cells (Wakelin *et al.*, 1981). Overall, the absorbance values for *A. lwoffii* biofilms were lower ($p<0.0001$) in the static model than *A. baumannii* (Figure 4.4B). This may be due to *A. lwoffii* growing to a lower cell density than *A. baumannii*, rather than a reduced capacity to form biofilm. There was some variation between strains, for example, clinical isolate AL04 formed the most biofilm of all the *A. lwoffii* tested, whereas the environmental strain AL28 formed less. Similarly, strain level variation was observed for *A. baumannii*, where AB20, NCTC 10303, formed the most biofilm and AB25 the least. NCTC 10303 is a clinical *A. baumannii* strain isolated from urine. The relative lack of biofilm mass formed by *A. lwoffii* strains may mean it is easier to treat and remove from hospital surfaces.

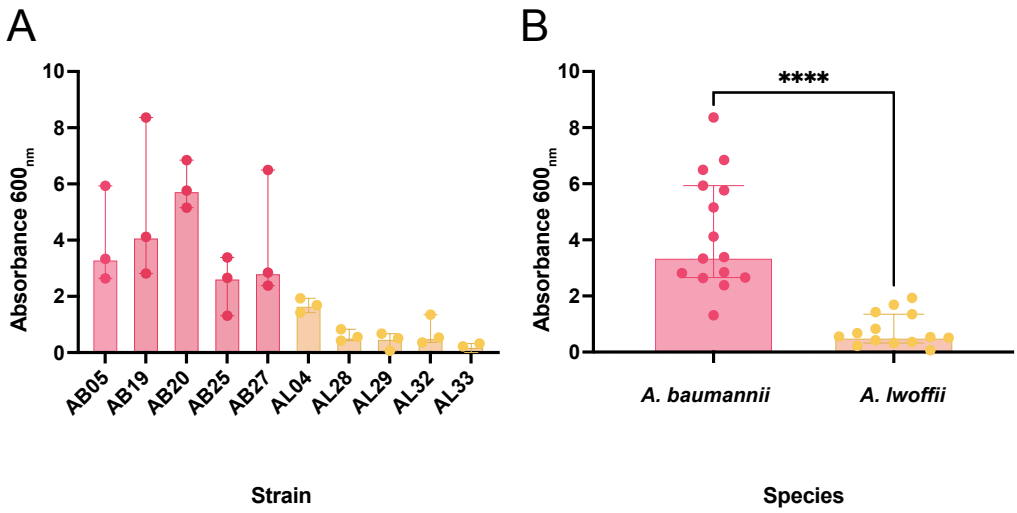


Figure 4.4: Biofilm formed by *A. baumannii* (AB, pink) and *A. lwoffii* (AL, yellow) after 48 hours under static conditions. A: Biofilm was stained with crystal violet and the absorbance at 600_{nm} was measured for each strain. Of the strains chosen, AL04, AB05, AB20 and AL29 are clinical strains. AL32 and AL33 are environmental. This experiment was independently repeated three times, bar height represents mean values. B: Static biofilm formed grouped by species. An unpaired T-test was used to determine statistical significance (**** p=<0.0001). The raw data used to create this graph is from panel A.

4.5.2 Biofilm formed under laminar flow

In infection, biofilm is not always formed statically, such as in lung infections where there is fluid, in this case mucus, which moves via cilia. Similarly biofilm formation, leading to gastritis, has been reported for *Acinetobacter*, including *A. lwoffii*, as well as *Helicobacter pylori* (Rathinavelu, Zavros, & Merchant, 2003). As with the lungs, there is a constant flow of secretions and stomach acid. Therefore, biofilm formation was also measured under laminar flow, using the Bioflux system (Benoit *et al.*, 2010), which mimics *in vivo* conditions

more closely. Flow cells were seeded with up to 11% percent coverage of cells at a 0 hour time point. The percentage coverage of the slide with biofilm is shown in Figure 4.5A. Biofilm formed quickly under laminar flow and in both species, after 12 hours, varying levels of biofilm were observed. By 36 hours, the biofilm measured covered $> 90\%$ of the slide in 4 out of 5 *A. baumannii* and 3 out of 5 *A. lwoffii* strains. As with the static biofilm model, strain level variation was observed. However, overall there was no significant differences in the amount of biofilm formed by *A. lwoffii* strains compared to *A. baumannii* strains and this may be due to the high levels of variability in the experiment. When the biofilms were disturbed after 48 hours, with a pulse of 5 dyne/cm^2, only strongly attached biofilm remained. This is shown in the final column of Figure 4.5A,B. As with the other time points, under flow conditions, *A. lwoffii* was as good as *A. baumannii* at forming biofilm.

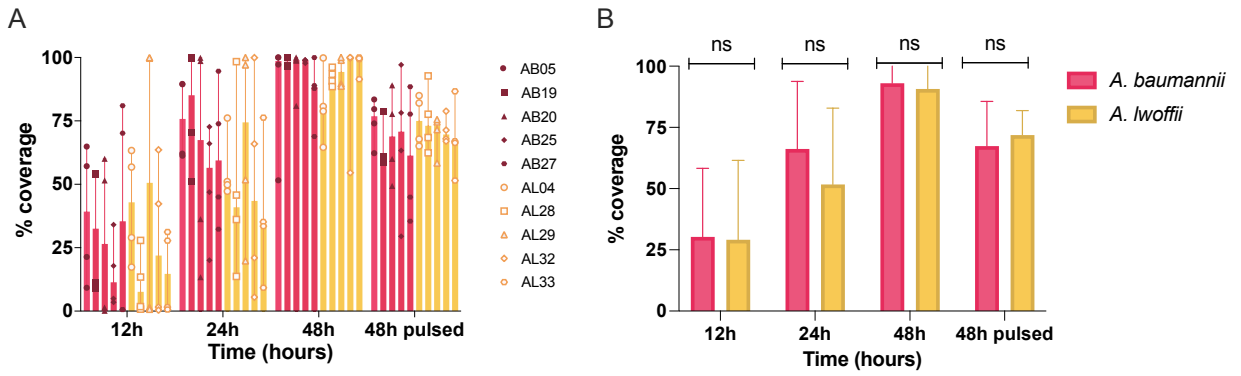


Figure 4.5: Biofilm formed under laminar flow. A: Percentage coverage of biofilm formed by *A. baumannii* (AB, pink) and *A. lwoffii, yellow* (AL) was measured at 37°C in a Bioflux model. Bacterial cells were seeded onto a flow cell, which was exposed to a constant flow of nutrients to more closely mimic infection conditions. Experiment comprised of two biological repeats. B: Mean values of biofilm coverage per species are plotted, using individual data from panel A. Of the strains chosen, AL04, AB05, AB20 and AL29 are clinical strains. AL32 and AL33 are environmental. Statistical significance was tested using an unpaired T test with Welch's correction. Data for this figure was gathered by Dr. Emma Holden, The Quadram.

To determine if there was a significant difference in the amount of biofilm formed when comparing *A. baumannii* and *A. lwoffii* more broadly, the mean values of the raw data were plotted in Figure 4.5B. When doing unpaired T tests with Welch's correction, it was clear that biofilm formation was not significantly different between the two species in a Bioflux model (p=0.9, 0.1, 0.6 and 0.3 for 12, 24, 48 and 48 hours pulsed, respectively). This indicates that *A. lwoffii* may be able to form comparable amounts of biofilm in more clinically relevant conditions.

4.5.3 The presence of biofilm- associated genes in the laboratory strains

To determine if the differences seen in the static biofilm assay can be explained by the presence of biofilm genes in the whole genome sequences, the laboratory strains were interrogated using a custom "vandb" database in ABricate, created using genes published in Hamidian *et al.,* (2022). This database was not comprehensive and did not include all biofilm-associated genes, for example quorum sensing genes. In *A. lwoffii* only two isolates encoded a biofilm associated gene, *pgaC* - AL04 and AL54. AL04 was tested for biofilm capacity with crystal violet and out of the *A. lwoffii* tested, it formed the most biofilm in static conditions, Figure 4.4. The results were not as clear, however, for biofilm formation under laminar flow, Figure 4.5.

Furthermore, all strains of *A. baumannii* tested contained the biofilm associated genes *pgaABCD* and *csuABC*. In addition to that, AB06, AB17, AB19, AB20, AB22 and AB25 also encoded *blp*. *A. baumannii* AB19 and AB20 formed the most biofilm, which agrees with the genomic data showing they harbour more biofilm-associated genes than the other *A. baumannii* strains.

4.5.4 Biofilm associated genes across all whole genome sequences

When expanding the search for biofilm associated genes to all the available genomes it became clear that on average the *A. lwoffii* genomes searched only coded for one biofilm gene - *pgaC*, whereas *A. baumannii* genomes coded for more (average of 8 genes), Figure 4.6. To determine if, despite the dataset size difference, there was a significant difference in biofilm associated genes a random permutation and Welch's T-test was conducted, where *A. lwoffii* coded for fewer biofilm associated genes than *A. baumannii* ($p = <0.0001$).

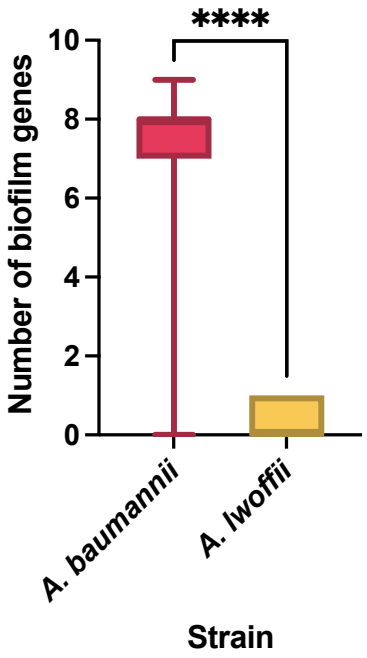


Figure 4.6: Number of biofilm- associated genes found across *A. baumannii* and *A. lwoffii*. *A. baumannii* is shown in pink and *A. lwoffii* is in yellow. Whiskers show the minimum and maximum number of biofilm associated genes found in the whole genome sequences and the middle horizontal line is the mean. Statistical significance was determined using a T-test with Welch's corrections (**** = p<0.0001).

Table 4.2 shows the percentage presence of each biofilm associated genes within the whole genome sequences, *A. baumannii* encodes more of the *pga* operon than *A. lwoffii*, including additional biofilm associated genes, such as *blp*.

4.5.5 Minimum biofilm eradication concentrations

Once bacteria, such as *Acinetobacter*, are existing in a biofilm lifestyle they are much harder to treat (Espinal, Martí, & Vila, 2012), therefore the concentration of antibiotics needed to eradicate biofilm formed by *A. baumannii* and *A. lwoffii* strains was tested. To determine

Table 4.2: Presence of biofilm associated genes in *A. baumannii and A. lwoffii* whole genome sequences

Gene	% *A. baumannii*	% *A. lwoffii*
pgaC	100	37
pgaA	100	0
pgaB	99	0
pgaD	99	0
csuC	90	0
csuB	89	0
csuA	89	0
blp	82	0
bap	1	0

the minimum biofilm eradication concentrations (MBEC) to a range of compounds, the two strains (AB20 and AL04) that formed the most dense, static biofilms were grown in a 96 well plate with pegs, allowed to form biofilm for 24 hours, and then the biofilm on the pegs were subjected to antibiotic or biocide treatment. The growth of cells after treatment was assessed to determine the MBEC.

Table 4.3: Minimum biofilm eradication concentrations for *A. lwoffii* AL04 and *A. baumannii* AB20

	AL04			AB20		
Drug	MBEC (μg/mL)	MIC (μg/mL)	Fold Change	MBEC (μg/mL)	MIC (μg/mL)	Fold Change
Cefotaxime	256	2	8	8192	16	10
Chlorhexidine	0.0017%	0.0008%	-1	>1%	0.0035%	>9
Ciprofloxacin	16	0.06	9	128	2	7
Meropenem	0.06	0.03	2	>128	0.25	>10
Oxacillin	8	16	-1	>4096	512	>3
Tetracycline	256	0.5	10	1024	2	10
Triclosan	0.5	0.5	1	>128	1	>8
Rifampicin	8	0.5	5	64	4	5

Table 4.3 displays the MBEC results. Both *A. lwoffii* AL04 and *A. baumannii* AB20 form biofilm on peg lids that are less susceptible to antibiotics than planktonic cells. The biofilms formed needed 10-fold higher concentrations of tetracycline to eradicate viable cells in both species, whereas for rifampicin only a 5 fold increase from MIC was observed. When focusing on the biocides (chlorhexidine and triclosan), AL04 biofilm had an MIC two fold higher than the MBEC for chlorhexidine and the same value in both lifestyles for triclosan, indicating that the biofilms did not display decreased susceptibility compared to planktonic cells. This was not the case for AB20 where, as with the antibiotics, the MBEC was many fold higher than the MIC. Therefore, in strains that formed the most dense biofilm, from both species, the biofilm lifestyle made them less susceptible to a range of different compounds, including drugs used to treat *Acinetobacter* infections (ciprofloxacin, meropenem and tetracycline).

4.6 Virulence and infection

Thus far this chapter has focused on differences in the biology between *A. baumannii* and *A. lwoffii*. The next section seeks to determine any differences in the ability to cause infection between the two species. As mentioned previously both species can cause a range of human infections, including bacteraemia. In order to look at this, the sensitivity of *Acinetobacter* strains to complement proteins was measured and a human monocyte tissue culture *in vitro* experiment was conducted to determine if strains could attach to, get inside or survive within macrophages. Additionally, virulence was also measured in an *in vivo Galleria mellonella* wax worm larvae model. Finally, the presence of genes related to virulence was searched for in the whole genome sequences.

4.6.1 Growth and survival in the presence of human serum

Human serum is 55% of the volume of whole blood (Aylward, 2019) and is comprised of complement proteins, which can directly kill bacteria and attach to them to recruit phagocytic cells (Carroll & Sim, 2011). To determine if both *A. lwoffii* and *A. baumannii* can survive and grow in human serum two experiments were conducted. The first experiment measured active growth at 37°C in 50% human serum and 50% LB and included heat-inactivated serum conditions, where complement was absent, to determine if the growth was dependent on a lack of complement proteins. Figure 4.7 shows the differences in growth between the two *A. baumannii* (AB05, AB18) and *A. lwoffii* (AL04, AL28) strains. AL04 grew better in heat-inactivated serum (without complement proteins) than normal human serum, but managed to grow in both. However the other *A. lwoffii* strain tested, AL28, provided inconsistent readings, due to clumping, possibly to protect the cells from serum proteins, so it is challenging to compare its growth. The *A. baumannii* strains both grew in normal and heat-inactivated serum.

When analysing mean generation time, all strains had a slower generation time in both heat-inactivated and normal human serum, than compared to LB (Table 4.4). Furthermore, both *A. baumannii* strains had faster mean generation times than the *A. lwoffii* strain AL04. It was not possible to compute mean generation time in serum for *A. lwoffii* AL28 due to its lack of growth. Finally, in all three strains with mean generation times, generation times were slower when complement proteins were in the serum. To determine if any of the differences in mean generation time were statistically significant a Welch's ANOVA was done in Graphpad Prism to compare each strain per condition. This showed that AB18 had a faster mean generation time in heat-inactivated serum than AB05 ($p=0.0098$) but otherwise the differences were not statistically significant between the strains in each condition.

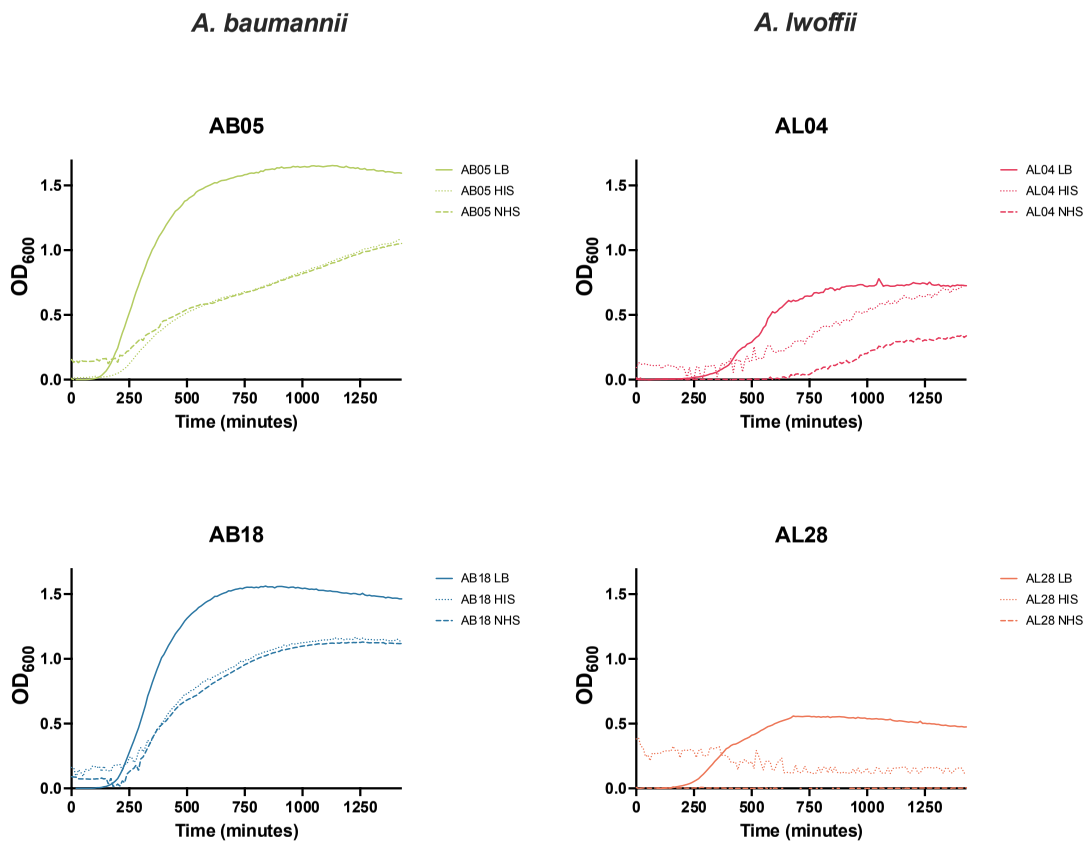


Figure 4.7: Growth in the presence of human serum. *A. baumannii* AB05 and AB18 and *A. lwoffii* AL04 and AL28 were grown for 24 hours in the presence of 50% human serum and 50% LB and LB only (solid line). AL04 and AB05 are clinical and AB18 and AL28 are non-clinical strains. HIS = heat inactivated serum (dotted line), NHS = normal human serum with complement proteins (dashed line). The experiment was independently repeated three times and the mean values were plotted.

Table 4.4: Mean generation times (hours) of strains grown in LB and human serum at 37°C

Strain	LB Mean generation time ± standard error	Heat-Inactivated Serum Mean generation time ± standard error	Normal Human Serum Mean generation time ± standard error
AL04	0.926 ± 0.26	3.257 ± 1.284	4.611 ± 1.728
AL28	0.492 ± 0.066	*	*
AB05	0.910 ± 0.088	2.507 ± 0.04	3.972 ± 1.62
AB18	0.691 ± 0.067	1.385 ± 0.012	1.495 ± 0.12

* due to the clumping of AL28 in serum it was not possible to compute accurate mean generation times, see Figure 4.7 for growth curves.

In addition to measuring active growth of strains in serum, their ability to survive in serum was also tested. Here, 1x10^6 *A. lwoffii* and *A. baumannii* cells were diluted 1 in 10 in normal human serum and incubated at 37°C with gentle rocking (20 rpm). At 0, 1.5, 3 and 24 hours, serial dilutions were performed to measure the CFU/mL of the strains.

Both *A. baumannii* and *A. lwoffii* strains tested could survive in human serum, Figure 4.8. For *A. lwoffii* (AL04) and *A. baumannii* (AB05 and AB18) there was an increase in CFU/mL counts after three hours (180 minutes), where cells were not being killed by high concentrations (90% v/v) of human serum. For *A. lwoffii* AL28 CFU/mL counts were decreasing by three hours but there was still viable cells and by 24 hours across all four strains the CFU/mL counts were similar in serum to in LB. These data combined with growth in human serum show that *A. lwoffii* AL28 is more susceptible to serum than other *A. lwoffii* and *A. baumannii* strains tested.

In summary, both *A. baumannii* strains tested could survive and grow in the presence

of human serum, including serum with intact complement proteins, whereas only one of the two *A. lwoffii* strains could survive and actively grow in serum. Additionally strains grew better in heat-inactivated serum. This indicates that, of the strains tested, the *A. baumannii* isolates are better at surviving in human serum.

