MACQUARIE
University

Multidrug resistance mechanisms in the human pathogen *Acinetobacter baumannii*

Liping Li

This thesis is presented as a partial fulfilment to the requirements for a Doctorate of Philosophy

Department of Chemistry and Biomolecular Sciences
Macquarie University, NSW, Australia
September 2018

Table of contents

List of figures

List of tables

List of supplementary information in Excel files

Declaration

I declare that the work in this thesis was conducted by me under the supervision of distinguished Prof. Ian T. Paulsen. The assistance and contribution of others have been appropriately acknowledged. The work presented here has not been previously submitted for any other degree.

Liping Li

Acknowledgements

My sincere gratitude goes to my principle Ph.D. supervisor Prof. Ian T. Paulsen. Ian has provided a platform where I have freedom to pursue my dream in Science and the opportunity to explore bacteria world. Like many other research programmes, my journey was not smooth, and there were times when I was thinking of giving up. Luckily, thanks to Ian's continuing patience, support and guidance, I could persevere through the toughest time and complete this thesis.

I am sincerely grateful to my co-supervisor Dr. Karl A. Hassan. Karl has provided me with hands-on support in experimental design, troubleshooting, manuscript preparation, and motivation. When I was still very new to bacterial drug efflux pump field, Karl has always been very patient in listening and answering any questions I had, which encouraged me to explore these mysterious drug transporters without hesitation. Same as Ian, Karl has always been there for me throughout this journey. Because of Ian and Karl, I could travel all the way to where I'm today.

I'm very grateful to have met Dr. Amy K. Cain at Paulsen group in the beginning of my Ph.D. Amy later went to the Wellcome Trust Sanger Institute for Postdoctoral training in Prof. Julian Parkhill's group. One of the major challenges during my Ph.D. was TraDIS. Fortunately, on the beginning of the fourth year of my Ph.D., through Amy, Paulsen group could collaborate with Parkhill's group and Baker's group, which enabled our antimicrobial TraDIS project to become feasible (Chapter 6). Thank you so much, Amy.

I feel very lucky to work in this multicultural research group, from where I met many lovely friends and colleagues of different nationality. Even though I am far away from my homeland, the people here made me feel at home. The postdoctoral researchers and fellow research students helped me to settled in this group, assisted me for numerous lab works, and made my Ph.D. journey full of fun and memorable moments.

Last but not the least, I thank my family members and friends, especially my parents who love me unconditionally, and respect and support my decision to travel overseas alone to pursue my dream.

Abstract

Infections caused by the nosocomial multidrug resistant human pathogen *Acinetobacter baumannii* are a global public health issue. Without new and potent antibiotics or alternative therapies for infectious diseases caused by *A. baumannii* and other drug resistant pathogens, the World Health Organization has predicted there will be a return of the pre-antibiotic era in the near future. This thesis investigates the effects of antimicrobial agents on *A. baumannii*, using two global approaches: RNA-Seq and TraDIS (transposon directed insertion site sequencing). Another novel high throughput technique, which combines Biolog phenotype microarrays and qPCR, was developed for the characterization of drug resistance phenotypes of putative drug efflux pumps.

Drug efflux pumps are cytoplasmic membrane transport proteins. They confer antimicrobial resistance through exporting the chemical agents out of the host cell. High expression levels of these proteins are frequently associated with increased bacterial drug resistance. More than 50 genes were annotated as putative drug efflux pumps in *A. baumannii* ATCC17978 strain. In this study, seventeen of these genes were heterologously cloned and highly expressed in *Escherichia coli*, and the drug susceptibility changes of the *E. coli* clones were screened through the combination of Biolog Phenotype Microarrays and qPCR. This multiplexed phenotype analysis approach identified potential drug resistance phenotypes of three novel drug efflux genes.