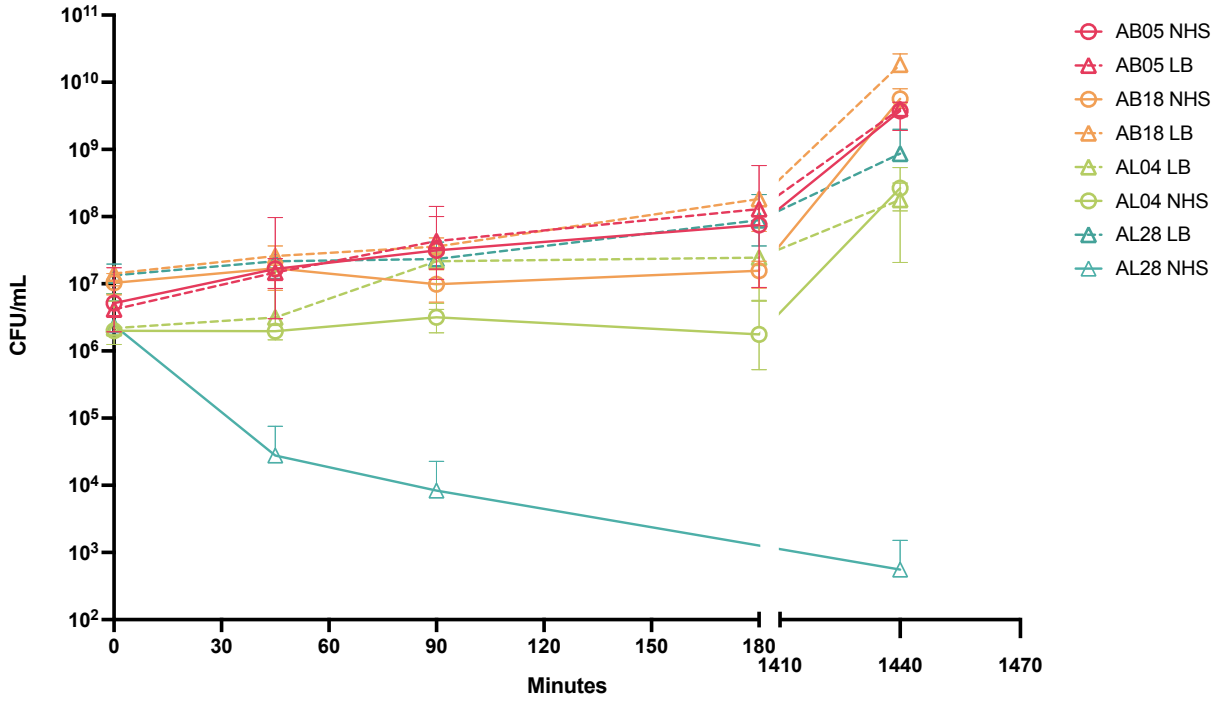


Figure 4.8: Survival in the presence of human serum. *A. baumannii* AB05 (pink), AB18 (orange) and *A. lwoffii* AL04 (green) and AL28 (blue) cells were added to 90% normal human serum (uninterrupted lines) or LB (dashed lines) and grown with gentle agitation at 37°C. CFU counts were taken at 0, 0.5, 1.5, 3 and 24 hours (45, 90, 180 and 1,440 minutes) to determine cell survival. As before, AL04 and AB05 are clinical and AB18 and AL28 are non-clinical strains. The experiment was independently repeated three times and median values with error range are shown. A two-way ANOVA with Tukey's multiple comparisons was carried out using Graphpad Prism and of the strains tested there was no significant differences in the survival of strains in human serum. AL28 in NHS at 180 minutes had a median CFU/mL of 0, it was below the limit of detection of the experiment, hence the missing data point. Additionally, at 0 minutes there are discrepancies in starting CFU/mL due to the limit of detection of the experimental set up.

4.6.2 Attachment, uptake and survival in human macrophages

In addition to measuring survival in human serum, the ability of *A. lwoffii* and *A. baumannii* strains to attach to, exist within and survive/ actively grow within human macrophages was determined. This is relevant because in a bloodstream infection, you would expect these organisms to encounter immune cells, such as macrophages. Briefly, THP-1 cells were cultured and stimulated with PMA to differentiate into macrophages. These macrophages were incubated with each strain at an MOI of 100 for 2 hours and then extracellular bacteria were washed away using PBS to measure attachment. Uptake of bacteria was measured by treating the macrophages with gentamicin to kill extracellular bacteria after 2 hours. Finally, survival was measured 6 hours after the bacteria were initially added to the cells - again gentamicin was used to remove extracellular bacteria after two hours. Survival was also measured after 24 hours, but by this time point the macrophage cells were lysed by the bacteria, which had then grown in the media, therefore planktonic growth of bacteria in the media and not the intracellular proliferation of cells was being measured (data not shown).

Of the six *Acinetobacter* strains tested, *A. baumannii* strains (AB05, AB18 and AB20) could attach to and get inside macrophages but limited growth within macrophages was seen. CFU counts were similar despite the prolonged incubation for survival. The *A. lwoffii* strains were poorer at attaching to the macrophages, but there was some evidence of them within macrophages after 4 hours but not after the longer incubation for survival, Figure 4.9. This shows, that of the strains tested, *A. lwoffii* was less able to cause intracellular infections in macrophages than *A. baumannii*. When comparing strains using a one-way ANOVA with multiple comparisons the results seen were not statistically significant, apart from when comparing SE01 (the positive control) to the *Acinetobacter* strains.

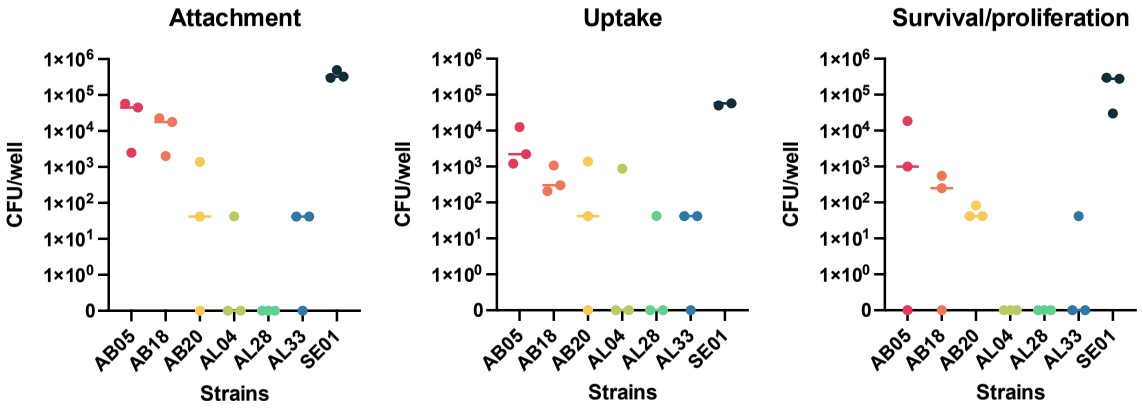


Figure 4.9: Infection of macrophages with *A. baumannii* (AB) and *A. lwoffii* (AL). The attachment, uptake and survival of bacterial cells was measured in human macrophages. Attachment and uptake were measured after four hours exposure to bacteria and survival/proliferation was measured at six hours. The experiment was repeated biologically three times. *Salmonella enterica* serovar Typhimurium (SE) was used a positive control. AB05, AB20 and AL04 are clinical strains, AL33 is an environmental strain. The isolation origins of AB18 and AL28 are unknown. Median values are shown as coloured lines on the plots. *A. lwoffii* AL04, AL28 and AL33 were less able to attach to, persist within the macrophages. A one-way ANOVA with Tukey's comparisons test was done to determine significance and none of the *Acinetobacter* conditions were significantly different.

4.6.3 *Galleria mellonella* model of infection

A. baumannii and *A. lwoffii* were differentially able to survive in human serum and macrophages and therefore an *in vivo*, *Galleria mellonella*, model was chosen to determine any differences in virulence. *G. mellonella* has an innate immune system functionally similar to that of vertebrates (Asai *et al.*, 2023) and has been used previously to highlight differences in virulence between *Acinetobacter* species (Peleg *et al.*, 2009). Therefore, *G. mellonella* larvae were inoculated with 10^6 colony forming units and their survival was measured over four days.

In a wax worm model, of the limited number of strains tested *A. baumannii* killed the *Galleria* more quickly than *A. lwoffii*, although there were differences between strains, where AB05 (*A. baumannii* AYE) was more virulent than AB18 (*A. baumannii* AII8), Figure 4.10. There was little difference observed between the *A. lwoffii* strains, despite one being a clinical isolate (AL04) and one not (AL28) and they were not significantly different from the experimental negative control of PBS.

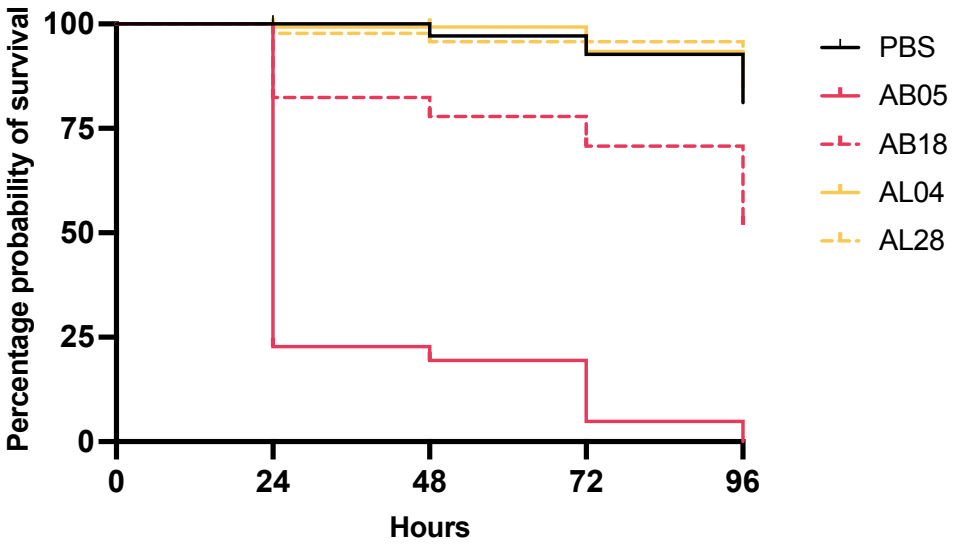


Figure 4.10: Survival of *Galleria mellonella* infected with *A. baumannii* and *A. lwoffii*. Survival was measured over 4 days (96 hours) after inoculation with either *Acinetobacter lwoffii* (AL) in yellow or *Acinetobacter baumannii* (AB) in pink. AB05 and AL04 are clinical strains, whereas AB18 and AL28 are not. The experiment was independently repeated four times and the probability of survival is plotted as a Kaplan–Meier curve, where a Log-Rank test was used to find statistical significance in Graphpad Prism. Of all the conditions tested, only AB05 was significantly more virulent than the other strains ($P<0.0001$). PBS is the injury control.

To determine if the differences in killing between strains were statistically significant, a log rank (Mantel Cox) test was conducted on two groups at a time using Graphpad Prism. This showed that *A. baumannii* AB05 is statistically better at killing *Galleria* larvae than all other conditions including PBS ($p < 0.0001$), even when compared against *A. baumannii* AB18. Furthermore, *A. baumannii* AB18 was statistically better at killing *Galleria* than PBS, AL04 and AL28 ($p < 0.0001$). Finally, there was no statistically significant differences found between the PBS and *A. lwoffii* AL04 and AL28 groups. Therefore, these *A. lwoffii*

strains could not kill *Galleria* at a rate any faster than the PBS-treated worms died (all p values >0.5). In the *Galleria* model of infection, *A. lwoffii* strains were less virulent than *A. baumannii*.

4.6.4 The presence of virulence associated genes

Work in this chapter showed that *A. lwoffii* is less virulent both *in vitro* and *in vivo*. Therefore to determine if there were any genetic signatures that could explain this, the genomes of both the laboratory strains, and genomes available on NCBI, were searched for the presence of virulence genes.

As with biofilm associated genes, virulence genes were searched for using ABRicate with custom database (vandb) created in this study using genes listed in Hamidian *et al.,* (2022). This resulted in a database of 27 genes ranging from siderophores and virulence regulatory genes and included biofilm associated genes. It is important to note that this database is not comprehensive and some virulence genes are omitted due to it being based upon data solely from the Hamidian *et al.,* (2022) paper. Biofilm gene presence discussed in Section 4.5.4. The results for virulence genes are shown in Table 4.5.

Table 4.5: The presence of virulence associated genes in *A. baumannii* and *A. lwoffii* sequences

Gene	Product/function	% *A. baumannii*	% *A. lwoffii*
blc	Outer membrane protein	100	89
gacA/S	Virulence regulator	100	100
wzb	Capsule biosynthesis	100	92
gigA	Virulence regulator	100	100
ptk	Capsule biosynthesis	100	95

bmfR	Virulence regulator	100	100
bmfS	Virulence regulator	100	100
smpA	Outer membrane protein	100	100
galU	Capsule biosynthesis	100	97
ABUW_2185	Siderophore	99	0
ABUW_2183	Siderophore	99	0
ABUW_2184	Siderophore	99	0
ABUW_2186	Siderophore	99	0
ABUW_2189	Siderophore	99	34
ABUW_2188	Siderophore	99	34
ABUW_2187	Siderophore	99	0
ompA	Outer membrane protein	86	0
bauA	Siderophore	83	0

Every *A. lwoffii* sequence contained the presence of *bfmRS, gacA/S* and *gigA* virulence regulatory systems as well as *smpA* a virulence gene encoding an outer membrane lipoprotein. Capsule biosynthesis virulence genes *galU, ptk* and *wzb* were found in 97%, 95 % and 92% of sequences, respectively. Another outer membrane protein encoding gene, *blc*, was found in 89% of the genomes. Finally, two siderophores (ABUW_2188 and ABUW_2189) were found in 34% of sequences.

A. baumannii encoded more genes than *A. lwoffii* from the vandb database, however this is at least in part due to the fact that the database is comprised of *A. baumannii* sequences. For example, *A. baumannii* had eight siderophores, in comparison to *A. lwoffii*, which only had two. All *A. baumannii* sequences tested encoded *bfmRS, gacA/S, gigA* and *smpA*, like *A. lwoffii*. In addition to those genes, 100% of sequences also contained *blc*, an outer membrane protein associated with virulence, and capsule biosynthesis genes involved in

virulence *wzb, ptk* and *galU*. Therefore, it is possible that despite causing fewer bloodstream infections than *A. lwoffii*, *A. baumannii* may be more virulent.

A. baumannii whole genome sequences had significantly more virulence associated genes, where *A. lwoffii* sequences (n= 38) had a mean of 9 virulence genes found per sequence and *A. baumannii* (n= 4,809) had a mean of 18 genes per genome sequence, Figure 4.11. To determine if this result was due to the different dataset sizes, as with other bioinformatics analyses, a random permutation test followed by a Welch's T-test was done to determine statistical significance. *A. baumannii* genomes code for significantly more virulence genes than *A. lwoffii* genomes, p <0.0001.

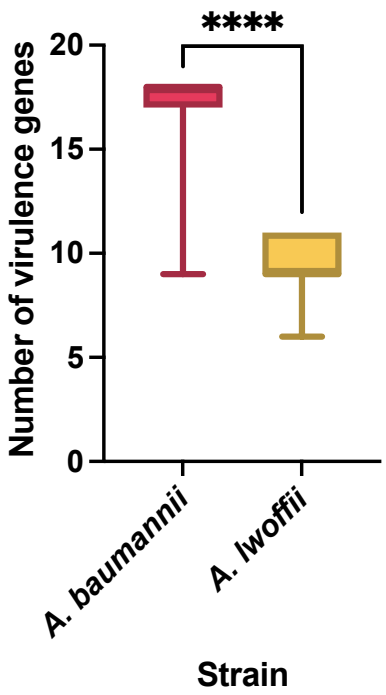


Figure 4.11: Number of virulence-associated genes found across *A. baumannii* and *A. lwoffii*. *A. baumannii* is shown in pink and *A. lwoffii* is in yellow. Whiskers show the minimum and maximum number of virulence associated genes found in the whole genome sequences and the middle horizontal line is the mean. Statistical significance was determined using a T-test with Welch's corrections (**** = p<0.0001).

4.7 Discussion

It has been well documented that both *A. baumannii* and *A. lwoffii* can cause infections in humans, including bloodstream infections. However, most studies focus on *A. baumannii* and closely-related species of the *acb* complex, when discussing infection-causing *Acinetobacter* and therefore far less is known about *A. lwoffii* and how it may cause infections. Additionally, far less is known generally about the biology of *A. lwoffii*, likely due to the fact it is easier to treat than other *Acinetobacter* infections.

The main aims of this chapter were to use phenotypic experiments and to compare genome sequences from *A. baumannii* and *A. lwoffii* to try to understand the biology, including the virulence, of these two organisms better. It is known that *Acinetobacter* is can be found in soil environments, as well as within/on humans and therefore it is not surprising that it can grow at a range of temperatures (Munoz-Price & Weinstein, 2008; Parte *et al.*, 2020). Due to the fact that there was little difference in growth rate of either species between the temperatures tested, all subsequent experiments were done at 37°C unless stated otherwise. There was, however, a difference in final cell density between *A. baumannii* and *A. lwoffii* and therefore caution is needed when interpreting some of the phenotypic data. For example, the absorbance for *A. lwoffii* static biofilm was lower than *A. baumannii* but this could be explained by the cells growing to a lower cell density.

To better understand the cellular morphology of either species, scanning electron micrographs were taken. The *A. lwoffii* cells displayed a longer cellular morphology, possibly due to ineffective cell division. Previous work has shown that in *Bacillus subtilis* nutrient limitation can change where septation in cell division occurs (Rothfield & Zhao, 1996), therefore it is possible that *A. lwoffii* was not grown in optimal media (LB) and needed additional substrates to grow, such as iron which is found in the blood.

The focus then turned to how either species may form biofilm, where biofilm is a more physiologically relevant lifestyle for bacteria. When comparing if either species can form biofilm and the extent of this, *A. baumannii* formed more biomass, at least in static conditions. Biofilm is a more relevant condition as bacteria do not naturally grow in monospecies planktonic culture (Jefferson, 2004). It is well known that *A. baumannii* can form biofilm, which is thought to play a role in their ability to persist and then to cause infection (Longo, Vuotto, & Donelli, 2014), however comparatively less information exists for *A. lwoffii*. It is known that *A. lwoffii* can form more biofilm when incubated with bacteria isolated from marine algae to form a multispecies biofilm (Burmølle *et al.*, 2006). Additionally, a study has shown that *A. lwoffii* can strongly enhance *Legionella pneumophila* biofilms (Guerrieri *et al.*, 2008). Therefore, it could be that *A. lwoffii* does form biofilm but the conditions of the static experiment (single species) were not optimised to show this. Additionally, the biofilm absorbance data could be normalised by CFU growth data in future experiments to make sure that the lack of biofilm mass seen by crystal violet staining is not an artefact of *A. lwoffii* growing to a lower final cell density in the laboratory.

Biofilm formation differed under laminar flow, where both species could form similar amounts of biofilm using this model. The data were more varied both within and between species for these experiments. This could be due to biofilm not uniformly covering the entire slide and therefore microscopy images taken at different points could have different amounts of bacterial load. It is clear, however, that biofilm does form under laminar flow in both species over time and certain strains are more adapted to this than others.

It is accepted that a biofilm lifestyle can protect dormant cells and reduce susceptibility to antimicrobials, despite this biofilm is not linked to a worse clinical outcome, at least in *Acinetobacter* lung infections (Wang, Huang, *et al.*, 2018). Despite biofilm not being linked to disease outcome, there are strong links between biofilm

formation and the susceptibility of cells to antibiotics (Caraher *et al.*, 2007). The MBEC data in this chapter follows the same pattern, where generally higher concentrations of antibiotics and biocides are needed to eradicate established biofilms than to treat planktonic cells. This has implications for the way susceptibility is measured in the clinic and indicates that traditional MIC tests are not perfect for gauging a bacterium's response to drug treatment. This is especially true when the bacteria will not be treated as a monospecies but likely in a multispecies or even multi-kingdom environment (Chevalier, Ranque, & Prêcheur, 2018). Previous work has shown that *A. baumannii* can work synergystically with *K. pneumoniae* to protect it from antibiotics (Semenec *et al.*, 2023).

Whilst it was known that *A. lwoffii* and *A. baumannii* can cause bloodstream infections, relatively little is known about how these opportunistic pathogens differ with respect to virulence. The data in this work showed that of the strains tested *A. lwoffii* was less virulent in a *G. mellonella* model. This result may be affected by the fact that *A. lwoffii* grows to a lower cell density and therefore the circumvent this challenge a higher initial inoculate could be used where virulence factors play a bigger role than bacterial growth. Alternatively, you could also do CFU counts from the *Galleria* to measure bacterial burden of infection. For the *in vivo Galleria* model, wax worm larvae was chosen due to their presence of an innate immune system, similar to vertebrates, and virulence in *G. mellonella* often mirrors that found in mammalian infection models (Asai *et al.*, 2023). However, to more appropriately model a bloodstream infection a murine model would be better e.g. in Leshkasheli *et al.* (2019). Murine models are more relevant because mice, like humans, are mammals. Mice are also genetically similar to humans and have similar organs, which enables different types of infection to be studied, such as lung, skin or bloodstream infections (Harris *et al.*, 2017).

To mimic clinically relevant conditions the survival and growth in human serum of both species was measured. This was done with normal human serum and heat-inactivated

serum because it is already known that there are strain-level differences in susceptibility to human complement proteins in *A. baumannii* (Kamuyu *et al.*, 2022) but this has not yet been evaluated in *A. lwoffii*. Both *A. baumannii* strains tested grew as well in serum with and without complement, however for AL04 (*A. lwoffii*), the presence of complement proteins reduced growth significantly. Additional experiments conducted on these strains in a skin wound model by Dr. Freya Harrison at the University of Warwick showed the same results with respect to complement sensitivity (data not shown). Around 50% of *A. baumannii* strains express CipA, a protein which binds to plasminogen allowing for the degradation of fibrinogen and the cleavage of complement protein C3b (Koenigs *et al.*, 2016). CipA allows for the bacteria to disseminate and to avoid killing by the alternative complement pathway (normally complement protein C3b sticks to the surface of bacterial cells and leads to the membrane attack complex where the bacterial cell is lysed) (Koenigs *et al.*, 2016). In order to survive in the presence of serum, CipA is not enough, in fact a recent study found that 50 genes are essential for the survival of *A. baumannii* in serum (Sanchez-Larrayoz *et al.*, 2017). Therefore, *A. lwoffii* may not express the required proteins for surviving in serum with complement proteins or it could be that *A. lwoffii* strains are capable of surviving in human serum as well as *A. baumannii* strains but the two chosen in this study were not particularly good at it, although the clinical isolate (AL04) was better than the type strain (AL28). This is surprising because the data from UKHSA shows that of the two, *A. lwoffii* causes more bloodstream infections (United Kingdom Health Security Agency, 2021), perhaps indicating this result may be specific to the strains tested in this work.

The ability of bacteria to cause infection is often measured *in vitro* in tissue culture. *A. baumannii* can be phagocytosed by macrophages (Qiu *et al.*, 2012) but conversely some cells successfully survive and even replicate within macrophage vacuoles (Sato *et al.*, 2019; Sycz *et al.*, 2021). Macrophages and bacterial cells can both drive the process of uptake, for example bacteria have pathogen-associated molecular patterns (PAMPs), such as LPS,

which can interact with pattern recognition receptors (PRRs) on macrophages, stimulating phagocytosis (Wan, Zhou, & Zhu, 2018). Additionally, some bacteria can aid the process of internalisation, for example in mycobacteria where pathogenic species use a serum factor to aid macrophage entry (Schorey, Carroll, & Brown, 1997). Whilst little is known about the response of *A. lwoffii* to human immune cells, we know that virulence factors in *A. baumannii*, such as Omp33-36, enable adhesion to host cells, which in turn aid macrophage uptake where they can modulate autophagy allowing *A. baumannii* to survive intracellularly (Tiku, 2022). This work focused on macrophages, however there are different immune cells that *Acinetobacter* may encounter during an infection situation, for example neutrophils (Lazaro-Diez *et al.*, 2017). Despite only testing one cell line, the *A. baumannii* strains in this work could attach to and get inside macrophages, but the *A. lwoffii* strains could not, therefore it is possible that *A. lwoffii* is more susceptible to killing by the human immune system than *A. baumannii*.

Virulence and biofilm formation have previously been linked to RND systems (Richmond *et al.*, 2016), and as mentioned in Chapter 3.1, *A. lwoffii* encodes fewer RND systems than *A. baumannii*. In this Chapter, *A. lwoffii* strains generally formed less biofilm and were less virulent in a *G. mellonella* model of infection. The lack of 'adaptive' efflux systems, such as AdeABC/ AdeFGH in *A. lwoffii*, which are often overexpressed in *A. baumannii* in response to external stimuli (Yoon, Courvalin, & Grillot-Courvalin, 2013; Coyne, Rosenfeld, *et al.*, 2010), may account in part for the reduced capacity to form biofilm and virulence.

In addition to phenotypic experiments, the whole genome sequences were searched for the presence of biofilm and virulence genes to compare the species. The virulence and biofilm database used was manually curated from Hamidian *et al.* (2022) based upon *A. baumannii* sequences, therefore this database was biased to highlight *A. baumannii* genes. *A. lwoffii* did contain fewer genes associated with virulence and biofilm within the database, but it

could be that *A. lwoffii* contains a complement of currently undescribed genes that encode proteins for these purposes. 89-90% of *A. baumannii* sequences contained *csu* genes, for Csu pili and these genes were absent in *A. lwoffii*. Csu pili enable *Acinetobacter* to colonise abiotic surfaces and form biofilm (Pakharukova *et al.*, 2018). Work in Section 4.5 showed that *A. lwoffii* can form biofilm, so it is likely that Csu pili are part of a network of biofilm formation, maturation and dispersal genes. Of the siderophores characterised, *A. baumannii* encodes more than *A. lwoffii*, which indicates that whilst siderophores might be useful in this environment, *A. lwoffii* must use other mechanisms, or currently unknown siderophores, to survive and sequester iron.

In summary, this work showed that *A. lwoffii* was less virulent both *in vitro* and *in vivo*, however the infection assays were all done with monospecies, single strain cultures. In the future, it would be interesting to determine whether *A. lwoffii* can better form biofilm, establish infections and be more virulent with the addition of other bacterial species or diverse *A. lwoffii* strains, an example of this in another species is in lung infections caused by *Pseudomonas aeruginosa*, where one in three patients have mixed strain populations causing infection (Diaz Caballero *et al.*, 2023).

4.8 Conclusions

This is the largest study to date on the biology of *A. lwoffii* and uses *A. baumannii* as a comparison organism because they cause the same types of infections in humans. This work has shown that despite belonging to the same genus, these species differ considerably in the laboratory and in infection models. *A. lwoffii* grows more poorly, forms less biofilm mass under static conditions and is less virulent *in vivo* and *in vitro*.

However, *A. lwoffii* does cause more cases of bacteraemia than *A. baumanii*, (United

Kingdom Health Security Agency, 2021) and questions remain as to how it manages to cause infections in humans and the mechanisms underpinning this.

4.9 Further work

As with Chapter 3, a limitation of this work was the limited number of *A. lwoffii* whole genome sequences available. To make these conclusions more robust, redoing the analyses with a larger dataset would help to minimise the risk of skewing the data due to unusual sequences.

From the growth kinetics data it is clear that the laboratory enviornment was not as well suited to *A. lwoffii* as it was to *A. baumannii* and therefore subsequent work could be done to try to optimise the culture conditions of this bacterium as it may affect some of the phenotypic experiments if the bacteria are nutrient deprived beforehand.

In order to expand the scope of this work it would be interesting to look at multispecies biofilms of either species and if there is any evidence of co-infection of *A. lwoffii* (or *A. baumannii*) with another organism. Co-infection may explain why on its own *A. lwoffii* seems to be less virulent than *A. baumannii* but still causes many bloodstream infections. Additionally, repeating experiments with more strains will also help to uncover if the phenotypes seen here are general to *A. lwoffii* or specific to the few strains tested.

4.10 Key findings

- Both species can readily form biofilm under laminar flow but *A. baumannii* is better at forming biofilm under static conditions
- *A. lwoffii* is less able to infect macrophages than *A. baumannii*
- *A. lwoffii* is less well adapted to surviving in human serum, both with and without complement, compared to *A. baumannii*
- *A. lwoffii* is less virulent in a *Galleria mellonella* model than *A. baumannii*

Chapter Five

RND pumps across the *Acinetobacter* genus.

5.1 Foreword

The work in this chapter has been published: Darby, E. M., Bavro, V. N., Dunn, S., McNally, A., & Blair, J. M. A. (2023). RND pumps across the genus *Acinetobacter*; AdeIJK is the universal efflux system. *Microbial Genomics, 9*(3).

5.2 Background

RND transporters are both directly useful by pumping out toxic chemicals, such as antibiotics, and in doing this, they can also allow the cell time to evolve resistance mutations (Darby, Trampari, *et al.*, 2023; Papkou *et al.*, 2020).

Table 5.1 shows the nine RND transporters characterised across the *Acinetobacter* genus to date (Kornelsen & Kumar, 2021). All but one, *czcA*, are hydrophobic and

amphipathic (HAE) RND transporters, whereas *czcA* is a heavy metal efflux (HME) transporter. Most RND systems found have been within *A. baumannii* genomes, given that this is the most well studied species within the genus. Of particular note are *adeB, adeG* and *adeJ*, whose tripartite complexes contribute to multidrug resistance by extruding compounds from the cytoplasm to outside the cell (Kornelsen & Kumar, 2021). AdeIJK is constitutively expressed, rarely overexpressed, providing intrinsic levels of resistance to antibiotics (Fernando, Xu, *et al.*, 2014), whereas AdeFGH and AdeABC are often overexpressed in the presence of antibiotics and can mediate multidrug resistance (Yoon, Courvalin, & Grillot-Courvalin, 2013; Shi *et al.*, 2020; Lari, Ardebili, & Hashemi, 2018; Coyne, Rosenfeld, *et al.*, 2010; Coyne, Guigon, *et al.*, 2010).

Table 5.1: RND genes characterised across *Acinetobacter*

RND gene	Compounds exported	Species	References
adeB	Aminoglycosides, trimethoprim, chloramphenicol, fluoroquinolones, tetracyclines, pentamide	*A. baumannii*	(Magnet *et al.,* 2001), (Adams *et al.,* 2018)
adeE	Amikacin, ceftazidime, chloramphenicol, ciprofloxacin, erythromycin, meropenem, rifampicin, tetracycline	*A. pittii*	(Chau *et al.,* 2004)
adeG	Chloramphenicol, trimethoprim, tetracycline-tigecycline, clindamycin, fluoroquinolones	*A. baumannii*	(Coyne *et al.,* 2010)

adeJ	Beta lactams, chloramphenicol, tetracycline, erythromycin, lincosamides, fluoroquinolones, fusidic acid, novobiocin, rifampicin, trimethoprim	*A. baumannii*	(Damier-Piolle *et al.,* 2008)
adeY	Unknown	*A. baylyi*	(Chu *et al.,* 2006)
abeD	Ceftriaxone, gentamicin, rifampicin, tobramycin, benzalkonium chloride	*A. baumannii*	(Srinvasan *et al.,* 2015)
arpB	Amikacin, tobramycin	*A. baumannii*	(Tipton *et al.,* 2017)
acrB	Colistin, tobramycin, acriflavin	*A. nosocomialis*	(Subhadra *et al.,* 2018)
czcA	Heavy metals e.g. copper	*A. baumannii*	(Williams *et al.,* 2016)

Table reproduced from (Kornelsen & Kumar, 2021)

Whilst some of the RND pumps across *Acinetobacter* have been characterised, for example pumps in *A. lwoffii* and *A. baumannii* were determined earlier in this thesis, the full spectrum of RND pumps and the average number per species is currently unknown. In other Gram negative species the number of RND pump varies from one in *Neisseria gonorrhoeae* to 12 in *Pseudomonas aeruginosa* (Colclough, Alav, *et al.*, 2020). Furthermore, the number of RND pumps can also differ within a species (Horiyama, Yamaguchi, & Nishino, 2010; Nemec *et al.*, 2007; Lin, Ling, & Li, 2009; Pugh *et al.*, 2023).

5.3 Aims and hypotheses

The complement of RND genes within the *Acinetobacter* genus is not known. The aims of this chapter are:

1. Develop a novel method to detect RND proteins within Gram negative bacterial genomes
2. Quantify the number of RND proteins across each species belonging to the *Acinetobacter* genus
3. Determine RND systems common among the majority of species
4. Determine if the number of RND transporters correlates with the ability to cause infection in humans

From these aims, the hypothesis that disease-causing *Acinetobacter* encode more RND systems than environmental species, in order to survive in different niches, will be tested.

5.4 The development of a novel database to search for RND proteins

The number of inner membrane RND pump transporters is known to differ between different bacterial genera. Therefore, a method was developed to quickly find the number of RND proteins in a given genome sequence. This was done by determining what residues are common across all RND pumps and using this information to search available genomes. To that end, the sequences of 24 known RND pump genes from 16 different species were aligned to determine the residues conserved in >80% of the sequences. Due to the degree

of differences between HAE and HME pumps, two alignments were created (found at https://doi:10.1099/mgen.0.000964 - Supplementary file 1), which led to the creation of two conserved residue files, with which BLASTp could search genomes to determine the presence and type of RND transporters. Many conserved residues were in the areas known to be involved in the proton motive force, such as D408 and T978 (*E. coli*, AcrB numbering (Su, Li, *et al.*, 2006; Takatsuka & Nikaido, 2006). The files used to search for the presence of RND proteins are in appendix B.

To validate that this method worked, it was applied to genomes from 10 Gram negative bacterial species, Table 5.2.

Table 5.2: BLASTp results for the average number of RND proteins within a range of Gram negative species' genomes

Species	Accession	Number of predicted HAE proteins	Number of predicted HME proteins	Total RND number in literature (reference)
Acinetobacter baumannii ACICU	GCA_000018445.1	5	1	6 (Kornelsen *et al.,* 2021)
Acinetobacter baumannii AYE	CU459141.1	4*	1	6 (Kornelsen *et al.,* 2021)
Campylobacter jejuni NCTC 11168	GCA_900475265.1	2	0	2 (McNeil *et al.*, 2019)
Escherichia coli K12	NC_000913.3	6	1	7 (Anes *et al.*, 2015)
Haemophilus influenzae NCTC 8143	GCA_001457655.1	1	0	1 (Zwama et al., 2019)
Klebsiella pneumoniae ATCC 43816	CP064352.1	8	1	9 (Alav *et al.,* 2021) (Zulfiqar *et al.,* 2012) (Ni *et al.*, 2020)
Neisseria gonorrhoeae FA1090	NC_002946.2	1	0	1 (Hagman *et al.*, 1997)

Pseudomonas aeruginosa PAO1	GCA_000006765.1	10	1	11 (McNeil *et al.*, 2019)
Shigella flexneri 5a M90T	CP037923	5	1	6 (Pasqua *et al.*, 2019)
Salmonella enterica serovar Typhimurium SL1344	FQ312003.1	6	0	5 (Alav *et al.*, 2021)

* AB AYE results were missing ArpB due to sequence dissimilarity with the RND proteins used in our alignments. This is discussed further below. *S. enterica* Typhimurium is shown separately at the bottom because an additional RND efflux protein, to those described in the literature, was found.

For all sequences apart from *Salmonella* and *A. baumannii* AYE, the number of RND proteins highlighted by this search equated to the number proposed to be present in the genomes in the literature, validating the method. For example, *N. gonorrhoeae* is described to encode only one RND pump protein, and the same was true using this search (Golparian *et al.*, 2014). For *E. coli* all seven known RND proteins in this strain were detected, including the HME protein, CusA. CusA was identified in both the HAE and HME searches, but was only included once in Table 5.2. In addition to finding the known *S. enterica* RND proteins, an additional HAE protein, annotated as OqxB by Prokka (Seemann, 2014), was also highlighted by the BLASTp search for *S. enterica*.

In *A. baumannii* AYE, however, the BLASTp searches only identified 5 out of 6 known RND proteins. Both HAE and HME searches failed to highlight ArpB, an RND-like protein that is involved in opaque to translucent colony formation switching (Tipton, Farokhyfar, & Rather, 2017). ArpB was added to both the HAE and HME alignments, but due to differences in the sequences of the other RND proteins compared to it, the number of conserved residues reduced dramatically across the aligned proteins and rendered the method unable to then detect any RND proteins successfully via BLASTp. Phylogenetic trees based upon the alignments with ArpB are shown in Figures 5.1 and 5.2 and show that ArpB clusters separately from the other proteins. Interestingly, when using BLASTp to estimate the number of HAE RND proteins across 100 sequences of *A. baumannii* (including *Acinetobacter baumannii* ACICU), in 95% of the sequences all 5 proteins were detected, Table 5.3. This suggests that ArpB can be identified by the search and that the sequence of ArpB in *A. baumannii* AYE differs from that of other *A. baumannii* sequences. When directly comparing the amino acid sequence of ArpB from AYE and ArpB from ACICU, they are only 24.5% identical with a coverage of 94%.

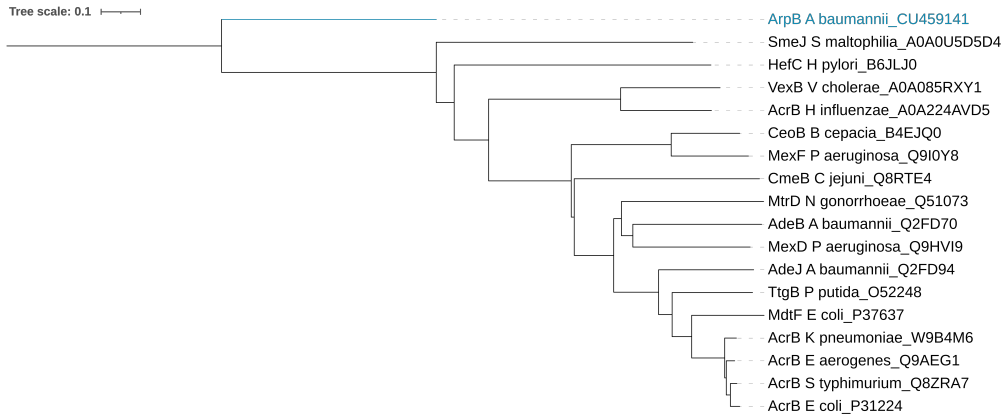


Figure 5.1: Phylogenetic tree of HAE pumps with ArpB (blue). HAE proteins from Gram negative species were downloaded and aligned using MAFFT. Tree was created using Treeio.

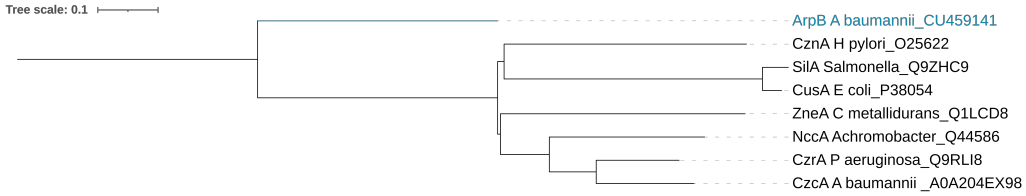


Figure 5.2: Phylogenetic tree of HME pumps with ArpB (blue). HME proteins from Gram negative species were downloaded and aligned using MAFFT. Tree was created using Treeio.

Table 5.3: Percentage of HAE and HME proteins found in 100 *A. baumannii* sequences

Number of HAE proteins	Percentage of sequences (%)
4	3
5	95
6	1
7	1
Number of HME proteins	Percentage of sequences (%)
1	67
2	27
3	5
4	1

5.5 RND systems within *A. baumannii*

Given that the method could highlight HAE and HME RND proteins across a range of Gram negative species, the method was applied to a panel of 100, random, *A. baumannii* sequences deposited on NCBI. Of the 100 *A. baumannii* sequences analysed, 96% encoded five HAE RND proteins, 3% had four and 2% had six or seven. In the three sequences that only had four proteins, RND protein AdeB was missing and a subsequent targeted BLASTn search for *adeB* yielded no results. The additional RND protein in the other two sequences (GCA_000302135.1 and GCA_000301875.1) is currently not characterised and when looking more closely at the seventh RND protein identified in GCA_000301875.1, it seems to be a truncated RND protein with amino acid sequence similarity (99%) to AdeB and found next to ArpB.

In the 100 *A. baumannii* sequences the number of HME pumps also differed within the species. 67% of sequences had only 1 heavy metal pump protein – CzcA. Although another inner membrane protein (CzcD) exists in the Czc system, its sequence is much shorter than the HME proteins in the alignment, so it was not found using the BLASTp search. The remaining sequences had an additional one to three proteins encoded in their genomes. In total, 27% had a total of two HME proteins, CzcA and a protein annotated as CusA. Furthermore, 5% had three HME proteins where the third protein was also labelled as CzcA, but was not found with CzcD, so it is likely to be annotated incorrectly and could represent a third distinct heavy metal efflux system in *A. baumannii*. Finally, one sequence (GCA_000307895.1) had four HME proteins and three of them were annotated as CzcA (genomic positions 4668–7796, 6237–9395 and 12251–15379 bp) and one as CusA. Of the three CzcA, two were found in an operon with other Czc proteins and the other one was a protein with an amino acid sequence similar (100% identity) to CzcA/CusA family proteins but not located near to other Czc protein-encoding genes.

In summary, a method was developed and validated to detect RND transporters in Gram negative bacterial genome sequences. This method could find both HAE and HME proteins and was used to give a snapshot of the RND proteins present across the *Acinetobacter* genus.

5.6 RND systems across *Acinetobacter*

5.6.1 The number of RND proteins across the genus

The BLASTp method developed in Section 5.4 was further applied to all the species within the *Acinetobacter* genus, in order to test the hypothesis that encoding more RND systems

is linked with an increased ability to cause infection. Depending on the number of available genomes per species, between 1 and 4 sequences were tested for the presence of RND proteins. Again, these genomes were downloaded from NCBI and randomly selected where possible. Genome sequence lists for the genus and *A. baumannii* datasets can be found at https://doi:10.1099/mgen.0.000964.

The number of RND proteins in species of *Acinetobacter* ranged from between 2 to 9, Figure 5.3 column 1, and this correlated with whether that species is known to cause human infection. Using a point biserial Pearson's correlation, the number of RND proteins and ability to cause infection was positively correlated (r_{pb}=0.375, n=65, p=0.002), which was statistically significant. Therefore, infection causing species tended to encode more RND genes than those that have not been reported to cause infection. Whilst there are likely to be more *Acinetobacter* species that have the capacity to cause human infection (particularly in immunocompromised individuals), the heatmap documents those published to date. In fact, since this work was completed *A. haemolyticus* has also been noted as being an important human pathogen, despite not being shown as such on Figure 5.3.