A protein synthesis inhibitor, tigecycline, is one of the last line therapeutic options for *A. baumannii* infection. RNA-Seq was used to analyse how *A. baumannii* physiologically responds to sub-inhibitory concentrations of tigecycline. The transcriptomic data showed an increase in expression of many genes involved in ribosome biosynthesis and assembly, and decrease in expression of genes involved in peptide synthesis, consistent with tigecycline-induced ribosomal stalling after translational initiation. Decreased expression of genes involved tricarboxylic cycle, cell respiration and cell division was observed, which is consistent with tigecycline's bacteriostatic effect. Tigecycline induced increased expression of genes involved in exogenous DNA uptake, mobile

genetic element translocation, and DNA mismatch repair, suggesting this antibiotic may promote gene mutation and lateral gene transfer.

Biocides are critical for disinfection in hospitals and are commonly used in personal hygiene products. Antibiotic resistance in bacterial pathogens has been intensively studied, but comparatively little is known about resistance to biocides or the mode of action of biocides. The potential modes of action and resistance mechanisms of ten clinically important biocides were assessed with TraDIS using a highly-saturated transposon Tn*5* mutant library. Like the tigecycline data, the TraDIS analysis suggested that these biocides have pleiotropic effects on the bacterial host. Lipooligosaccharide and capsular polysaccharide biosynthesis genes, and drug efflux genes appear to be important fitness factors in *A. baumannii* against biocides. Collapse of the cytoplasmic membrane proton motive force is likely to be a downstream antibacterial effect of multiple biocides including silver nitrate, which could potentially cause malfunction of membrane transport, cell division and various pleiotropic physiological effects.

Antimicrobial drug resistance in pathogenic bacteria, such as *A. baumannii*, is a multifactorial phenomenon. The contemporary high throughput technologies, for example the ones that were used in this thesis, has enabled us to decipher this problem at a global scale. Knowledge of finer details of antimicrobial mode of actions and bacterial drug resistance will further shed light on finding appropriate solutions for multidrug resistance infections.

Contributions

Chapter 2

Rapid multiplexed phenotypic screening identifies drug resistance functions for three novel efflux pumps in *Acinetobacter baumannii*

(Accepted for publication in the Journal of Antimicrobial Chemotherapy on 31st January 2016)

The work was conceptualized by Hassan and Paulsen. All experimental work including design and troubleshooting was conducted by Li. All the data analyses were performed by Li. The manuscript was written by Li, with significant contributions and guidance from Hassan and Paulsen, and some suggestions from Brown.

Chapter 3

An ace up their sleeve: a transcriptomic approach exposes the AceI efflux protein of *Acinetobacter baumannii* and reveals the drug efflux potential hidden in many microbial pathogens

(Accepted for publication in Frontiers in Microbiology on 22nd April 2015)

This review paper was written by Hassan, Elbourne and Paulsen, with Li and Gamage's contributions to the section of "Regulatory proteins acting on PACE efflux pumps".

Chapter 4

Fluorescence-based flow sorting in parallel with transposon insertion site sequencing identifies multidrug efflux systems in *Acinetobacter baumannii*

(Accepted for publication in mBio on 12th August 2016)

The conceptualization of this work was by Hassan and Paulsen. Hassan, Huang, Liu conducted flow sorting and sample collections. Li prepared the library samples for the TraDIS analysis and generated the control data set for TraDISort. TraDIS sequencing was conducted in Sanger centre. Hassan and Cain processed and analysed the data. Hassan, Cain and Paulsen wrote the manuscript.

Chapter 5

Tigecycline translational inhibitive effects on *Acinetobacter baumannii*, a transcriptomics view

The work was conceptualised by Paulsen, Hassan and Li. All experiments were conducted by Li, with the exception of tigecycline TraDIS data that was provided by Cain, Bionett, Baker and Parkhill. All the transcriptomic and TraDIS data analyses were performed by Li. The manuscript was written by Li, with significant contributions from Paulsen and Hassan.

Chapter 6

Transposon Directed Insertion-site Sequencing revealed potential modes of action and resistance determinants of ten biocides in *Acinetobacter baumannii*

Conceptualisation of this work was by Paulsen, Hassan and Li. All experimental work including design and troubleshooting was conducted by Li, using a transposon mutant library provided by Baker. TraDIS sequencing and data processing were conducted by Cain and Parkhill's group. All the data analyses were performed by Li. The manuscript was written by Li, with significant contributions from Paulsen and Hassan.