Previous work has shown that not all species have the same number of total RND components; for example in *A. baumannii* around 20% of all isolates are missing the OMF AdeC and up to 25-30% have been shown to be missing the RND protein AdeB (Chu, Chau, & Houang, 2006; Lin, Ling, & Li, 2009; Coyne, Courvalin, & Périchon, 2011). This is also evident in this data; whilst the number of RND proteins differs across the *Acinetobacter* genus, the number of RND proteins also differs between members of the same species. For example, in *A. colistiniresistens* sequences there were between 3 and 5 HAE RND proteins and between 1 and 4 HME RND proteins. The average of the total RND proteins, 7, detected in the four *A. colistiniresistens* sequences tested is shown in Figure 5.3. Furthermore, this variation is seen in other species for example *A. bereziniae*, where 3-4 HAE and 1-3 HME proteins were found as well as in *A. haemolyticus, A. pittii, A. proteolyticus, A. tandoii* and

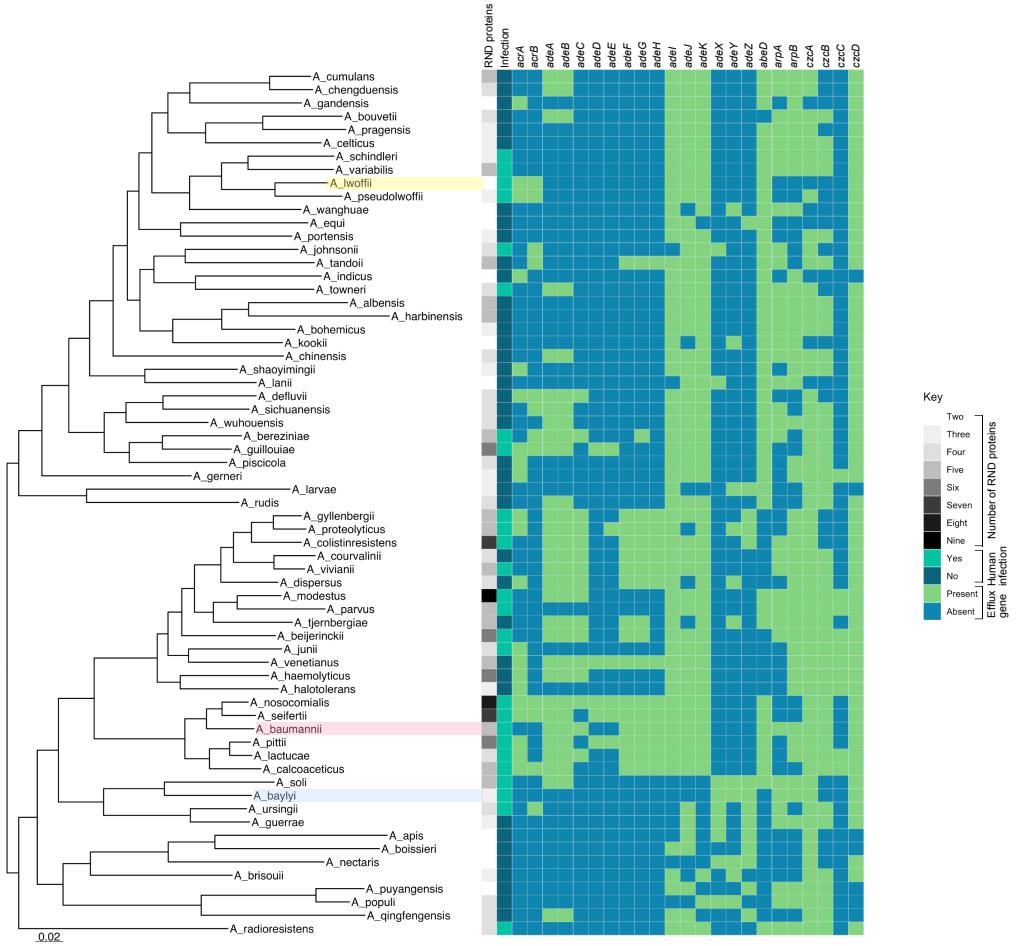


Figure 5.3: The presence of RND pumps across *Acinetobacter*. Heatmap of *Acinetobacter* genus with characterised RND genes presence/absence and the number of RND proteins. For column 1, number of RNDs, the mean average of the total number of (both HME and HAE) RND efflux proteins for each species was determined, to 1 significant figure, where the greater the number of proteins, the darker grey the colour. For column 2, if a species has been shown in the literature to cause infection it is turquoise. Subsequent columns 3-25 are highlighted green if the efflux gene was found in the reference sequences using ABRicate. *A. lwoffii* is highlighted yellow, *A. baumannii* is pink and *A. baylyi* is blue. Generally, species that encode more RND pumps are associated with causing human infection. For example, *A. vivianii* and *A. courvalinii* have the same number of ABRicate RND genes (green boxes) but *A. vivianii* has more RND proteins (darker grey box) and is associated with human infection.

above in the 100 *A. baumannii* sequences.

5.6.2 Presence of RND efflux pump genes across *Acinetobacter*

To gain a broader picture of what RND genes, including periplasmic adaptor protein (PAP) genes and outer membrane factor (OMF) genes, are present in the genus *Acinetobacter*, genomes from all validated *Acinetobacter* species were searched for the presence of these genes using a custom database of *Acinetobacter* RND genes in ABRicate (AciRND, available at: https://github.com/emd803/Gene-Databases). Figure 5.3 shows a heatmap, where the presence (green) and absence (blue) of RND genes is mapped onto a phylogenetic tree of the genus. The average number of RND proteins, as determined by the novel RND residue BLASTp search, is also plotted in greyscale, column 1.

The average number of RND proteins sometimes over or underestimates the number compared to the RND genes found by ABRicate, this is because ABRicate searched only one reference sequence and the number of RND proteins is the average of up to four sequences searched by BLASTp. When directly comparing the same sequence using BLASTp to search for proteins with conserved RND residues and ABRicate to highlight all characterised RND genes, BLASTp finds the same or more efflux pump proteins compared to ABRicate in 38 species. In the remaining 27 species, BLASTp found 75% of the RND proteins highlighted by ABRicate. Therefore, a combination approach of both methods provides the best resolution when looking for RND genes and proteins. In total BLASTp found 274 proteins with conserved RND residues and ABRicate found 272 characterised RND transporter genes.

Parts of the metal ion efflux system, Czc, are also common across the genus where *czcD* and *czcA*, coding for the inner membrane proteins, are found in almost all species. In

contrast, other RND systems, such as *adeABC* and *adeFGH*, are commonly found in a clade comprised of *A. baumannii* and closely related species, which have a higher propensity to cause human infection.

Notably, almost all *Acinetobacter* species encode *adeIJK* and it is striking, that those that do not, encode the *adeXYZ* operon instead. This is true in all but three species, *A. colistiniresistens, A. gyllenbergii* and *A. proteolyticus*, which encode genes that are similar to both *adeK* and *adeZ* according to the ABRicate search. Indeed, when looking more closely at these, it seems that they have a full *adeIJK* operon, but also an additional RND operon with an OMF that is 69-71% identical to *adeK*, found with a PAP and RND protein. In addition to the three species that encoded parts of *adeIJK* and *adeXYZ*, *A. cumulans* encoded two complete copies of *adeIJK*.

5.7 *A. baumannii adeIJK* and *A. baylyi adeXYZ* are homologous efflux systems

Originally, *adeXYZ* was described in *A. baylyi* and stated to have high sequence similarity to *adeIJK* from *A. baumannii* (Chu, Chau, & Houang, 2006; Roca *et al.*, 2011). The fact that the absence of *adeIJK* in Figure 5.3 seemed to match almost perfectly to the presence of *adeXYZ* suggested that these pumps may be divergent examples of the same pump, rather than distinct systems. Therefore, the sequence, genomic location, function and structure of *adeIJK* from *A. baumannii* AYE and *adeXYZ* from *A. baylyi* ADP1 were compared. In addition, another *adeIJK* from a more phylogenetically distant *Acinetobacter* (*A. lwoffii* 5867) was included for context.

5.7.1 Genetically, *adeIJK* and *adeXYZ* are similar

In *A. baumannii* AYE, *A. lwoffii* 5867 and *A. baylyi* ADP1, the *adeIJK* and *adeXYZ* operons are found in the same genomic location, with conserved regions up and immediately downstream of the operon, Figure 5.4. The genes flanking the *adeIJK*/*XYZ* operons encode PAP2 phosphatase family proteins and YbjQ family proteins. Despite the high level of conservation around the operons, downstream from the OMF the sequences differ dramatically. Neighbouring genes that could be annotated by Prokka are also included in Figure 5.4, however there are discrepancies where homologous genes are annotated differently in different species.

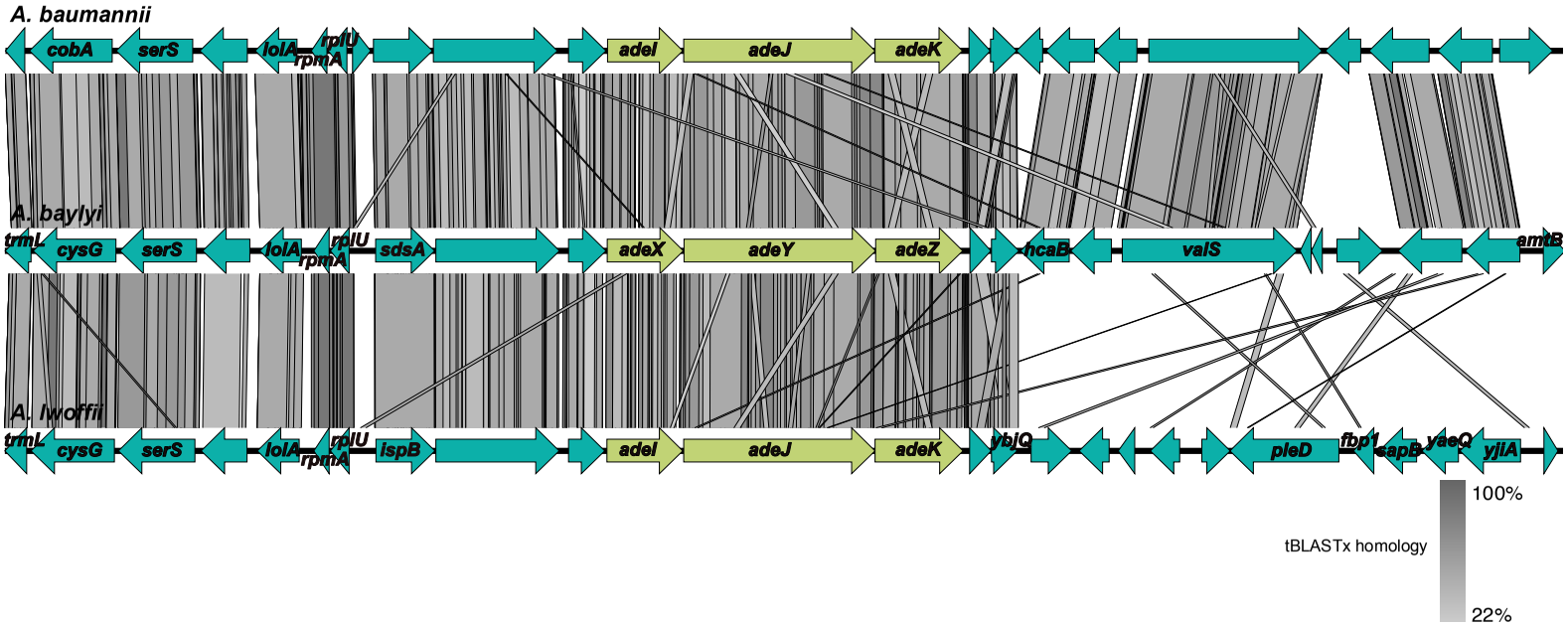


Figure 5.4: The genomic context of *adeIJK* and *adeXYZ*. Figure was created in Easyfig (v.2.2.5) using *A. baumannii* AYE, *A. baylyi* ADP1 and *A. lwoffii* 5867 sequences +/- 10 Kb from the *ade* operons, annotated using Prokka. The grey scale shows tBLASTx homology and arrows refer to coding regions within the genome. Immediately around *adeIJK/XYZ* is conserved but differs further downstream of the OMF. *A. baumannii* and *A. baylyi* are more similar downstream of the OMF, compared to *A. lwoffii*. The two genes immediately after each OMF are genes which encode YbjQ family proteins and this is conserved in all three species (Abau: HKO16_14475,HKO16_14480, Abay: KJPEBFEI_02742, KJPEBFEI_02743, Alwo: NCTC5867_02643, *ybjQ*. The gene immediately upstream of AdeI/X is a gene encoding a PAP2 phosphatase protein (Abau:HKO16_14455, Abay:KJPEBFEI_02738, Alwo:NCTC5867_02647).

The periplasmic adaptor protein genes, *adeI* and *adeX*, in *A. baumannii, A. lwoffii* and *A. baylyi* are between 62-70% similar. The sequence of *adeI* from *A. baumannii* is more similar to *adeX* from *A. baylyi* than *adeI* from *A. lwoffii*. The same pattern can be seen for the RND (*adeJ/Y*) and OMF (*adeK/Z*) genes, where the nucleotide sequences from *A. baumannii* for each component of the tripartite pump are more like the sequences from *A. baylyi* than *A. lwoffii*, despite them sharing the nomenclature with *A. lwoffii*, Figure 5.5.

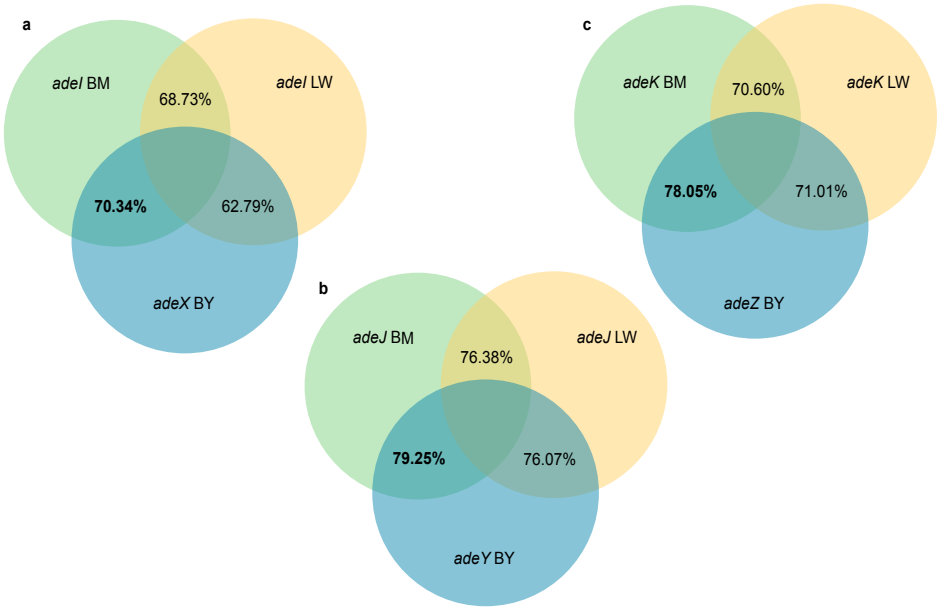


Figure 5.5: Percentage identity between *Acinetobacter* RND genes. Percentage identity was determined by a MAFFT (v.7) alignment of the PAP, RND and OMF genes, which was analysed by Sequence Manipulation Suite. BM- *A. baumannii* (green) LW- *A. lwoffii* (yellow) BY- *A. baylyi* (blue) a – PAP b – RND c- OMF. In bold are most similar pair for PAP, RND and OMF based upon nucleotide % identity.

Given that *adeIJK/XYZ* is found in all *Acinetobacter*, but *adeABC* and *adeFGH* are found only in a subset of species, the recombination levels and polymorphisms in and around all three *ade* operons were analysed. To determine if there were any recombination hotspots and polymorphisms around *adeIJK*, Gubbins was used to infer recombination levels

and polymorphisms across the whole genomes of 100 *A. baumannii* sequences. Figure 5.6 shows the recombination predictions and polymorphisms for *adeABC* (e and f), *adeFGH* (c and d) and *adeIJK* (a and b) across the sequences. Of the three systems, there is no signature of recombination seen around the *adeIJK* genes across these sequences, indicating it is an ancestral operon common across all genomes tested. High levels of recombination were seen around *adeABC* and because it was found in *A. baumannii* and other infection-causing species (Figure 5.3) but not all *Acinetobacter*, it is likely that this operon is in a recombination hotspot where genes have been acquired by horizontal gene transfer. Polymorphisms are also shown in Figure 5.6 (red and blue blocks), where more are seen around AdeABC. Further to this, the level of recombination and polymorphisms is also low around *adeFGH*.

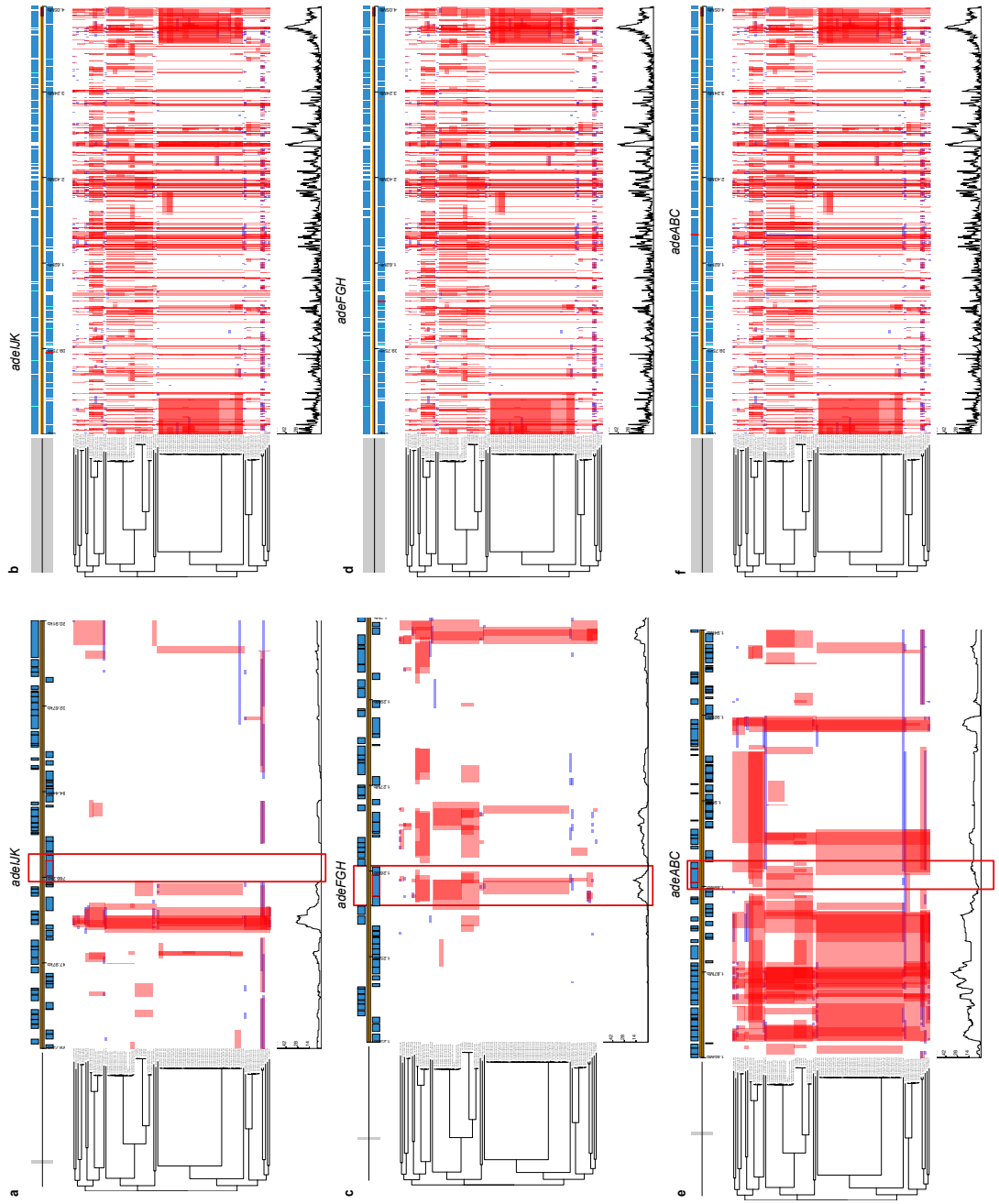


Figure 5.6: Recombination levels around *adeABC*, *adeFGH* and *adeIJK*.

100 *A. baumannii* genomes were aligned against reference *A. baumannii* AYE and the presence of polymorphisms and recombination was determined using Gubbins. Parts a, c and e are zoomed in parts of the genome at each *ade* operon, showing the levels of SNPs (red and blue squares, red are ancestral SNPs) and recombination levels (the black line on the bottom, the higher the peak the more recombination). The right hand side, b, d and f, show the entire genome and the position of each different *ade* operon, which is highlighted red underneath the label. All figures have an associated phylogenetic tree created by Snippy to show the relatedness of the *A. baumannii* sequences. *adeIJK* (a) has fewer SNPs and recombination than *adeABC* (e) and *adeFGH* (c) indicating it is highly conserved.

5.7.2 The phenotypic impact of *adeIJK* and *adeXYZ* expression is the same

To determine whether the substrate profile of AdeIJK and AdeXYZ are the same, *adeIJK* from *A. baumannii* AYE and *adeXYZ* from *A. baylyi* ADP1 were cloned and expressed in *A. baumannii* ATCC 17978 Δ*adeAB* Δ*adeFGH* Δ*adeIJK* and susceptibility to known substrates of different Ade systems was measured. The effect of expression of AdeIJK and AdeXYZ in ATCC 17978 Δ*adeAB* Δ*adeFGH* Δ*adeIJK* was identical with both conferring decreased susceptibility to chloramphenicol, ciprofloxacin, clindamycin, tetracycline and rifampicin.

Initially, these plasmids were expressed in ATCC 17978 lacking only *adeIJK* (Δ*adeIJK*), which increased the ethidium bromide (EtBr) MIC from 1 to 64 µg/mL (data not shown). However, in the triple pump deletion background Δ*adeAB* Δ*adeFGH* Δ*adeIJK*, expression of neither AdeIJK nor AdeXYZ altered susceptibility to EtBr (Table 5.4) suggesting AdeIJK does not export ethidium bromide, which has been previously shown (Damier-Piolle *et al.*, 2008). The basis for this difference in relation to strain background is not fully understood but has been reported previously (Damier-Piolle *et al.*, 2008).

Table 5.4: Broth microdilution MIC results (μg/mL) for AdeIJK and AdeXYZ when expressed in *A. baumannii* ATCC 17978 Δ*adeAB* Δ*adeFGH* Δ*adeIJK*

Strain	Chloramphenicol	Ciprofloxacin	Clindamycin	Tetracycline	Ethidium Bromide	Rifampicin
RND systems known to export	AdeABC, AdeFGH, AdeIJK	AdeABC, AdeFGH, AdeIJK	AdeFGH, AdeIJK	AdeABC, AdeIJK	AdeABC	AdeIJK
Presumed efflux routes	DBP	DBP	DBP	DBP/PBP	DBP	PBP
WT	64	16	64	2	>256	4
KO	8	8	4	0.25	1	2
p*adeIJK*	512	128	8	8	1	8
p*adeXYZ*	512	128	8	8	1	8

WT = *A. baumannii* ATCC 17978, KO = *A. baumannii* ATCC 17978 Δ*adeAB* Δ*adeFGH* Δ*adeIJK* + empty pVRL2 vector, p*adeIJK* = ATCC 17978 Δ*adeAB* Δ*adeFGH* Δ*adeIJK* + Abaum *adeIJK* pVRL2, p*adeXYZ* = ATCC 17978 Δ*adeAB* Δ*adeFGH* Δ*adeIJK* + Abayl *adeXYZ* pVRL2. DBP= distal binding pocket. PBP = proximal binding pocket.

5.7.3 AdeJ from *A. baumannii* is structurally similar to AdeY from *A. baylyi*

Since AdeIJK from *A. baumannii* and AdeXYZ from *A. baylyi* are genetically and functionally similar, their respective RND-transporters were examined to determine if they shared similar structural features, using comparative analysis of the experimentally available structures and making homology models of the missing ones. Again, *A. lwoffii* AdeJ was included for context. Protein modelling work in this Section was done by Prof. Vassiliy Bavro at the University of Essex.

The high-fidelity homology modelling of AdeJ from *A. lwoffii* (76.38% identity) and AdeY from *A. baylyi* (79.25% identity) was enabled by recent determination of the experimental structures of AdeJ from *A. baumannii* (*Ab*AdeJ) in both apo- and eravacycline-bound forms (7M4Q.pdb; and 7M4P.pdb respectively (Zhang, Morgan, *et al.*, 2021). The amino acid sequence of AdeJ from *A. baumannii* (*Ab*AdeJ), *A. lwoffii* (*Al*AdeJ) and *A. baylyi* AdeY align without any gaps, allowing for one-to-one positional correspondence between them (Figure 5.7), with the sole exception of a single residue insertion after position 602 (*Ab*AdeJ numbering) in both *A. lwoffii* AdeJ and AdeY, which maps to the protein surface (PC1 sub-domain), and thus should not directly affect drug binding. There is also a high level of conservation with other members of the RND-transporter family, including AdeB (Ornik-Cha *et al.*, 2021; Morgan, Glaza, *et al.*, 2021), AcrB (Eicher *et al.*, 2012; Nakashima *et al.*, 2011) and MtrD (Lyu *et al.*, 2020). Indeed, the very close geometry of these transporters, with the superposition of the AdeJ structure and the ligand-occupied T-conformers AcrB (7M4P:B and 4DX5:B respectively), yielded a strikingly low RMSD of 1.17Å, allowing for direct comparison and interpretation of their binding pockets (Figure 5.8). As the current knowledge of substrate recognition within RND pumps derives primarily from AcrB, this close relation allowed for an

unambiguous assignment of the binding determinants between the pumps. Accordingly, the analysis of the residues lining the proximal and the distal binding pockets, which are implicated in the processing of the high-molecular weight (including macrolides and rifampins) and lower-molecular weight/planar compounds (e.g. tetracyclines, fluoroquinolones and beta-lactams) respectively (Eicher *et al.*, 2012; Ornik-Cha *et al.*, 2021; Nakashima *et al.*, 2011; Tam *et al.*, 2021), identified high levels of positional conservation of the residues previously described as forming the recognition determinants of these binding pockets (Vargiu & Nikaido, 2012; Eicher *et al.*, 2012; Nakashima *et al.*, 2011) (annotated in Figure 5.7) between the AdeJ and AdeY, fitting with the antimicrobial susceptibility data in Table 5.4 and suggesting an identical substrate profile.

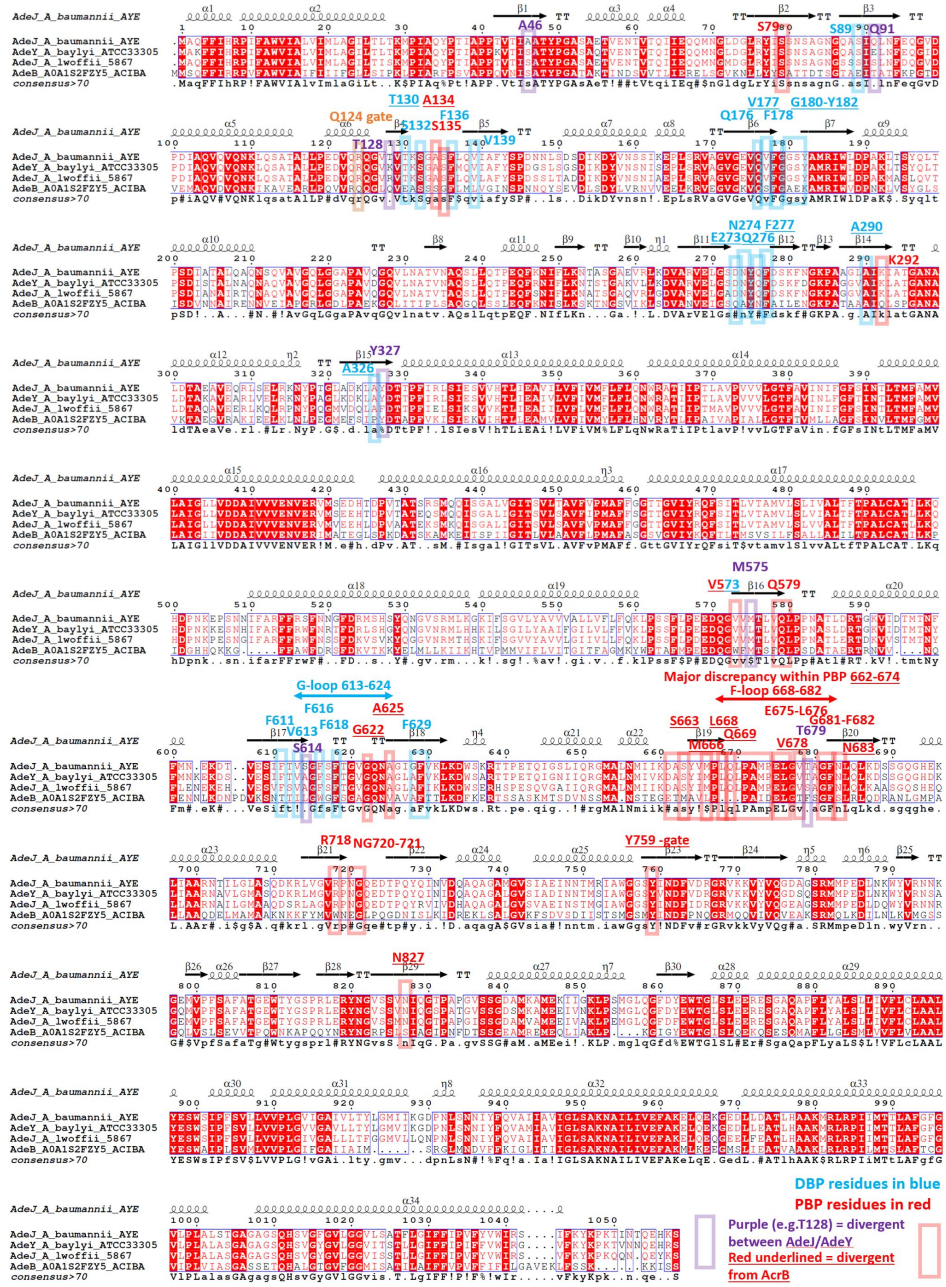


Figure 5.7: Sequence alignment of AdeJ/Y and *Ab*AdeB, highlighting key determinants in the DBP (blue text) and PBP (red text). The alignment shows the close relationship and positional equivalence between AdeJ and AdeY. Residues within the drug-binding pockets that are divergent are highlighted in purple. Secondary structural elements derived from the experimental *Ab*AdeJ structure 7M4P.pdb. Consensus sequence displayed as a bottom row. Figure prepared with ESpript 3.0 and made by Prof. V. N. Bavro.

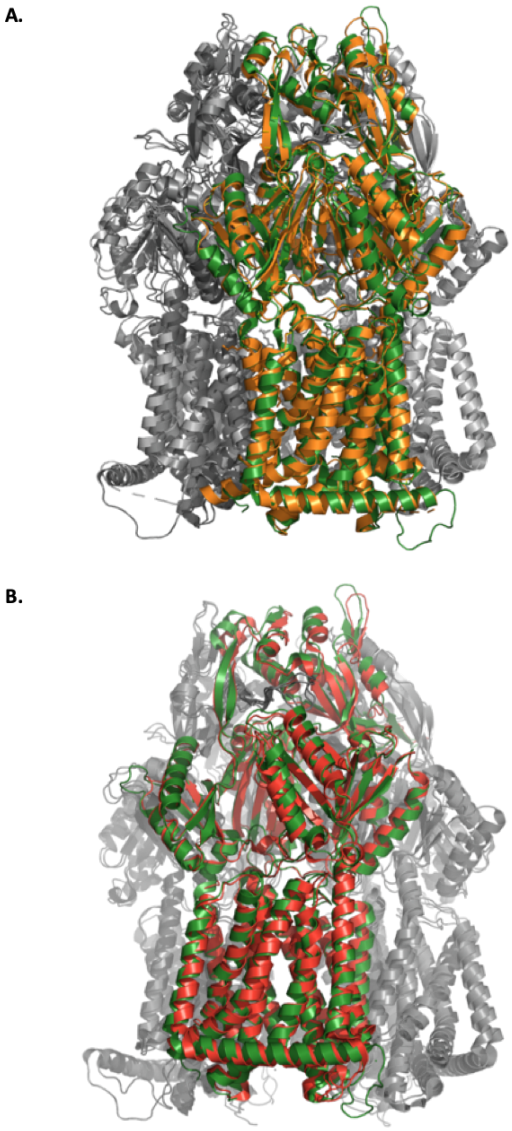


Figure 5.8: Structural similarity of AdeJ to other RND pumps. A - *Ab*AdeJ (green) superposed over *Ab*AdeB (orange) showing the close relation between the two pumps. AdeB is based on the experimental structure PDB ID 7KGI resulting in an RMSD of under 1 Angstrom (0.998Å) over the C-alphas. B - *Ab*AdeJ (green) superposed over *E. coli* AcrB (red), with an RMSD of 1.17Å based on the experimental structure PDB ID 4DX5. Figure created by Prof. V. N. Bavro.

The PBP is generally conserved amongst the RND transporters, except for the

residue range 660-688 (*Ab*AdeJ numbering), which forms the bottom section of the PBP and covers the so-called F-loop (Figure 5.7). Strikingly, while this region displays near-complete conservation between the AdeJ and AdeY (the only minor exception of T679, which in *A. lwoffii* is represented by a conservative substitution to serine), it shows major deviation from both the *Ab*AdeB (Zhang, Morgan, *et al.*, 2021; Morgan, Glaza, *et al.*, 2021; Su, Morgan, *et al.*, 2019) and *E. coli* and *Salmonella* AcrB (Murakami, Nakashima, Yamashita, & Yamaguchi, 2002; Johnson, Fais, *et al.*, 2020). The AdeJ/Y PBP also features a diagnostic V573, which is strictly conserved amongst them, but not present in other RND-transporters, providing a clear differentiation of the AdeJ and AdeY pumps. Another prominently conserved residue within the AdeJ/Y-subfamily is found in the front of the PBP, corresponding to the R718 in *Ab*AdeJ (Figure 5.9, panel B). It is notable, that the R718 is conserved in AcrB/MtrD/MexB transporters, but not in the paralogous AdeB, which suggests closer relation of the AdeJ/Y to the former. Taken together the above conservation analysis suggests a common mode of substrate recognition in the PBP of the AdeJ and AdeY, once again highlighting their close relationship to each other.

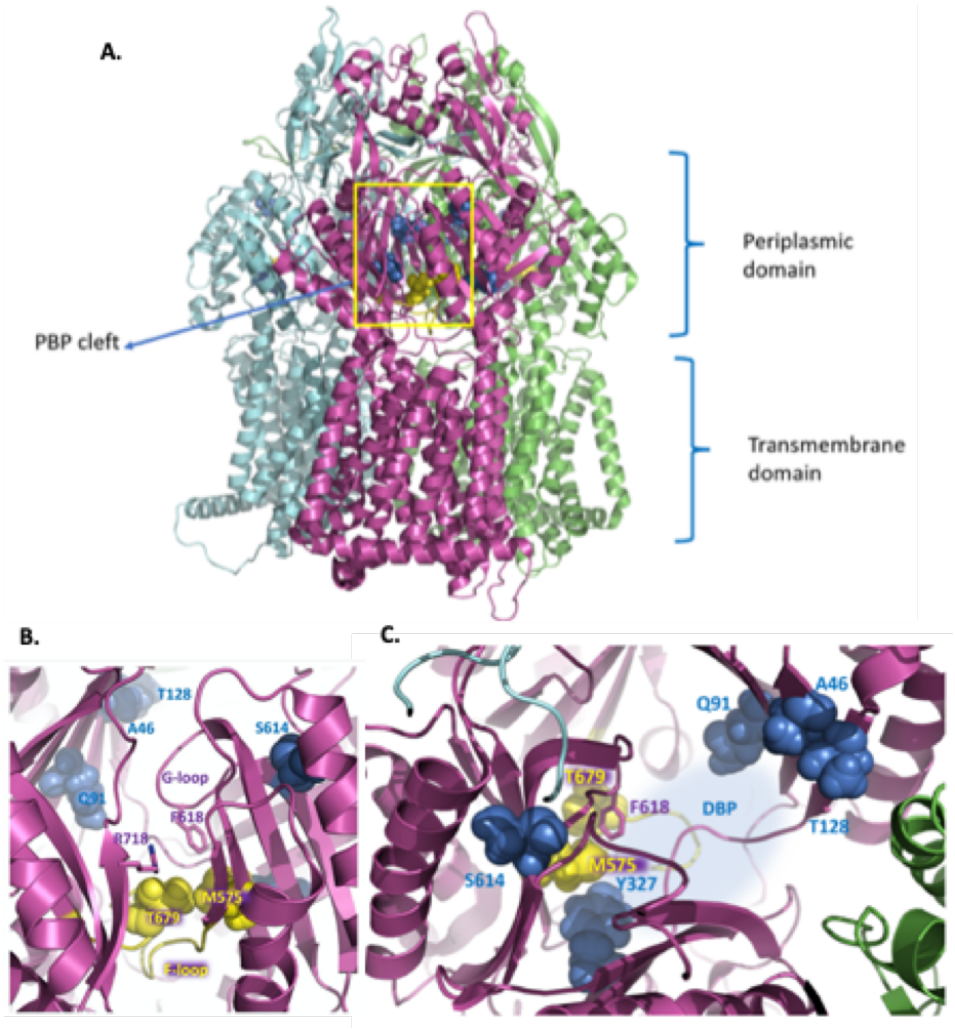


Figure 5.9: *Ab*AdeJ trimer model (A), PBP features (B) and DBP features (C). A - General view of the AdeJ trimer (based on the experimental structure of *Ab*AdeJ PDB ID 7M4P), highlighting the PBP (yellow) and DPB (blue) residue positions that show discrepancy between the members of the AdeJ/Y. B - Main features of PBP, and the prominent/divergent residue positions are shown based on the experimental structure of *Ab*AdeJ (7M4P.pdb; chain C). Divergent residues are shown in space-fill. Those belonging to the PBP are coloured yellow; the ones belonging to the DBP are blue. C - Main features of the DBP, seen from the top of the protomer. Some elements of the top of the pocket and the funnel domain have been removed. Figure created by Prof. V. N. Bavro.

The PBP and DBP are separated by the flexible G-loop (covering residue range 613-624 in *Ab*AdeJ), which contains a conserved phenylalanine (F618), which is involved in drug-binding in the DBP of both the newly-determined AdeJ fluorocycline-bound structures (Zhang, Morgan, *et al.*, 2021; Morgan, Zhang, *et al.*, 2022), and in AdeB (Morgan, Glaza, *et al.*, 2021). Amongst the AdeJ/Y the whole of the G-loop is strictly conserved, which yet again suggest similar binding properties in the upper part of the PBP and the front part of the DBP.

Moving to the DBP, the *Ab*AdeJ residues F136, F178, Y327, V613, F618, and F629 are universally conserved across AdeJ/Y, AcrB/MtrD and AdeB-transporters. Again, amongst the AdeJ/Y family members, there are very few substitutions (Figure 5.7), but it is striking that three out of four that are present (A46, Q91 and T128), are clustered together at the back of the pocket (Figure 5.9, panel C), forming a plausible interaction site, hinted by the apparent covariation of the residues occupying positions 46 and 128. While in *A. baumannii* this pair is represented by A46/T128, in *A. lwoffii* and *A. baylyi* it is instead a S46 in combination with either R128 or K128 respectively. The last DBP residue to show variation within the AdeJ/Y subfamily is Y327 (F327 in *A. lwoffii*), and is found at the bottom of the pocket, opposite side across from the A46/T128 pair (Figure 5.9, panel C). Intriguingly, it is in direct contact with M575, itself a variable residue, forming the front of the PBP, and also interacting with the variable PBP residue T679 mentioned above.

In summary, the structural analysis of the AdeJ and AdeY transporters reveals that they share a number of distinguishing features that are common across them as an "AdeJ" family, but distinctive enough to set them apart as a separate group within the wider RND-family (i.e., AdeB, AcrB, MtrD). These include major changes in the PBP, including the entire F-loop region, which is very much divergent from other RND transporters. At the same time, the overall high conservation of the DBP, combined with the presence of the R718 residue, helps to explain their identical substrate profile (Table 5.4), while suggesting a

closer functional alignment to AcrB/MtrD/MexB transporters than to the paralogous AdeB.

Thus, based upon the genetic, structural and phenotypic similarities between the two systems, *adeXYZ* should be named *adeIJK* and to this end the heatmap in Figure 5.10, has been amended to combine *adeXYZ* and *adeIJK*, now showing the presence of *adeIJK* in every described *Acinetobacter* species.

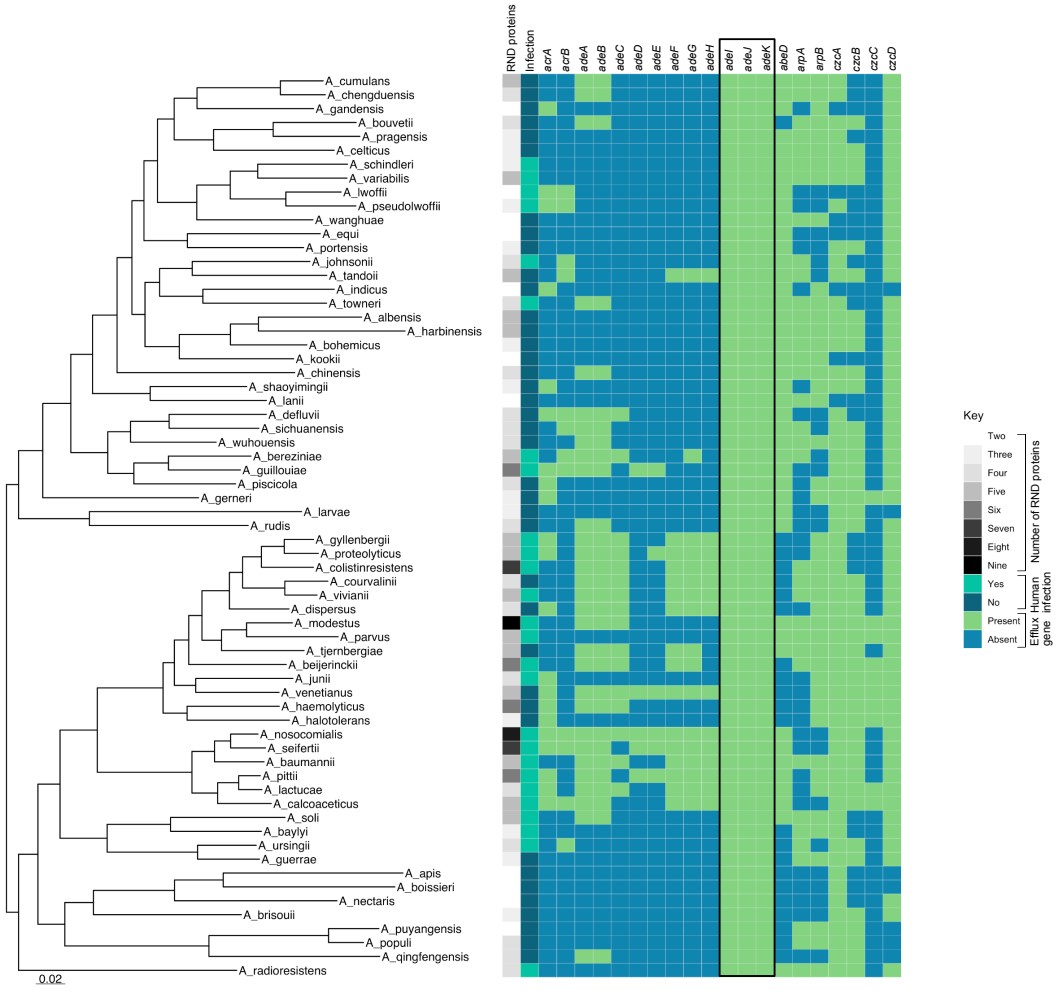


Figure 5.10: AdeIJK is the ancestral efflux pump across the *Acinetobacter* genus. Heatmap of *Acinetobacter* genus with characterised RND genes presence/absence and the number of RND proteins. For column 1, number of RNDs, the mean average of the total number of (both HME and HAE) RND efflux proteins for each species was determined, to 1 significant figure, where the greater the number of proteins, the darker grey the colour. For column 2, if a species has been shown in the literature to cause infection it is turquoise. Subsequent columns 3-25 are highlighted green if the efflux gene was found in the reference sequences using ABRicate. *adeIJK* and *adeXYZ* columns were amalgamated (black box) in this heatmap because they are the homologues, for clarity.

5.8 Discussion

The number of RND efflux genes per genome differs at both the genus and species level. Novel RND pumps are being characterised continually and it is likely there are many yet to be discovered. Due to the broad roles that RND pumps have within cells, fully understanding the complement of systems within a given species is important, especially when trying to understand MDR phenotypes in pathogens. A method has been developed to screen for HAE and HME RND proteins from bacterial genomes based upon conserved residues, which highlighted both known RND proteins and some uncharacterised ones in a variety of Gram negative species. However, there were limitations to the method as very divergent systems, such as ArpB, may not be detected. A combination approach of searching for known efflux genes and using the conserved residue RND protein search provides the best insight into the complement of RND systems in a given species.