Chapter 1 Introduction

Since their discovery in the 1930s, antibiotics have tremendously improved modern medicine and greatly influenced life on Earth. The most profound impacts of antibiotics are that various infectious diseases, which were previously life threatening, can be prevented and treated by antibiotics, and infections that may have arisen during surgical intervention for conditions such as serious heart disease, cancers, and organ failure requiring transplants, are now preventable using prophylactic antibiotics. This has resulted in a dramatic reduction in the morbidity and mortality associated with infectious disease, and human life expectancy in general has since been extended (*1*). The World Health Organization (WHO) still recognises many antibacterials as essential medicines (*2*). Unfortunately, since the clinical introduction of antibiotics, there has been a rapid emergence of multiple-drug resistant (MDR) or even pan-drug resistant (PDR) pathogens. Today these highly resistant pathogens can render entire classes of antibiotics ineffective, which threatens to return medicine to the 'pre-antibiotic era' (*3, 4*).

Six significant highly resistant nosocomial pathogens, namely *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, were acronymically dubbed 'the ESKAPE pathogens' by the Infectious Diseases Society of America, because they are capable of 'escaping' the biocidal action of antibiotics and leave clinicians with very limited therapeutic options (*5, 6*). Furthermore, the growing number of elderly patients, patients in neonatal intensive care units, and patients undergoing surgery, transplantation, and chemotherapy results in an enlarging pool of immunocompromised individuals who will be at risk of these infections. The traditional response to emerging resistance was the periodic introduction of new compounds. However, there has been a substantial decline in novel antimicrobials approved to market, due in part to a significant retraction of investment towards antibacterial research and development by the major pharmaceutical companies (*7*). Therefore, it is urgent and critical to fully understand the physiology of these nosocomial pathogens and their mechanisms of drug resistance, so new strategies can be devised to overcome the global problem of

bacterial MDR. The major focus of this thesis is on drug resistance mechanisms of one ESKAPE pathogen, *A. baumannii*.

1.1. Taxonomy and Ecology of *A. baumannii*

A. baumannii are Gram-negative bacteria with cells 1.5 μm in length and morphology varying from coccoid to coccobacillary, depending on the growth phase (*8*). *A. baumannii* is classified as a member of the *Acinetobacter* genus within the γ-Proteobacteria. Species within this genus are strictly aerobic, nonfermenting, nonfastidious, catalase-positive, oxidase-negative bacteria with a DNA GC content between 39% and 47% (*8*).

At present, there are 42 *Acinetobacter* species characterized and assigned with valid names (*9*). Many *Acinetobacter* species appear to be very pervasive, as they have been recovered from a wide range of environments, including soil and fresh water (*8*). For instance, *A. baylyi* known for its metabolic versatility and high transformability was first isolated from an active sludge plant in Victoria, Australia (*10*). *A. rudis* was isolated in northern Israel from raw milk during a study of seasonal psychrotrophic bacterial flora composition of the milk (*11*). Various *Acinetobacter* species were found as fuel oil degraders such as *A. oleivorans*, *A. haemolyticus*, *A. johnsonii* and *A. venetianus* (*12, 13*). Apart from environmental members, there are fifteen species identified so far that have been isolated from human clinical specimens. They include *A. pittii*, *A. nosocomialis*, *A. guillouiae*, *A. bereziniae*, *A. seifertii*, *A. junii*, *A. lwoffii*, *A, parvus*, *A. radioresistens*, *A. schindleri*, *A. soli*, *A. ursingii*, *A. haemolyticus*, *A. johnsonii* and *A. baumannii* (*14-18*). *A. baumannii* is the most important human pathogenic species of *Acinetobacter*, as it is the predominant species recovered from clinical specimens during infection outbreaks, whereas other species are only sporadically detected (*8, 18*). Whether *A. baumannii* exists in a natural reservoir outside the hospital is still unclear.

A. baumannii MDR strains are frequently recovered from clinical samples taken from patients hospitalized in intensive care unit (ICU) wards where antibacterial use is common and intensive. For

instance, *A. baumannii* has been isolated from respiratory tract and urinary tract samples of ICU patients, as well as exudates and abscesses from surgical or wound sites, and from blood specimens (*8*). *A. baumannii* also tends to asymptomatically colonize on different body sites of the ICU patients, such as the groin, axilla, pharynx and rectum (*19*). In addition to antibiotics, *A. baumannii* strains have been found to be resistant to different antiseptics, possibly due to the selective pressure of biocide treatment of hospital inanimate surfaces (*20*). Furthermore, it was shown that *A. baumannii* could survive on inanimate surfaces for as long as 30 days (*21*). Variours *A. baumannii* reservoirs in hospital have been identified, including healthcare workers' hands and gowns, ward curtains, mattresses, humidifiers, multidose vials of medication, intravenous nutrition, inadequately sterilized reusable arterial pressure transducers, and computer keyboards (*8*). The prolonged survival of *A. baumannii* in clinical settings is likely linked to its high levels of desiccation tolerance, and antibiotic and antiseptic resistance. Compared to hospital settings, the rate of *A. baumannii* carriage in the community is relatively low. Severe community-acquired *A. baumannii* pneumonia and bacteremia have been reported in tropical climate areas such as Singapore, Hong Kong, and northern Australia (*22-24*). Genomic analysis of community-acquired isolates has revealed that they are genetically and phenotypically distinct from nosocomial isolates and do not have high levels of antibiotic resistance (*25-27*).

1.2. Clinical manifestation and virulence of *A. baumannii*

A. baumannii infects immunocompromised patients especially those hospitalized in ICU wards. It can cause a range of infections include pneumonia, surgical site infections, urinary tract infections, bloodstream infections, skin/soft tissue infections, and post-neurosurgical meningitis (*8, 18*). Occasionally, it has also been reported to be associated with endophthalmitis, necrotizing fasciitis and central nervous system infections (*8*).

A. baumannii was previously thought to be an uncommon cause of healthcare associated infections. However, over the past few decades it has started to gain public attention as an important nosocomial

pathogen. In 1999, a study showed that within three months 400 patients in 15 hospitals in New York City were infected by carbapenem-resistant *A. baumannii*, 12% of the isolates were resistant to all standard antibiotics (*28*). Carbapenem antibiotics have become one of the most efficient treatments in controlling nosocomial Gram-negative infections since the 1980s. During 2002 and 2004, 102 U.S. soldiers from Iraq/Kuwait and Afghanistan were identified with MDR or PDR *A. baumannii* bloodstream infections. *A. baumannii* isolates recovered from these patients were resistant to carbapenem, ciprofloxacin, amikacin and cefepime (*29*). In 2003, the first outbreak of carbapenem-resistant *A. baumannii* was reported in Australia, after meropenem use was dramatically increased in the hospital (*30*). Alarmingly, in 2014 an outbreak of severe *A. baumannii* infections in immunocompetent patients caused by two extensively drug resistant (XDR) and hyper-virulent strains was reported in the United States (*31*).

1.3. Therapeutic options for *A. baumannii* treatment

Prior to the early 1990s, various antibiotics were effective in the treatment of *A. baumannii* infections. They included aminoglycosides (amikacin, tobramycin, gentamycin, kanamycin and netilmicin), extended-spectrum penicillins (piperacillin, ticarcillin, ampicillin, and carbenicillin, in combination with sulbactam), broad-spectrum cephalosporins (cefotaxime, cefepime, ceftriaxone and ceftazidime), carbapenems (imipenem), fluoroquinolones (levofloxacin and ciprofloxacin) and tetracyclines (tetracycline, minocycline and doxycycline) (*8*). Although effective in eradicating *Acinetobacter* spp., because of their high toxicity, polymyxins (colistin and polymyxin B) were only occasionally used in serious *A. baumannii* infections (*8*).