The number of RND proteins differs between species of *Acinetobacter* and also within species. In *Acinetobacter*, there were between two and nine RND proteins in a given sequence, where *A. nosocomialis* and *A. modestus* had the most RND genes. Generally, species that cause human infection encoded more RND proteins. Previous work has shown that by over-expressing the RND genes *adeABC*, *A. baumannii* is more virulent in the lungs of a mouse (Yoon, Balloy, *et al.*, 2016) and the role of RND pumps in infections has also been noted in other Gram negative bacteria including *N. gonorrhoeae* (MtrCDE), *Salmonella enterica* (AcrAB-TolC), *Pseudomonas aeruginosa* (MexAB-OprM), *Campylobacter jejuni* (CmeABC) and *Vibrio cholerae* (VexB/D/K) (Jerse *et al.*, 2003; Buckley *et al.*, 2006; Hirakata *et al.*, 2002; Lin, Sahin, *et al.*, 2003; Bina *et al.*, 2008). Subsequent work is needed to fully elucidate if there is a link between the number of RND proteins and a species' ability to cause infection in humans across the *Acinetobacter* genus. At the time this study was conducted, sixty-four *Acinetobacter* species were characterised, however since then further *Acinetobacter* species

have been described. Furthermore, when looking at RND proteins within the *A. baumannii* species, almost all sequences had the five characterised RND proteins but there was some variation, with some sequences lacking AdeB, which has been shown previously in a subset of *A. baumannii* isolates (Chu, Chau, & Houang, 2006; Lin, Ling, & Li, 2009).

The RND system *adeIJK* was found to be present across *Acinetobacter* and sequences without it encoded *adeXYZ*, which led to the investigation to determine if these systems are actually homologues. The MIC data showed that both AdeIJK and AdeXYZ can export the same compounds including tetracycline, chloramphenicol and clindamycin which have previously been identified as substrates as AdeIJK (Kornelsen & Kumar, 2021). Exact values were not directly comparable to previous studies as different expression systems were used. The pVRL vectors are high copy number plasmids explaining why some MICs increased above that of the wild-type strain. Very high levels of expression of AdeIJK have previously been shown to be toxic in both *A. baumannii* and *E. coli* (Damier-Piolle *et al.*, 2008) and correspondingly we were unable to successfully express AdeIJK in *E. coli* despite testing a range of vectors.

Homology modelling supported the data suggesting identical substrate profiles to the structure of AdeJ and AdeY, and in particular their binding pockets were highly similar.

The structural analysis confirmed the common structural features of AdeJ/Y, which justifies isolating them as the same subfamily of RND transporters. Indeed, while the overall architecture, and correspondingly the structure of the drug-binding pockets is conserved across AdeJ/Y, they show unique features clearly distinguishing them even from the closely related RND relatives.

The differentiating features of AdeJ/Y include the organisation of the principal drug-binding pockets, with, in particular the base of the PBP displaying clear differences from other members of RND family in residue ranges 660-688, including the F-loop. Indeed, the

F-loop is different not only between the AdeJ/Y and AdeB, but also between AdeJ/Y and AcrB/MtrD. It seems likely this discrepancy may be contributing to the differential substrate efflux efficiencies between AdeB and AdeJ/Y in Table 5.4 (Sugawara & Nikaido, 2014).

The PBP also features a diagnostic V573, which is only present within the AdeJ/Y subfamily, providing differentiation from other transporters. In addition, AdeJ/Y also display some hybrid features, linking them to the canonical MDR transporters. These include the presence of an arginine (R718 in AdeJ/Y), which forms front part of the PBP (substrate channel 2 exit) (Zwama, Yamasaki, *et al.*, 2018; Tam *et al.*, 2021), and which is conserved across AcrB/MtrD/MexB (R717 in AcrB) (Lyu *et al.*, 2020; Nakashima *et al.*, 2011; Sennhauser *et al.*, 2009), where it has been implicated in the binding of the macrolides and rifamicyns (Lyu *et al.*, 2020; Zwama & Nishino, 2022; Trampari *et al.*, 2019). This critical residue is not conserved in AdeB however, suggesting that AdeJ/Y likely process their substrates similarly to AcrB/MtrD rather than to AdeB. This is further supported by the analysis of DBP, where only 4 residues have been shown to have limited variability within the analysed AdeJ/Y structures. As mentioned above, three out of four variable residues (A46, Q91 and T128) are clustered together at the back of the pocket and display positional covariation, suggesting functional interaction between them and formation of a plausible interaction site, which could be responsible for distinctive DBP ligand coordination and warrant further investigation beyond the remit of the current study. The additional conservation of the residues within the DBP, including the critical ligand-binding residues such as AcrB F610 (corresponding to AbAdeJ F611), across AcrB/MtrD/MexB, but not AdeB once again suggests closer relation of AdeJ/Y to the former.

The clear distinction of AdeJ/Y from AdeB, and the closeness of the former to AcrB/MtrD/MexB, suggest that the AdeJ/Y represent the basal efflux pumps within the genus, while AdeB paralogues may have been acquired within the genus *Acinetobacter*.

Together, the genetic, structural and phenotypic data presented shows that AdeIJK and AdeXYZ are in fact divergent homologues of the same system and we propose that *adeXYZ* should be named *adeIJK*. As shown in Figure 5.10, this highlights the presence of *adeIJK* in every *Acinetobacter* species studied to date indicating it is under high selection pressure providing an important function. This further agrees with our recombination analysis, which showed the lowest levels of recombination around *adeIJK*, when compared to *adeABC/adeFGH*. A recent study also looked into recombination within antibiotic resistance genes and found *adeJ* and *adeK* but not *adeI*, were affected by recombination (Hernández-González, Mateo-Estrada, & Castillo-Ramirez, 2022). This is contrast to what we report, and whilst this work was being conducted another paper was published showing that *adeIJK* genes are highly conserved across *Acinetobacter*, supporting the results found here with respect to recombination and polymorphisms around *adeIJK* (Naidu *et al.*, 2023). The reason for the presence of *adeIJK* across all species in the genus might be due to the wide roles that *adeIJK* carries out in *Acinetobacter*, for example its ability to protect against antibacterial host-associated fatty acids and modulate the bacterial cell membrane (Jiang, Hassan, *et al.*, 2019). Furthermore, *adeIJK* plays a role in virulence, biofilm formation, surface motility and can export a broad range of compounds, including clinically relevant antibiotics, providing intrinsic resistance (Yoon, Balloy, *et al.*, 2016; Yoon, Nait Chabane, *et al.*, 2015; Knight *et al.*, 2018). It is therefore possible to say that *adeIJK* is the defining *Acinetobacter* pump, and to belong to the genus a species will encode a version of *adeIJK*. Important disease-causing *Acinetobacter* may also encode *adeABC*, which when overexpressed increases antibiotic resistance in *A. baumannii* (Yoon, Courvalin, & Grillot-Courvalin, 2013). Therefore, it is advantageous in some species to have the combination of *adeIJK*, providing intrinsic resistance, and *adeABC*, synergistically providing even higher resistance. It is uncommon to for *adeIJK* to be overexpressed, implying the functions it carries out are important and need to be tightly regulated (Yoon, Courvalin, & Grillot-Courvalin, 2013; Damier-Piolle *et al.*, 2008) and that it is not a "drug

efflux pump" but a pump crucial for homeostasis within the bacterial cell, in other words it has a more fundamental role in *Acinetobacter* than antibiotic efflux.

5.9 Conclusions

In summary this work showed that AdeIJK is the ancestral RND efflux pump conserved across *Acinetobacter* and it therefore plays an important biological role. In addition, species that were associated with causing human infection often encoded additional RND efflux pump systems, such as AdeABC and AdeFGH. Efflux pumps expression is often tightly regulated to enable bacteria to respond to external pressures, such as the presence of toxic compounds. Therefore it is advantageous to have more of these systems, which protect against distinct substrates, to increase the bacterium's chances of survival - such as in a host environment.

5.10 Further work

Whilst this work was being conducted, more *Acinetobacter* species have been isolated and therefore, although comprehensive, this work does not reflect every species of *Acinetobacter* currently. In order to overcome this, the data analysis could be expanded to include the newly discovered *Acinetobacter* species.

It has already been shown that *A. baumannii* AdeIJK expression increases virulence in a *Galleria mellonella* model (Saranathan *et al.*, 2017). However, the role of AdeIJK and virulence in more complex infection assays, such as murine models has yet to be done. The advantage of a mouse model is that mice have both an adaptive and innate immune response. Furthermore, you can recover bacterial loads from distinct areas in mice to see

how the infection spreads, such as enumerating the colony forming units (CFUs) found in the gall bladder or spleen of infected mice (McConnell, Actis, & Pachón, 2013).

Additionally, we know that AdeIJK is rarely overexpressed in clinical isolates of *A. baumannii* and the overexpression of AdeABC or AdeFGH is favoured (Yoon, Courvalin, & Grillot-Courvalin, 2013). However, we don't know in *Acinetobacter* species that only encode AdeIJK if the same is true. It would be interesting to compare the expression levels of *adeIJK* from a number of different *Acinetobacter*, all with different complements of RND systems, to determine if it is always as tightly regulated.

Given that AdeIJK, although found in all species of *Acinetobacter*, can be different at the amino acid level between species, it would also be interesting to compare the function of AdeIJK from different *Acinetobacter* species. For example, if AdeIJK from *A. baumannii* is better at the efflux of certain compounds than AdeIJK from *A. lwoffii*. A way to achieve this would be to clone different *adeIJK* sequences into expression vectors and express them in a strain of *A. baumannii* lacking its major efflux pump transporters.

Finally, this work focused entirely on PAP, RND and OMF genes and proteins. It failed to include the presence and diversity of the genes that encode the regulators of these systems. For example, we know that *adeN* encodes the negative repressor for *adeIJK* expression (Rosenfeld *et al.*, 2012), but we do not know the prevalence of *adeN* across *Acinetobacter* and how well conserved it is. Other important repressor proteins for controlling expression of RND systems, such as EnvR, which represses transcription of *acrAB* in *Salmonella* (Blair, Siasat, *et al.*, 2023) and *Escherichia coli* (Hirakawa *et al.*, 2008) have been characterised and we know that efflux regulators can have a high degree of sequence variation within a species, but this has only been looked at for *E. coli* and *Salmonella* (Colclough, Scadden, & Blair, 2019). In order to get a more complete view of RND efflux across *Acinetobacter*, it would be pertinent to include the regulator genes *baeS,*

baeR, adeN, adeR, adeS, adeL, soxR, arpR, acrR, copR and *copS* to the heatmap, to understand more of the potential mechanisms behind efflux.

Further to this, although a number of repressor protein encoding genes have been discovered, there are still fundamental gaps in understanding what induces the transcriptional regulation of RND pumps in *Acinetobacter*. Some research exists, for example sucrose stress seems to increase the action of BaeSR in increasing expression of *adeIJK*, and furthermore osmotic stress has also been implicated in BaeSR increasing the expression of *adeABC* (Lin, Lin, Yeh, *et al.*, 2014; Kornelsen & Kumar, 2021). Nevertheless it is currently not understood, what controls *adeN* expression and therefore a reduction in the expression of *adeIJK*. Further work into what binds to the repressor protein AdeN to regulate *adeIJK* would be useful to further understand how efflux can be impaired to reduce the development of antibiotic resistance.

5.11 Key findings

- RND proteins can be found within whole genome sequences based upon their conserved residues
- *Acinetobacter* species encode differing numbers of RND transporters, both across the genus and within individual species
- AdeIJK and AdeXYZ are actually homologous RND systems
- AdeIJK is the ancestral RND efflux system in *Acinetobacter* and it is found in all species within the genus

Chapter Six

Overall discussion and conclusions

This work is a comprehensive comparison between *Acinetobacter baumannii* and *Acinetobacter lwoffii*, including a whole genus overview of RND efflux systems. *A. lwoffii* is an emerging pathogen that causes more cases of bacteraemia than *A. baumannii* in England and Wales (United Kingdom Health Security Agency, 2021), but relatively little is known about it. Chapter 3 showed that *A. lwoffii* is more susceptible to antibiotics, contains fewer resistance genes, more DNA defence systems and can less readily evolve resistance to clinically relevant antibiotics used to treat *Acinetobacter*. Additionally, Chapter 4 showed that the biology of these two organisms differ and *A. lwoffii* is less virulent compared to *A. baumanii*. Finally, Chapter 5 showed that *A. lwoffii* had fewer RND efflux pumps compared to *A. baumannii*.

It is interesting that *A. lwoffii* has remained susceptible and not evolved resistance because it causes infections often treated in hospitals, where resistance is often selected for by the prescribing of antibiotics. To investigate this, the presence of resistance genes and capacity to evolve resistance mutations was determined. The difference in susceptibility between *A. lwoffii* and *A. baumannii* can be explained by both the lack of acquired resistance genes in *A. lwoffii* genomes and also the reduced capacity to evolve resistance mutations,

for example in the target of antibiotics. Efflux is known to underpin antibiotic resistance mechanisms, both by allowing a cell time to acquire mutations and by directly exporting toxic substrates outside of the cell (Papkou *et al.*, 2020; Silva, Sundar, & Khare, 2023). Additionally, it has been recently shown that efflux mutations often precede canonical drug mutations (Silva, Sundar, & Khare, 2023). Therefore, the fact that *A. lwoffii* only has one tripartite RND system likely puts it at a disadvantage evolutionarily for acquiring drug resistance and limits its capacity to mutate efflux systems (in the way *A. baumannii* does). In addition to a difference in RND-efflux makeup, *A. lwoffii* encoded significantly more DNA defence systems than *A. baumannii*, which may show it is more conservative about the DNA it accepts and maintains within its genome, thus explaining the lack of ARGs. *A. baumannii*, in contrast, is well known to accept diverse DNA sequences, including from different species (Traglia *et al.*, 2019; Smith, Gianoulis, *et al.*, 2007). It is possible that by not acquiring novel efflux pumps and ARGs, such as those encoded on plasmids, *A. lwoffii* may not evolve antibiotic resistance in the same and rapid way *A. baumannii* has. There is already an instance of a plasmid maintained within *A. lwoffii* with an RND and PAP gene on it (Naidu *et al.*, 2023). This is particularly concerning as this plasmid belongs to a *rep* group, which has the capacity to disseminate across the species. It is therefore essential that more work is done to understand the threat of RND pumps on mobile genetic elements, such as plasmids, and how these may confer antibiotic resistance.

From the UKHSA report it is clear that *A. lwoffii* causes more bloodstream infections than *A. baumannii* (United Kingdom Health Security Agency, 2021), however in this work we were unable to demonstrate that the *A. lwoffii* strains tested were better at causing infection, *in vitro* and *in vivo*, than the *A. baumannii* strains. Again, these results may reflect the fact that *A. lwoffii* only encodes one RND system - AdeIJK. RND pumps have diverse roles, including being important for survival in nutrient limiting environments such as during infection, (Fernando & Kumar, 2013). Additionally, *Salmonella enterica* serovar

Typhimurium lacking one of its RND transporters (AcrB) is less virulent in a mouse model as well as cultured intestinal and macrophage cells (Wang-Kan *et al.*, 2017). RND pumps have also been shown to be important in a biofilm lifestyle (Alav, Sutton, & Rahman, 2018), something *A. lwoffii* could do to a lesser extent than *A. baumannii*.

When looking more broadly across the *Acinetobacter* genus to contextualise whether *A. lwoffii* with one RND system (AdeIJK) or *A. baumannii* with three (AdeABC, AdeFGH and AdeIJK) were outliers it became clear that generally *Acinetobacter* that cause infection have more RND systems. Additionally, this work showed that for a species to be part of the *Acinetobacter* genus, it must have a version of AdeIJK, where AdeIJK was likely inherited from the last common ancestor of *Acinetobacter*, indicating it has a biologically important role. Whilst the biological role of many RND pumps is unknown, AdeIJK has been shown to modulate lipids in the cell membrane and contribute to cell homeostasis, which may explain why it has not been lost from any *Acinetobacter* species to date (Jiang, Hassan, *et al.*, 2019). The number of RND efflux pumps in a given species can differ substantially, indicating that their importance to a bacterium is dependent on many factors, including lifestyle. For example *Neisseria gonorrhoeae* only has one RND transporter but is a host-restricted bacterium and this pump allows it to export host-derived antimicrobial peptides, which is very important for the cell's survival (Handing *et al.*, 2018). Whereas *Escherichia coli* can have six RND pumps and is found in a range of environments and body sites (Colclough, Alav, *et al.*, 2020; Hagman *et al.*, 1997; Pugh *et al.*, 2023). Therefore, perhaps the addition of efflux pumps in certain members of the *Acinetobacter* genus allows them to survive in more diverse environments, which typically lends itself to causing infections, where the bacterium must deal with pH, temperature and nutrient changes, such as iron availability.

As with all studies, this work has its limitations. Strains can differ hugely within the same species, for example in *E. coli* certain sequence types can have different efflux

gene components compared to strains from a different sequence type (Pugh *et al.*, 2023). Therefore, in order to make more meaningful comparisons between the two species there would need to be more whole genome sequences (and strains to test) available, especially for *A. lwoffii*. In addition to this, although there are sequence typing schemes for *A. baumannii* to help divide genomes based upon similarity of housekeeping genes, there is no scheme for *A. lwoffii*, so it is more challenging to understand how diverse the strains are, and how representative they are of the species. Furthermore, due to the lack of prior research about *A. lwoffii* there are still knowledge gaps about its basic biology; in the lab it grew to a lower final density of cells than *A. baumannii* and this is likely due to the nutrient conditions not being optimised for this species. Additionally, there are no knockout strains (or methods for creating knockouts) for *A. lwoffii*, and therefore to more comprehensively understand the roles of efflux transporters in this organism, it would be beneficial to remove AdeIJK and determine what happens to the fitness of *A. lwoffii*. Studies have done this in *A. baumannii*, and shown that with the deletion of either *adeABC* or *adeIJK* it becomes more susceptible to antibiotics (Amin *et al.*, 2013).

In the future it would be interesting to do a study which takes *A. lwoffii* and *A. baumannii* from multiple body sites of patients both healthy and those with an infection and sequence these strains with metadata, such as if treatment was given and for how long. This would provide more information firstly about the rates of commensal/ healthy carriage of these species and also the types of infection caused. In this work we focused the discussion on bacteraemia infections based upon data from the UKHSA report (United Kingdom Health Security Agency, 2021), however other studies have shown *A. lwoffii* causing peritonitis (Tas, Oguz, & Ceri, 2017), endocarditis (Ahmadi *et al.*, 2009) and catheter-related infections (Tega *et al.*, 2007). Similarly, in sampling infections it also might be possible to do culturing to find incidences of co-infection, which would enable researchers to understand more about the biology of *A. lwoffii* infections in humans. In

addition to focusing on *A. lwoffii* from the clinical setting, to better understand the diversity of this species, sampling should also occur in the environment, for example we know *A. lwoffii* can be found in gold mines (Walter *et al.*, 2020), as a fish pathogen (Kozińska *et al.*, 2014) and even degrade oil (Nakar & Gutnick, 2003).

In summary, this work is important because *A. lwoffii* is the largest cause of *Acinetobacter*-derived bacteraemia in England and Wales and rates are increasing. Whilst there is research on understanding *A. baumannii*, particularly because it is often multidrug resistant, there remains a significant knowledge gap when it comes to *A. lwoffii* biology and virulence. This work helps us to understand why some bacteria are not evolving drug resistance and begin to understand more about susceptibility. Whilst currently *A. lwoffii* infections are responsive to antibiotic treatment, this may not always be the case if *A. lwoffii* develops antibiotic resistance mechanisms - either through horizontal gene transfer or the evolution of drug resistance mutations, such as in the presence of ciprofloxacin. An open question remains as to why some bacterial species are constrained in developing resistance and what the evolutionary trade-off, of this retained susceptibility is and whether it is likely evolutionarily that *A. lwoffii* will develop antibiotic resistance and through what mechanisms this will occur. This work provides a unique situation where the change (or lack of) from susceptibility to resistance may be monitored in an opportunistic bloodstream pathogen. Antibiotic resistance is a huge public health issue and by researching the myriad of mechanisms that have led to this crisis, solutions can be implemented to protect public health.