At present, the agents that remain as active treatments for *A. baumannii* infections include imipenem, meropenem, doripenem, sulbactam, amikacin, tobramycin, colistin, polymyxin B, minocycline and tigecycline. Of these, meropenem was approved by the U.S. Food and Drug Administration (FDA) in 1996, doripenem and tigecycline were approved in 2007 and 2005 respectively (*32*). Carbapenems are still the treatment of choice for serious MDR or XDR *A. baumannii* infections. However, increasing levels of carbapenem resistance among *A. baumannii* isolates makes the already limited

therapeutic options for *A. baumannii* even more challenging for clinicians, often leaving polymyxins as the only choice, because most carbapenem-resistant isolates tend to be MDR or XDR (*33*). Unfortunately, there has recently been an occurrence of infections caused by colistin and carbapenem resistant strains (*34*). The only two remaining effective aminoglycosides, amikacin and tobramycin, are not normally used as monotherapy due to their toxicity, instead they are usually used in combination with other drugs like carbapenems and colistin. Tigecycline represents another therapeutic option, however tigecycline resistant MDR isolates emerged soon after its clinical introduction (*35*).

1.4. Drug resistance mechanisms in *A. baumannii*

Antibiotics target various crucial cellular macromolecules to induce cell death or inhibit cell growth. The major classes of antibiotics usually target either cell wall biosynthesis, protein biosynthesis, or DNA replication (*36*).

The Gram-negative bacterial cell wall is composed of three layers, including the inner membrane (IM), outer membrane (OM) and periplasm between these two membranes. The peptidoglycan layer within the periplasmic space imparts mechanical strength and serves as a major structural barrier, and thus enables bacteria to survive osmotic stress that is encountered in most environments (*37*). *β*-lactam antibiotics can actively block the active sites of penicillin-binding proteins (PBP) which are crucial for crosslinking of peptidoglycan and supplying covalent connectivity to the cell wall meshwork, and therefore impair cell wall synthesis (*37*). Glycopeptide antibiotics, such as vancomycin, also act as cell wall synthesis inhibitors, but they target peptidoglycan precursors and block their incorporation into the growing cell wall (*38*). Inhibition of cell wall synthesis does not directly cause lysis-dependent cell death, which requires other cellular activities. For instance, bacterial cell lysis induced by β-lactam antibiotics requires both inhibition of peptidoglycan biosynthesis, and active cellular autolysins, enzymes that hydrolyze peptidoglycan matrix (*39*).

Drugs such as macrolides (*40*), aminoglycosides and tetracyclines (*41*) can inhibit protein synthesis. Translation of mRNA into protein involves three sequential phases, namely translation initiation, polypeptide chain elongation and termination, and involves the ribosome and a range of cytoplasmic accessory factors (*42*). The ribosome is composed of two ribonucleoprotein subunits, the 50S and 30S subunits. This group of antibiotics can be categorized into two subclasses: the 50S inhibitors and 30S inhibitors. For instance, the 50S inhibitors includes macrolides, such as erythromycin, and amphenicols, such as chloramphenicol, which bind in the polypeptide export tunnel and thus restricts the diameter of the tunnel, resulting to peptidyl tRNA drop-off and abortion of polypeptide chain elongation (*43*). The 30S inhibitors includes tetracyclines, tigecycline and aminoglycosides, such as kanamycin (*44*). For example, tetracycline blocks access of aminoacyl-tRNA into A-site on 16S rRNA, a subunit of the 30S ribosomal subunit, which causes protein synthesis perturbation and mistranslation.

DNA replication inhibitors include quinolone antibiotics, such as nalidixic acid and ciprofloxacin, that target topoisomerase II and IV to inhibit reduction of topological strain during DNA synthesis and gene transcription, arresting DNA replication (*45*). This effect then induces the DNA stress response (SOS response) (*46*). The SOS response has been implicated in horizontal gene transfer (HGT, see below) (*47*), homologous recombination (*48*) and the induction of error-prone DNA polymerases (*49*). The induction of SOS response has also observed to be caused by beta-lactam antibiotics (*50, 51*). These side effects of antibiotic treatment have likely contributed to the rapid development of drug resistance and the genome plasticity observed in hospital associated pathogens.