Appendix One

Table A.1: List of *Acinetobacter* species sequences searched for the presence of RND transporters, with assembly accession codes

Species	Accession code
A. albensis	GCA_015209685.1
	GCA_900095025.1
A. apis	GCA_900197575.1
A. baumannii	GCA_000069245.1
	GCA_001433045.1
	GCA_900444815.1
	GCA_900493605.1
A. baylyi	GCA_000046845.1
	GCA_010577805.1
	GCA_010577925.1
	GCA_010577955.1
A. beijerinckii	GCA_000368985.1
	GCA_000369005.1
	GCA_000931715.1
A. bereziniae	GCA_001055215.1
	GCA_001500155.3
	GCA_006334885.1
	GCA_016502565.1

A. bohemicus	GCA_000367925.1
	GCA_008802275.1
	GCA_900116265.1
	GCA_905183085.1
A. boisseri	GCA_900096955.1
A. bouvettii	GCA_000368865.1
	GCA_001485025.1
	GCA_004209145.1
	GCA_902753875.1
A. brisouii	GCA_000368645.1
	GCA_000488275.1
	GCA_000931655.1
	GCA_000964015.1
A. calcoaceticus	GCA_002055515.1
	GCA_000818215.1
	GCA_000931735.1
	GCA_001510805.1
A. celticus	GCA_001707755.1
A. chengduensis	GCA_003664645.1
A. chinesis	GCA_002165375.2
A. colistiniresistens	GCA_000413935.1
	GCA_003227755.1
	GCA_007713425.1
	GCA_900406805.1

A. courvalinii	GCA_008802255.1
	GCA_014635545.1
	GCA_016502305.1
	GCA_016508605.1
A. cumulans	GCA_003024525.3
	GCA_003664635.1
	GCA_003664655.1
	GCA_012371365.1
A. defluvii	GCA_001704615.3
	GCA_013072655.1
A. dispersus	GCA_001753605.1
	GCA_009884975.1
A. equi	GCA_001307195.1
A. gandensis	GCA_001678755.1
	GCA_008802205.1
A. gerneri	GCA_000368565.1
	GCA_000430245.1
	GCA_000747725.1
A. guerrae	GCA_003611455.1
	GCA_009014115.1
	GCA_009372255.1
A. guillouiae	GCA_000829655.1
	GCA_002370525.2
	GCA_008996235.1
	GCA_009011835.1

A. gyllenbergii	GCA_000413855.1
	GCA_000414075.1
	GCA_000931695.1
	GCA_001682515.1
A. haemolyticus	GCA_001932555.1
	GCA_009899805.1
	GCA_009899945.1
	GCA_009899955.1
A. halotolerans	GCA_004208515.1
A. harbinesis	GCA_000816495.1
A. indicus	GCA_002938515.1
	GCA_002938785.1
	GCA_002938915.1
	GCA_013116605.1
A. johnsonii	GCA_001484935.1
	GCA_007989645.1
	GCA_009789205.1
	GCA_012271875.1
A. junii	GCA_001941805.1
	GCA_004123275.1
	GCA_012271855.1
	GCA_901873405.1
A. kookii	GCA_900096895.1
A. lactucae	GCA_002076935.1
	GCA_000399705.1
	GCA_000516615.2
	GCA_001415555.1

A. lanii	GCA_011191955.1
	GCA_011578285.1
A. larvae	GCA_001704115.1
A. lwoffii	GCA_000836095.1
	GCA_006538585.1
	GCA_014769185.1
	GCA_900444925.1
A. modestus	GCA_014636095.1
A. nectaris	GCA_000488215.1
A. nosocomialis	GCA_005281455.1
	GCA_000694975.1
	GCA_001056825.1
	GCA_002144915.1
A. parvus	GCA_000248155.2
	GCA_000368025.1
	GCA_000962795.1
	GCA_001485085.1
A. piscicola	GCA_002233755.1
	GCA_004152775.1
A. pittii	GCA_000191145.1
	GCA_900110525.1
	GCA_900495015.1
	GCA_900496515.1
A. populi	GCA_002174125.1
A. portensis	GCA_009372215.1
	GCA_010646905.1
A. pragensis	GCA_001605895.1

A. proteolyticus	GCA_000367945.1
	GCA_001753605.1
	GCA_002835245.1
	GCA_902505965.1
A. pseudolwoffii	GCA_002803605.1
	GCA_900088145.1
A. puyangensis	GCA_900096995.1
A. qingfengensis	GCA_001753595.1
	GCA_008693185.1
A. radioresistens	GCA_003258335.1
	GCA_003006715.1
	GCA_003594775.1
	GCA_006370545.1
A. rudis	GCA_000413895.1
	GCA_000829675.1
A. schindleri	GCA_001485065.1
	GCA_003069395.1
	GCA_010918895.1
	GCA_014204595.1
A. seifertii	GCA_001054375.1
	GCA_002795375.1
	GCA_004378605.1
	GCA_016064815.1
A. shaoyimingii	GCA_011174715.1
	GCA_011578045.1
A. sichuanensis	GCA_003024515.2

A. soli	GCA_001953195.1
	GCA_000760595.1
	GCA_002204165.1
	GCA_018863215.1
A. tandoii	GCA_000400735.1
	GCA_000760555.1
	GCA_002795165.1
	GCA_008867985.1
A. tjernbergiae	GCA_000488175.1
	GCA_000759995.1
A. towneri	GCA_017498585.1
	GCA_000760575.1
	GCA_004786115.1
	GCA_018501125.1
A. ursingii	GCA_000949815.1
	GCA_000949835.1
	GCA_001056675.1
	GCA_901873655.1
A. variabilis	GCA_000369625.1
	GCA_003938405.1
	GCA_009822135.1
	GCA_016607565.1
A. venetianus	GCA_000368585.1
	GCA_001484985.1
	GCA_001575095.1
	GCA_001577485.1

A. vivanii	GCA_016502725.1
	GCA_014635885.1
A. wanghuae	GCA_009557235.1
	GCA_009601085.1
A. wuhouensis	GCA_001696605.3
	GCA_002165345.2
	GCA_004209115.1
	GCA_004209325.1

Appendix Two

C.1: BLASTp databases for finding RND proteins based upon conserved residues.

>HAE_RND_CONSERVED_RESIDUES

--------XXXXFIXRPXXAXXXXXXIXXXGXXXXXXLPXXXYPXXXXPXXXXXXXYPGAXAXXXXXX
VXXXXEXXXXGXXXXXYXXSXXXXXGXXXXXXXFXXXTXXXXAXXXVXNXXXXXXXXLPXXXX
XXXXXXXXXXXXXXXXXXXXXXXX--XXXXXXXXXYXXXXXXXXXXXXXGVGXXXXXGX-XXAX
RXWXXPXXXXXXXLXXXDVXXAXXXXNXQXXXGXXXXXXXXXXXXXXXXXXXXXXLXXXXXX
XXXXXXXXXXGXXXXLXDXAXXXXGXXXXXXXXXXNGXXXXXXXIXXXXGANXXXXXXXXXXX
XXXXXXXXPXXXXXXXXYDXTXXXXXSIXXVXXTLXEAXXLVXXVXXXFLXXXRXXXIPXXXXPX
XXXGTFXXXXXXGFSXNXLTXXXXXLAIGXXVDDAIVVXENXXRXXXX-XGXXXXXAXXXXXXXX
XXXXXXXXXXLXAVFXPXAXXXGXXGXXXXXFXXTXXXXXXXSXXXXLXXXPXXXAXXLKXX---
XXXXXX-XXXXX----------XXXXXXXFXXXXXXYXXXXXXXXXXXXXXXXXXXXXXXXXXXX
XPXXXXPXEDXGXXXXXXXXPXXXXXXXXXXXXXXXXXXX--XXXXVXXXXXXXGXXXXX--XX
XNXXXXFXXLKXXXXRXXX-XXXXXXXXXXXX-XXXXXXXXX-XXXXXXXPXXX---XLGXXXGXX
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX----XXXXXXXXXXXXXXXXXXDXXXAXXXGVX
XXXIXXTXXXXX-GXXXXXXFXXXGRXXXVXXXXXXXXRXXXXXXXXXXVXXX----------
----------XGXXXPXXXXXXXXXXXXXXXXXRXNXXXXXXXXXXX--AXGXSXGXAXXXXXXXXXX
--LPX----GXXXXXXGXXXXXXXXXXXXXXXXXXXXXXXFLXLAAXYESXXXPXXXXXXVPXXXXG
AXXXXXX----------XXXXXXXXXXXGLXXXXGLXXKNAILIXEFAXXXX-XXGXXXXXAXXXAXXX
RXRPIXMTXXAXXXGXXPLXXXXGAGXXXXXXXGXXXXXGXXXXTXXXXXXXPXXXXXXXXXX
XXXXXXXXXX----------XXXXX

> HME_RND_CONSERVED_RESIDUES

MXXXXXXXXXXXRXXVXXXXXXXXXXXGXXXXXXXPXDAXPDXXXXQVXIXTXXXGXXXXXVEX
XXTXPXXXXXXXXXGXXXXRXXSXXGXSXXXXXFXXXXXXYXXRXXXXEXLXXXXXXLPXXXX
XXXXPXXTXXGXIXXXXXXXX----XXXXXX---XXXXXX----------LRXXQDWXXXXXLXXXXX

VAXXXXXGGXXXXXXXXXDXXXXXXXXXSXXXXXXAXXXXNXXXGXXXXXXXXXXXXXVRXXX
XXXXXXDXXXIXX-XXXXGXPXXXXDXAXVXXGXXXRXGXXXXXGXXEXXXGXXXXXXXXNXX
XVXXXVXXXXXXXXX-XLPXGVXXXXXXDRXXLXXXXXXXXXXXXLXEXXXXVXXXXXXFLXXXR
XAXXXXXXXPLXXXXXXXXMXXXXXXXNXMSLGXX--XXGXXXDXAXVXXENXXXRLXXXXXXX
-XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXIXXXXXPXXXXXGXEXXXFXPXAXXXXXAXXGA
XXLXXXXXPXXXXXXXXXXXX-XXXXXXXXXXXXXXYXXXLXXXXXXXXXXXXXAXXXXXXXXX
XXXXXGXXFXPXXXEGXXXXXXXXXPXXSXXXXXXXXXXXXXXXXX-XXPEVXXXXXXXGXXXX
XXDXXXXXXXXXXXXLXPXXXWX-XXXXXXXXXXXXXXXXXXXXGXXXXXXXPIXXRXXXXXX
GXXSXXXXKXXGXXXXXXXXXAXXIXXXXXXXXGXXXXXXXXXXGXXXXXXXXXRXXXAXXGX
XXXXXXXXXXXXXXGXXXXXXXXGXXRXXXXXRXXXXXRXXXXXXXXLXXXXXXXXX-----XXL
XXXAXXXXXXGXXXXXXENXXXXXXXXXXXXGRDXXSXVXXXXXXXXXXXXXXX-XXXXXXXXG
QFEXXXXAXXXLXXXXPXXXXXXFXXLXXXFXXXXXXXLXXXXXPXAXXGGXXXLXXXXXXXSX
XXXXGFIALXGVAXXXGXVMXXXXXXXXX----------XXXXXXXAXXXGAXXRXRPXXMTXXVXXX
GXXPXXXXXGXGXEVXXXXAXXXXGGXXXXXXLXLXXXPXXXXXXXXXXXXXXXXX----------
-

Appendix Three

Table C.1: Plasmids from *A. baumannii* and *A. lwoffii*

Species	Accession/ID
A. baumannii	AFDA02000009
A. baumannii	CP023021
A. baumannii	CP023024
A. baumannii	CP023030.1
A. baumannii	CP023032
A. baumannii	CP023035
A. baumannii	CP026340
A. baumannii	NC_010401
A. baumannii	NC_010402
A. baumannii	NC_010403
A. baumannii	NC_010605
A. baumannii	NC_019985
A. baumannii	NC_020524
A. baumannii	NC_020525
A. baumannii	NC_021727
A. baumannii	NC_021728
A. baumannii	NC_021732
A. baumannii	NZ_AEOY01000096
A. baumannii	NZ_AFDB02000005

A. baumannii	NZ_AFDL01000008
A. baumannii	NZ_AFDO01000021
A. baumannii	NZ_AP014650
A. baumannii	NZ_AYFH01000048
A. baumannii	NZ_AYFH01000057
A. baumannii	NZ_AYFZ01000080
A. baumannii	NZ_AYFZ01000083
A. baumannii	NZ_CM003313
A. baumannii	NZ_CM003315
A. baumannii	NZ_CM004453
A. baumannii	NZ_CP007714
A. baumannii	NZ_CP010399
A. baumannii	NZ_CP010782
A. baumannii	NZ_CP015365
A. baumannii	NZ_CP015484
A. baumannii	NZ_CP016302
A. baumannii	NZ_CP018144
A. baumannii	NZ_CP018862
A. baumannii	NZ_CP021344
A. baumannii	NZ_CP021783
A. baumannii	NZ_CP021784
A. baumannii	NZ_HG977525
A. baumannii	NZ_HG977529
A. lwoffii	AL04_p2
A. lwoffii	AL04_p3
A. lwoffii	AL04_p4
A. lwoffii	AL04_p5
A. lwoffii	AL23_p2

A. lwoffii	AL23_p3
A. lwoffii	AL23_p4
A. lwoffii	AL23_p5
A. lwoffii	AL23_p6
A. lwoffii	AL23_p7
A. lwoffii	AL23_p8
A. lwoffii	AL23_p9
A. lwoffii	AL44_p2
A. lwoffii	AL44_p3
A. lwoffii	AL44_p4
A. lwoffii	AL51_p10
A. lwoffii	AL51_p11
A. lwoffii	AL51_p12
A. lwoffii	AL51_p13
A. lwoffii	AL51_p14
A. lwoffii	AL51_p2
A. lwoffii	AL51_p3
A. lwoffii	AL51_p4
A. lwoffii	AL51_p5
A. lwoffii	AL51_p6
A. lwoffii	AL51_p7
A. lwoffii	AL51_p8
A. lwoffii	AL51_p9
A. lwoffii	CP019144
A. lwoffii	CP019145
A. lwoffii	CP019146
A. lwoffii	CP019147
A. lwoffii	CP019148

A. lwoffii	CP019149
A. lwoffii	CP019150
A. lwoffii	CP019151
A. lwoffii	CP019152
A. lwoffii	CP072550
A. lwoffii	CP072551
A. lwoffii	CP072552
A. lwoffii	CP072553
A. lwoffii	CP072554

Appendix Four

Table D.1: All mutational information for strains evolved in the presence of meropenem, ciprofloxacin and gentamicin.

Strain	CHROM	POS	TYPE	REF	ALT	EVIDENCE	FTYPE	STRAND	EFFECT	PRODUCT
AB18 Ancestor										
AL28 Ancestor										
Mero_AB18 WT1	HGMGGKAI_16	233	snp	A	G	G:14 A:0	rRNA	-	intergenic region	23S ribosomal RNA (partial)
	HGMGGKAI_16	242	snp	T	C	C:14 T:0	rRNA	-	intergenic region	23S ribosomal RNA (partial)
Mero_AB18 WT2										
Mero_AB18 M1	HGMGGKAI_1	416838	del	TA	T	T:18 TA:0	CDS	-	frameshift variant c.362delT p.Leu121fs	AdeN
	HGMGGKAI_5	211376	snp	A	G	G:20 A:0	CDS	-	missense variant c.416T>C p.Val139Ala	AdeJ
Mero_AB18 M2	HGMGGKAI_1	416914	del	GA	G	G:20 GA:0	CDS	-	frameshift variant c.286delT p.Ser96fs	AdeN
	HGMGGKAI_5	210956	snp	G	A	A:20 G:0	CDS	-	missense variant c.836C>T p.Ser279Leu	AdeJ
Mero_AB18 M3	HGMGGKAI_5	211377	snp	C	A	A:20 C:0	CDS	-	missense variant c.415G>T p.Val139Phe	AdeJ
Mero_AB18 M4	HGMGGKAI_1	416672	snp	C	T	T:20 C:0	CDS	-	missense variant c.529G>A p.Gly177Arg	AdeN

	HGMGGKAI_5	211320	snp	C	A	A:20 C:0	CDS	-	missense variant c.472G>T p.Val158Leu	AdeJ
	HGMGGKAI_366	387	snp	G	A	A:12 G:0				
Mero_AB18 M5	HGMGGKAI_2	309478	snp	G	C	C:20 G:0	CDS	+	missense variant c.1693G>C p.Val565Leu	Peptidoglycan D,D-transpeptidase PBP3
	HGMGGKAI_5	211386	snp	A	C	C:20 A:0	CDS	-	missense variant c.406T>G p.Phe136Val	AdeJ
	HGMGGKAI_5	256181	snp	A	G	G:14 A:0	CDS	-	missense variant c.44T>C p.Ile15Thr	hypothetical protein
	HGMGGKAI_6	15877	del	TTA	T	T:18 TTA:0	CDS	+	frameshift variant c.122_123delAT p.Tyr41fs	Peptidoglycan D,D-transpeptidase PBP2
Mero_AL28 WT1	JPHHDCAP_1	62161	snp	T	C	C:20 T:0				
	JPHHDCAP_3	26899	snp	C	A	A:15 C:0				
	JPHHDCAP_3	26943	snp	G	C	C:12 G:0				
	JPHHDCAP_3	41687	snp	T	C	C:26 T:0				
	JPHHDCAP_3	42008	snp	A	T	T:20 A:0	CDS	+	synonymous variant c.72A>T p.Ala24Ala	HigA1
	JPHHDCAP_4	76996	snp	C	T	T:12 C:0	CDS	-	missense variant c.238G>A p.Asp80Asn	CzcD
Mero_AL28 WT2	JPHHDCAP_3	41687	snp	T	C	C:16 T:0				
	JPHHDCAP_3	42008	snp	A	T	T:20 A:0	CDS	+	synonymous variant c.72A>T p.Ala24Ala	HigA1
Mero_AL28 M1										

Mero_AL28 M2	JPHHDCAP_2	1976693	del	CT	C	C:19 CT:0	CDS	-	frameshift variant c.46delA p.Arg16fs	PhoR
Mero_AL28 M3										
Mero_AL28 M4	JPHHDCAP_1	62161	snp	T	C	C:20 T:0				
Mero_AL28 M5										
Cipro_AB18 WT1										
Cipro_AB18 WT2										
Cipro_AB18 M1	HGMGGKAI_1	11670	snp	G	T	T:20 G:0	CDS	+	missense variant c.833G>T p.Arg278Leu	AdeL
	HGMGGKAI_3	314460	snp	C	A	A:20 C:0	CDS	-	missense variant c.235G>T p.Gly79Cys	GyrA
	HGMGGKAI_4	305711	del	CAATAA GCATTT CAGGC TT	C	C:18 CAATA AGCAT TTCAG GCTT:0	CDS	-	conservative inframe deletion c.616_633del AAGCCTGA AATGCTTATT p.Lys206_Ile211del	NusA
Cipro_AB18 M2	HGMGGKAI_1	671516	snp	C	G	G:20 C:0	CDS	-	missense variant c.88G>C p.Gly30Arg	AdeS
	HGMGGKAI_3	314453	snp	G	A	A:20 G:0	CDS	-	missense variant c.242C>T p.Ser81Leu	GyrA
	HGMGGKAI_9	19938	snp	T	A	A:20 T:0	CDS	+	missense variant c.996T>A p.Asn332Lys	ParC

Cipro_AB18 M3	HGMGGKAI_1	671516	snp	C	G	G:20 C:0	CDS	-	missense variant c.88G>C p.Gly30Arg	AdeS
	HGMGGKAI_3	314453	snp	G	A	A:20 G:0	CDS	-	missense variant c.242C>T p.Ser81Leu	GyrA
	HGMGGKAI_9	19938	snp	T	A	A:20 T:0	CDS	+	missense variant c.996T>A p.Asn332Lys	ParC
Cipro_AB18 M4	HGMGGKAI_1	11720	snp	T	A	A:20 T:0	CDS	+	missense variant c.883T>A p.Phe295Ile	AdeL
	HGMGGKAI_1	416694	del	ATCAGG AGATG	A	A:18 ATCAG GAGAT G:0	CDS	-	frameshift variant c.497_506del CATCTCCTG A p.Thr166fs	AdeN
	HGMGGKAI_1	1080609	snp	G	A	A:10 G:0	CDS	-	synonymous variant c.768C>T p.Gly256Gly	hypothetical protein
	HGMGGKAI_4	29576	snp	A	G	G:20 A:0				
	HGMGGKAI_4	215409	snp	G	A	A:20 G:0	CDS	+	stop gained c.377G>A p.Trp126*	RppH
Cipro_AB18 M5	HGMGGKAI_1	671171	del	CATTCC AAACCT G	C	C:18 CATTC CAAAC CTG:0	CDS	-	conservative inframe deletion c.421_432del CAGGTTTGG AAT p.Gln141_ Asn144del	AdeS

	HGMGGKAI_1	1080636	snp	G	T	T:20 G:0	CDS	-	missense variant c.741C>A p.Asp247Glu	hypothetical protein
	HGMGGKAI_1	1216275	snp	G	T	T:20 G:0	CDS	-	missense variant c.41C>A p.Ala14Asp	hypothetical protein
	HGMGGKAI_2	443324	snp	T	C	C:20 T:0				
	HGMGGKAI_2	443328	ins	T	TCA	TCA:18 T:0				
	HGMGGKAI_22	1525	snp	G	A	A:14 G:0	rRNA	-	intergenic region	23S ribosomal RNA (partial)
Cipro_AL28 WT1	JPHHDCAP_1	65985	snp	C	A	A:20 C:0	CDS	+	missense variant c.502C>A p.Pro168Thr	YfdX2
	JPHHDCAP_1	65990	snp	A	T	T:20 A:0	CDS	+	synonymous variant c.507A>T p.Ala169Ala	YfdX2
	JPHHDCAP_2	2669688	snp	T	A	A:10 T:0				
	JPHHDCAP_3	41687	snp	T	C	C:21 T:0				
	JPHHDCAP_3	42008	snp	A	T	T:21 A:0	CDS	+	synonymous variant c.72A>T p.Ala24Ala	HigA1
Cipro_Al28 WT2	JPHHDCAP_2	1859296	snp	G	A	A:12 G:0				
	JPHHDCAP_3	29282	snp	T	G	G:20 T:0	CDS	+	missense variant c.204T>G p.Asp68Glu	MerA
	JPHHDCAP_3	30025	snp	T	G	G:20 T:0	CDS	+	missense variant c.947T>G p.Val316Gly	MerA

	JPHHDCAP_3	31473	ins	A	AGTT	AGTT:18 A:0	CDS	+	disruptive inframe insertion c.30_32dup TTG p.Cys11dup	hypothetical protein
	JPHHDCAP_3	37827	snp	A	G	G:22 A:0	CDS	+	missense variant c.514A>G p.Arg172Gly	Hin2
	JPHHDCAP_3	41687	snp	T	C	C:26 T:0				
	JPHHDCAP_3	42008	snp	A	T	T:20 A:0	CDS	+	synonymous variant c.72A>T p.Ala24Ala	HigA1
	JPHHDCAP_4	76996	snp	C	T	T:12 C:0	CDS	-	missense variant c.238G>A p.Asp80Asn	CzcD
Cipro_AL28 M1	JPHHDCAP_2	387064	ins	T	TGA	TGA:20 T:0	CDS	+	frameshift variant c.136_137dup GA p.Asp46fs	AdeN
	JPHHDCAP_2	397197	del	AATATT ACTGA ATTTAA CT	A	A:16 AATAT TACTG AATTT AACT:0	CDS	+	disruptive inframe deletion c.1083_1100 delTATTACT GAATTTAAC TA p.Ile362_ Tyr367del	IlvA
	JPHHDCAP_2	1834404	snp	C	T	T:20 C:0	CDS	+	missense variant c.1354C>T p.Arg452Cys	GyrB

Cipro_AL28 M2	JPHHDCAP_2	1994600	snp	C	T	T:20 C:0	CDS	+	missense variant c.680C>T p.Ser227Leu	AtpB
	JPHHDCAP_2	2593005	snp	C	T	T:20 C:0	CDS	-	missense variant c.383G>A p.Arg128Gln	AdeJ
	JPHHDCAP_2	2702012	snp	C	A	A:20 C:0	CDS	+	missense variant c.242C>A p.Ser81Tyr	GyrA
	JPHHDCAP_3	41687	snp	T	C	C:26 T:0				
	JPHHDCAP_3	42008	snp	A	T	T:20 A:0	CDS	+	synonymous variant c.72A>T p.Ala24Ala	HigA1
	JPHHDCAP_4	76996	snp	C	T	T:12 C:0	CDS	-	missense variant c.238G>A p.Asp80Asn	CzcD
Cipro_AL28 M3	JPHHDCAP_2	1994600	snp	C	T	T:20 C:0	CDS	+	missense variant c.680C>T p.Ser227Leu	AtpB
	JPHHDCAP_2	2593005	snp	C	T	T:20 C:0	CDS	-	missense variant c.383G>A p.Arg128Gln	AdeJ
	JPHHDCAP_2	2702012	snp	C	A	A:20 C:0	CDS	+	missense variant c.242C>A p.Ser81Tyr	GyrA
	JPHHDCAP_2	2863918	ins	A	AT	AT:18 A:0				
	JPHHDCAP_4	76996	snp	C	T	T:12 C:0	CDS	-	missense variant c.238G>A p.Asp80Asn	CzcD

Cipro_AL28 M4	JPHHDCAP_2	1994600	snp	C	T	T:20 C:0	CDS	+	missense variant c.680C>T p.Ser227Leu	AtpB
	JPHHDCAP_2	2593005	snp	C	T	T:20 C:0	CDS	-	missense variant c.383G>A p.Arg128Gln	AdeJ
	JPHHDCAP_2	2702012	snp	C	A	A:20 C:0	CDS	+	missense variant c.242C>A p.Ser81Tyr	GyrA
Cipro_AL28 M5	JPHHDCAP_2	1994600	snp	C	T	T:20 C:0	CDS	+	missense variant c.680C>T p.Ser227Leu	AtpB
	JPHHDCAP_2	2593005	snp	C	T	T:20 C:0	CDS	-	missense variant c.383G>A p.Arg128Gln	AdeJ
	JPHHDCAP_2	2702012	snp	C	A	A:20 C:0	CDS	+	missense variant c.242C>A p.Ser81Tyr	GyrA
Gen_AB18 WT1										
Gen_AB18 WT2	HGMGGKAI_1	1442472	snp	C	A	A:14 C:0				
Gen_AB18 M1	HGMGGKAI_1	307851	snp	C	T	T:20 C:0	CDS	-	stop gained c.378G>A p.Trp126*	TetR
	HGMGGKAI_1	672102	snp	C	T	T:20 C:0	CDS	-	missense variant c.277G>A p.Asp93Asn	AdeR
	HGMGGKAI_7	806	snp	C	T	T:20 C:0	CDS	-	missense variant c.1543G>A p.Gly515Ser	FusA
	HGMGGKAI_366	246	snp	A	T	T:24 A:0				

Gen_AB18 M2	HGMGGKAI_1	671425	snp	T	C	C:20 T:0	CDS	-	missense variant c.179A>G p.Asp60Gly	AdeS
	HGMGGKAI_1	674000	snp	C	T	T:20 C:0	CDS	+	missense variant c.290C>T p.Pro97Leu	AdeB
	HGMGGKAI_7	703	snp	A	G	G:20 A:0	CDS	-	missense variant c.1646T>C p.Phe549Ser	FusA
Gen_AB18 M3	HGMGGKAI_1	672107	snp	G	A	A:20 G:0	CDS	-	missense variant c.272C>T p.Ala91Val	AdeR
	HGMGGKAI_1	674117	snp	G	A	A:20 G:0	CDS	+	missense variant c.407G>A p.Gly136Glu	AdeB
	HGMGGKAI_7	746	snp	A	T	T:20 A:0	CDS	-	missense variant c.1603T>A p.Phe535Ile	FusA
Gen_AB18 M4	HGMGGKAI_1	672107	snp	G	A	A:20 G:0	CDS	-	missense variant c.272C>T p.Ala91Val	AdeR
	HGMGGKAI_1	674117	snp	G	A	A:20 G:0	CDS	+	missense variant c.407G>A p.Gly136Glu	AdeB
	HGMGGKAI_7	746	snp	A	T	T:20 A:0	CDS	-	missense variant c.1603T>A p.Phe535Ile	FusA

Gen_AB18 M5	HGMGGKAI_1	671161	snp	G	A	A:20 G:0	CDS	-	missense variant c.443C>T p.Ala148Val	AdeS
	HGMGGKAI_4	215672	snp	G	T	T:20 G:0	CDS	+	stop gained c.70G>T p.Glu24*	PtsP
	HGMGGKAI_7	806	snp	C	T	T:20 C:0	CDS	-	missense variant c.1543G>A p.Gly515Ser	FusA
Gen_AL28 WT1	JPHHDCAP_2	557129	snp	T	G	G:12 T:0				
	JPHHDCAP_3	26899	snp	C	A	A:14 C:0				
	JPHHDCAP_3	30707	snp	G	T	T:20 G:0	CDS	+	missense variant c.1629G>T p.Lys543Asn	MerA
	JPHHDCAP_3	37827	snp	A	G	G:22 A:0	CDS	+	missense variant c.514A>G p.Arg172Gly	Hin2
	JPHHDCAP_3	41687	snp	T	C	C:30 T:0				
	JPHHDCAP_3	42008	snp	A	T	T:20 A:0	CDS	+	synonymous variant c.72A>T p.Ala24Ala	HigA1
	JPHHDCAP_4	76996	snp	C	T	T:20 C:0	CDS	-	missense variant c.238G>A p.Asp80Asn	CzcD
	JPHHDCAP_4	77095	snp	T	C	C:20 T:0	CDS	-	missense variant c.139A>G p.Lys47Glu	CzcD
	JPHHDCAP_4	77302	complex	ATC	TTT	TTT:20 ATC:0				
	JPHHDCAP_4	77316	snp	T	C	C:20 T:0				

	JPHHDCAP_4	77415	snp	G	C	C:20 G:0	CDS	+	synonymous variant c.93G>C p.Pro31Pro	MerR
	JPHHDCAP_4	77449	snp	G	A	A:20 G:0	CDS	+	missense variant c.127G>A p.Val43Ile	MerR
	JPHHDCAP_4	77472	snp	A	G	G:20 A:0	CDS	+	synonymous variant c.150A>G p.Lys50Lys	MerR
	JPHHDCAP_4	77698	snp	T	C	C:15 T:0	CDS	+	synonymous variant c.376T>C p.Leu126Leu	MerR
Gen_AL28 WT2	JPHHDCAP_1	41290	snp	A	C	C:20 A:0				
	JPHHDCAP_2	405216	snp	G	A	A:20 G:0	CDS	+	missense variant c.337G>A p.Gly113Ser	Ribokinase
	JPHHDCAP_3	8043	snp	A	C	C:20 A:0	CDS	-	missense variant c.409T>G p.Leu137Val	VirB4
	JPHHDCAP_3	21503	snp	C	A	A:20 C:0	CDS	+	missense variant c.630C>A p.Ser210Arg	Soj2
	JPHHDCAP_3	21513	snp	G	T	T:20 G:0	CDS	+	missense variant c.640G>T p.Val214Phe	Soj2
	JPHHDCAP_3	22311	snp	G	T	T:20 G:0	CDS	+	stop gained c.658G>T p.Glu220*	KorB
	JPHHDCAP_3	22783	snp	G	T	T:18 G:0				

	JPHHDCAP_3	28803	snp	C	G	G:20 C:0	CDS	+	synonymous variant c.189C>G p.Leu63Leu	MerC
	JPHHDCAP_3	37827	snp	A	G	G:10 A:0	CDS	+	missense variant c.514A>G p.Arg172Gly	Hin2
	JPHHDCAP_3	41687	snp	T	C	C:31 T:0				
	JPHHDCAP_3	42008	snp	A	T	T:20 A:0	CDS	+	synonymous variant c.72A>T p.Ala24Ala	HigA1
	JPHHDCAP_3	45049	snp	G	T	T:20 G:0				
	JPHHDCAP_3	51801	snp	T	G	G:20 T:0	CDS	-	missense variant c.488A>C p.Tyr163Ser	hypothetical protein
	JPHHDCAP_4	76996	snp	C	T	T:12 C:0	CDS	-	missense variant c.238G>A p.Asp80Asn	CzcD
Gen_AL28 M1	JPHHDCAP_1	62161	snp	T	C	C:20 T:0				
	JPHHDCAP_3	10760	snp	T	C	C:20 T:0	CDS	-	synonymous variant c.1395A>G p.Glu465Glu	TopA2
	JPHHDCAP_3	10799	snp	T	A	A:20 T:0	CDS	-	synonymous variant c.1356A>T p.Pro452Pro	TopA2
	JPHHDCAP_3	10827	snp	T	A	A:20 T:0	CDS	-	missense variant c.1328A>T p.Glu443Val	TopA2
	JPHHDCAP_3	11645	snp	T	A	A:16 T:0	CDS	-	synonymous variant c.510A>T p.Ser170Ser	TopA2

JPHHDCAP_3	15916	snp	A	T	T:20 A:0	CDS	+	missense variant c.674A>T p.Glu225Val	hypothetical protein
JPHHDCAP_3	22781	snp	A	C	C:13 A:0				
JPHHDCAP_3	35260	snp	G	T	T:20 G:0	CDS	-	missense variant c.492C>A p.Phe164Leu	Acr3
JPHHDCAP_3	37827	snp	A	G	G:10 A:0	CDS	+	missense variant c.514A>G p.Arg172Gly	Hin2
JPHHDCAP_3	40361	snp	T	G	G:20 T:0	CDS	+	missense variant c.759T>G p.Ser253Arg	ChrA1
JPHHDCAP_3	41687	snp	T	C	C:32 T:0				
JPHHDCAP_3	41695	snp	A	G	G:32 A:0				
JPHHDCAP_3	41710	snp	T	C	C:34 T:0				
JPHHDCAP_3	41764	snp	T	C	C:33 T:0	CDS	+	synonymous variant c.15T>C p.Arg5Arg	hypothetical protein
JPHHDCAP_3	42008	snp	A	T	T:20 A:0	CDS	+	synonymous variant c.72A>T p.Ala24Ala	HigA1
JPHHDCAP_3	43869	snp	C	A	A:20 C:0	CDS	+	missense variant c.1371C>A p.Asp457Glu	Kup2
JPHHDCAP_3	45003	snp	T	G	G:20 T:0				
JPHHDCAP_3	47250	snp	T	G	G:11 T:0	CDS	+	missense variant c.619T>G p.Phe207Val	DNA-cytosine methyltransferase

	JPHHDCAP_3	54268	snp	T	A	A:20 T:0	CDS	-	synonymous variant c.570A>T p.Pro190Pro	hypothetical protein
	JPHHDCAP_3	54400	snp	A	C	C:20 A:0	CDS	-	synonymous variant c.438T>G p.Gly146Gly	hypothetical protein
Gen_AL28 M2	JPHHDCAP_1	62161	snp	T	C	C:20 T:0				
	JPHHDCAP_2	405216	snp	G	A	A:20 G:0	CDS	+	missense variant c.337G>A p.Gly113Ser	Ribokinase
	JPHHDCAP_3	1505	del	AT	A	A:19 AT:0	CDS	-	frameshift variant c.540delA p.Gln180fs	hypothetical protein
	JPHHDCAP_3	13375	snp	A	T	T:12 A:0	CDS	+	missense variant c.195A>T p.Lys65Asn	hypothetical protein
	JPHHDCAP_3	13392	del	TTTATA AACAA TTACA AGAAA AAAAG CCTGA TTAAAT CAGGC TTTTTT TC	T	T:10 TTTAT AAACA ATTAC AAGAA AAAAA GCCTG ATTAAA TCAGG CTTTT TTTC:0	CDS	+	frameshift variant& stop lost& spliceregion variant c.213_*15 delTTATAA ACAATTAC AAGAAAA AAAGCCT GATTAAAT CAGGCTT TTTTTC p.Phe71fs	hypothetical protein
	JPHHDCAP_3	13487	snp	C	G	G:14 C:0	CDS	-	missense variant c.286G>C p.Asp96His	hypothetical protein

	JPHHDCAP_3	18247	snp	G	T	T:20 G:0				
	JPHHDCAP_3	20068	snp	C	A	A:20 C:0				
	JPHHDCAP_3	25339	snp	A	G	G:20 A:0	CDS	-	missense variant c.305T>C p.Ile102Thr	hypothetical protein
	JPHHDCAP_3	28771	snp	C	A	A:18 C:0	CDS	+	missense variant c.157C>A p.Leu53Met	MerC
	JPHHDCAP_3	28820	snp	G	T	T:18 G:0	CDS	+	missense variant c.206G>T p.Trp69Leu	MerC
	JPHHDCAP_3	28992	del	TCGCCG CTGCGG GCCGGA CAG	T	T:16 TCGCC GCTGC GGGCC GGACA G:0	CDS	+	frameshift variant c.380_399 delGCCGC TGCGGGC CGGACAG C p.Arg127fs	MerC
	JPHHDCAP_3	29092	snp	A	G	G:19 A:0	CDS	+	missense variant c.14A>G p.Lys5Arg	MerA
	JPHHDCAP_3	41687	snp	T	C	C:21 T:0				
Gen_AL28 M3	JPHHDCAP_3	41687	snp	T	C	C:26 T:0				
	JPHHDCAP_3	42008	snp	A	T	T:20 A:0	CDS	+	synonymous variant c.72A>T p.Ala24Ala	HigA1
	JPHHDCAP_4	76996	snp	C	T	T:12 C:0	CDS	-	missense variant c.238G>A p.Asp80Asn	CzcD

Gen_AL28 M4	JPHHDCAP_3	6887	snp	T	G	G:20 T:0	CDS	-	missense variant c.1565A>C p.Asn522Thr	VirB4
	JPHHDCAP_3	23369	snp	G	C	C:20 G:0	CDS	+	missense variant c.439G>C p.Val147Leu	hypothetical protein
	JPHHDCAP_3	37827	snp	A	G	G:10 A:0	CDS	+	missense variant c.514A>G p.Arg172Gly	Hin2
	JPHHDCAP_3	41687	snp	T	C	C:33 T:0				
	JPHHDCAP_3	42008	snp	A	T	T:20 A:0	CDS	+	synonymous variant c.72A>T p.Ala24Ala	HigA1
	JPHHDCAP_4	76996	snp	C	T	T:20 C:0	CDS	-	missense variant c.238G>A p.Asp80Asn	CzcD
	JPHHDCAP_4	77095	snp	T	C	C:20 T:0	CDS	-	missense variant c.139A>G p.Lys47Glu	CzcD
	JPHHDCAP_4	77121	snp	A	G	G:20 A:0	CDS	-	missense variant c.113T>C p.Leu38Pro	CzcD
	JPHHDCAP_4	77148	snp	T	C	C:20 T:0	CDS	-	missense variant c.86A>G p.Asn29Ser	CzcD
	JPHHDCAP_4	77225	snp	A	G	G:20 A:0	CDS	-	synonymous variant c.9T>C p.Gly3Gly	CzcD
	JPHHDCAP_4	77235	snp	A	G	G:20 A:0				
	JPHHDCAP_4	77253	ins	A	AT	AT:19 A:0				

	JPHHDCAP_4	77302	complex	ATC	TTT	TTT:20 ATC:0				
	JPHHDCAP_4	77316	snp	T	C	C:20 T:0				
	JPHHDCAP_4	77544	complex	ACCTG	TCCTA	TCCTA:16 ACCTG:0	CDS	+	missense variant c.222_226 delACCTG insTCCTA p.LysPro Glu74Asn ProLys	MerR
	JPHHDCAP_4	77641	snp	C	T	T:15 C:0	CDS	+	synonymous variant c.319C>T p.Leu107Leu	MerR
	JPHHDCAP_4	77698	snp	T	C	C:15 T:0	CDS	+	synonymous variant c.376T>C p.Leu126Leu	MerR
Gen_AL28 M5	JPHHDCAP_1	41290	snp	A	C	C:20 A:0				
	JPHHDCAP_2	917216	snp	G	A	A:20 G:0	CDS	-	missense variant c.562C>T p.Leu188Phe	SdhA
	JPHHDCAP_2	1075139	snp	C	T	T:20 C:0	CDS	-	missense variant c.961G>A p.Ala321Thr	hypothetical protein
	JPHHDCAP_3	19336	snp	T	G	G:20 T:0	CDS	+	missense variant c.279T>G p.Asp93Glu	hypothetical protein
	JPHHDCAP_3	21513	snp	G	T	T:20 G:0	CDS	+	missense variant c.640G>T p.Val214Phe	Soj2

JPHHDCAP_3	23082	complex	CAGATTATTCAGG	AATATAATTAAGT	AATATAATTAAGT:18CAGATTATTCAGG:0	CDS	+	stop gained c.152_164 delCAGATTATTCAG GinsAATATAATTAA GT p.ProAsp TyrSer Gly51*	hypothetical protein
JPHHDCAP_3	24888	snp	C	A	A:20 C:0	CDS	-	synonymous variant c.756G>T p.Ser252Ser	hypothetical protein
JPHHDCAP_3	28127	snp	T	G	G:20 T:0	CDS	+	synonymous variant c.180T>G p.Val60Val	MerT
JPHHDCAP_3	30540	del	AAGCTGGTCATCG	A	A:18AAGCTGGTCATCG:0	CDS	+	conservative inframe deletion c.1465_1476 delCTGGTCATCGAG p.Leu489_ Glu492del	MerA
JPHHDCAP_3	31044	del	TGCGGCAACTCGTCGAGCGTCGGC	T	T:16TGCGGCAACTCGTCGAGCGTCGGC:0	CDS	+	frameshift variant c.267_289 delGCAACTCGTC GAGCGT CGGCG CG p.Gln90fs	hypothetical protein
JPHHDCAP_3	34129	snp	A	T	T:20 A:0	CDS	-	missense variant c.578T>A p.Val193Glu	ArsH

	JPHHDCAP_3	34238	snp	T	A	A:20 T:0	CDS	-	missense variant c.469A>T p.Met157Leu	ArsH
	JPHHDCAP_3	37827	snp	A	G	G:10 A:0	CDS	+	missense variant c.514A>G p.Arg172Gly	Hin2
	JPHHDCAP_3	41687	snp	T	C	C:32 T:0				
	JPHHDCAP_3	42008	snp	A	T	T:20 A:0	CDS	+	synonymous variant c.72A>T p.Ala24Ala	HigA1

Table D.2: Susceptibility data (μg/mL) for all strains in the antibiotic evolution experiments.

Strain	Ampicillin	Ciprofloxacin	Eythromycin	Ethidium Bromide	Gentamicin	Meropenem	Moxifloxacin	Tetracycline
AB18 Ancestor	16	0.25	8	512	0.25	1	0.06	1
AL28 Ancestor	0.25	0.03	0.25	8	0.06	0.015	0.015	0.25
Mero_AB18 WT1	16	0.25	8	512	0.25	0.25	0.06	1
Mero_AB18 WT2	16	0.25	8	512	0.25	0.5	0.06	1
Mero_AB18 M1	128	1	32	512	0.5	4	0.25	4
Mero_AB18 M2	128	1	16	512	0.25	4	0.25	4
Mero_AB18 M3	256	1	32	512	0.5	4	0.25	4
Mero_AB18 M4	128	1	16	512	0.25	4	0.25	4
Mero_AB18 M5	64	0.5	16	512	0.25	2	0.12	1
Mero_AL28 WT1	0.25	0.03	0.25	8	0.06	0.03	0.015	0.25
Mero_AL28 WT2	0.25	0.03	0.25	8	0.06	0.03	0.015	0.25
Mero_AL28 M1	0.5	0.06	0.25	8	0.06	0.06	0.03	0.12
Mero_AL28 M2	0.5	0.06	0.25	8	0.06	0.06	0.03	0.12
Mero_AL28 M3	0.5	0.06	0.25	8	0.06	0.06	0.03	0.12
Mero_AL28 M4	0.5	0.06	0.25	8	0.06	0.06	0.03	0.12
Mero_AL28 M5	0.5	0.06	0.25	8	0.06	0.06	0.03	0.12
Cipro_AB18 WT1	16	0.25	8	512	0.25	1	0.06	1
Cipro_AB18 WT2	16	0.25	8	512	0.25	1	0.06	1
Cipro_AB18 M1	16	64	8	512	0.12	0.25	4	2
Cipro_AB18 M2	32	128	32	512	2	1	16	4
Cipro_AB18 M3	32	128	32	512	2	1	16	4
Cipro_AB18 M4	16	16	8	512	0.12	0.5	1	2
Cipro_AB18 M5	8	16	32	512	4	1	2	2
Cipro_AL28 WT1	0.12	0.03	0.25	8	0.06	0.015	0.015	0.12
Cipro_Al28 WT2	0.12	0.03	0.25	8	0.06	0.015	0.015	0.12
Cipro_AL28 M1	1	1	0.5	8	0.03	0.12	0.25	0.5
Cipro_AL28 M2	0.5	2	0.5	8	0.03	0.06	0.25	0.25
Cipro_AL28 M3	0.5	2	0.5	8	0.03	0.06	0.25	0.25
Cipro_AL28 M4	0.5	2	0.5	8	0.03	0.06	0.25	0.25
Cipro_AL28 M5	0.5	2	0.5	8	0.03	0.06	0.25	0.25
Gen_AB18 WT1	16	0.25	8	512	0.25	0.5	0.06	1
Gen_AB18 WT2	16	0.25	8	512	0.25	0.25	0.06	1
Gen_AB18 M1	8	2	32	512	32	1	0.5	4

Gen_AB18 M2	16	2	32	512	32	1	0.5	2
Gen_AB18 M3	16	2	16	256	64	1	0.5	2
Gen_AB18 M4	16	2	32	256	64	1	0.5	2
Gen_AB18 M5	16	2	16	512	32	1	0.5	2
Gen_AL28 WT1	0.25	0.06	0.25	8	0.06	0.03	0.015	0.12
Gen_AL28 WT2	0.12	0.03	0.25	8	0.06	0.03	0.015	0.12
Gen_AL28 M1	0.25	0.03	0.25	8	0.25	0.03	0.03	0.12
Gen_AL28 M2	0.25	0.03	0.25	8	0.25	0.03	0.03	0.12
Gen_AL28 M3	0.25	0.03	0.25	8	0.25	0.03	0.03	0.12
Gen_AL28 M4	0.25	0.03	0.25	8	0.25	0.03	0.03	0.12
Gen_AL28 M5	0.12	0.03	0.25	8	0.06	0.015	0.015	0.25

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