Bacteria have developed various MDR mechanisms that enable them to survive in hospital environments. The following sections will focus on describing the evidences of how *A. baumannii* has evolved to adapt to antibacterial challenges, and the drug resistance mechanisms currently identified in this organism.

1.4.3. Genetic view of *A. baumannii* responses and adaptations to drugs

It is well known that bacteria can evolve and develop capabilities in drug resistance, especially in hospital environments where there is typically strong antimicrobial selective pressure. There are three major ways, through which bacteria can be antibacterial resistant or tolerant. They include: 1) acquired resistance via HGT acquiring exogenous resistance determinants carried by mobile genetic elements (MGE) (*52*), or through mutations causing constitutive overexpression of drug resistance genes or target alteration to bypass drug recognition, 2) intrinsic resistance such as impermeability of bacterial cell wall, and 3) adaptive resistance through the regulation of the resistance genes in response to a drug (*53*). MGEs are segments of DNA that encode enzymes and proteins that mediate the movement of DNA within genomes (intracellular mobility) or between bacterial cells (intercellular mobility). Well studied MGEs include prophage, transposons, insertion sequences (IS), integrons, mobile introns and conjugative plasmids (Figure 1.1) (*54*). MGEs could facilitate DNA exchange between *A. baumannii* strains and among different bacterial species present in the same environment.

Significant variation in gene content relating to HGT, such as the sequences of transposons, prophage and integrons are constantly detected in the genomes of various nosocomial *A. baumannii* outbreak strains from geographically scattered hospitals (*55*). Resistance islands (RI), which are enriched in antibiotic resistance determinants carried by MGEs such as conjugative plasmids, transposons, prophages and mobile introns (i.e. class I introns) are a common hallmark of HGT in MDR pathogens. Compared to the remainder of the host chromosome, these RIs usually have different base composition (i.e. G+C content) and codon usage frequency, as the genes are likely to maintain these features from their previous host for some time after their acquisition. For instance, a large RI 86 kb in size, carrying as many as 45 resistance genes, has been identified in the MDR *A. baumannii* strain, AYE. Various parts of this large RI were predicted to have originated from *Pseudomonas*, *Salmonella*, and *Escherichia* (*27*). RI of large size have also been observed in many other clinical MDR strains of *A. baumannii* but have not been found in a community acquired strain (*26, 55, 56*), which suggests that antibiotic selection plays a key role in the plasticity of *A. baumannii* genome in

the hospital environments. Insertion sequences (IS) do not carry open reading frames (ORF) that encode resistance or virulence proteins as with many of the other classes of MGEs identified in the genomes of nosocomial pathogens, but they can mediate insertional mutations leading to chromosomal gene losses and overexpression (*57*). For instance, the carbapenem resistance phenotypes of MDR *A. baumannii* strains were often correlated with the overexpression of their intrinsic ß-lactamase encoding genes due to the insertion of an upstream IS*Aba1*, which provides promoter for the overexpression of bla_{ampC} or $bla_{OXA\text{-}23}$ (Table 1.1).

For effective clinical surveillance, prevention and treatment of *A. baumannii* infections, it is important to get insight into the regional and global dissemination patterns of this pathogen. Traditional molecular typing studies and recent whole genome sequencing studies have indicated that most hospital outbreaks worldwide are due to strains from one of three clonal lineages, which are commonly referred to as global (or European) clonal (EC or GC) lineages I, II, and III (*58, 59*). GCII clones are the most prevalent outbreak strains. Whole genome sequencing studies have shown that there are substantial differences in gene content among the strains of the same lineage (*55, 60*). These studies also proposed that there is a high probability for co-infections caused by multiple clonal *A. baumannii* strains on the same patient, and that there is likely to be a large reservoir of *A. baumannii* strains capable of colonizing patients circulating in the hospital environment. Therefore, even though *A. baumannii* typing schemes based on a few housekeeping genes are useful from a broad epidemiological view, the accessory resistance genes carried by RIs can result in strains from the same clonal lineage displaying varied drug resistance patterns, which impacts the development of treatment strategies.

In addition to the known resistance determinants, the RIs or MGEs also contain many genes annotated as hypothetical proteins and genes with only putative functions. It will be important to know whether these genes also play a role in MDR and MDR development. The following sections will introduce the mechanism of individual resistance determinants characterized and their contributions to *A. baumannii* drug resistance phenotypes.

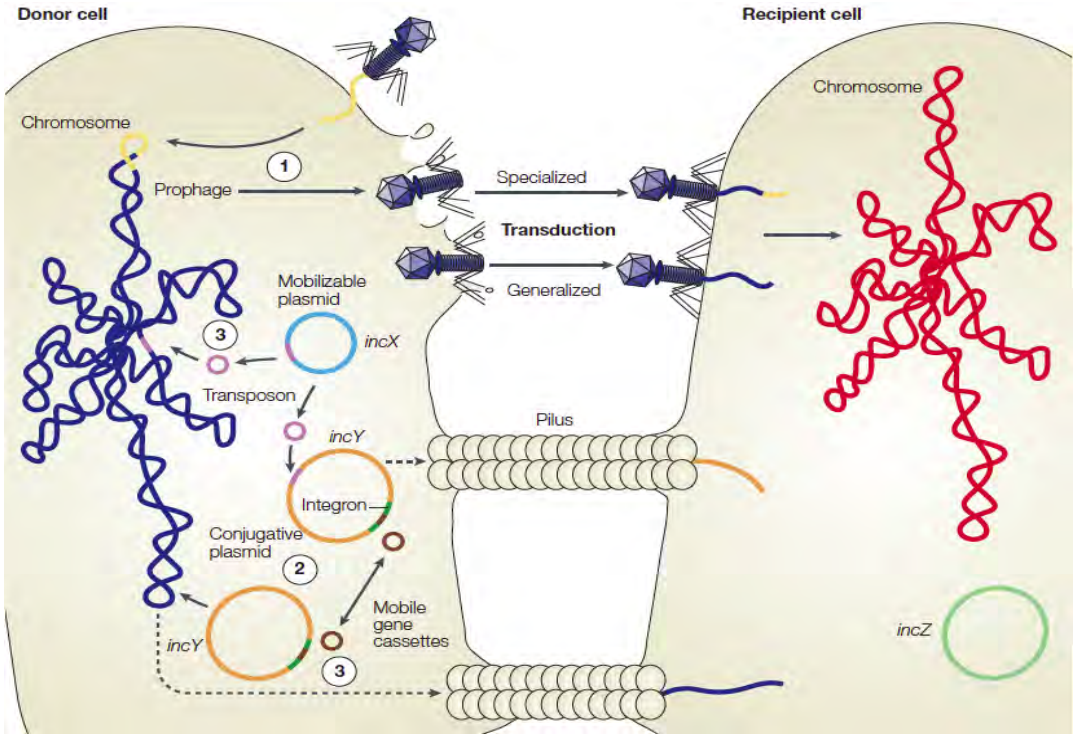

Figure 1.1. Horizontal DNA transfer between bacterial cells. (1) A temperate phage genome (yellow) inserts into a bacterial chromosome (dark blue) as a prophage. Afterwards, it either replicates occasionally packaging host DNA alone (generalized transduction), or with its own DNA (specialized transduction), lyses the cell, and infects a surrounding bacterial recipient cell in which the novel DNA recombines into the recipient host cell chromosome (red). (2) Conjugative plasmids (orange) and integrated conjugative elements (ICEs; not shown) use cell membrane apparatus such as pilus to connect with the recipient cell and to transfer themselves to the recipient cell, which is conjugation. Alternatively, a copy of a small, multicopy plasmid or defective genomic island or a copy of the entire bacterial chromosome can be transferred to a naive cell, in which these genetic elements either insert into the chromosome or replicate independently if compatible with the resident plasmids (light green). (3) Transposons (pink) integrate into new sites on the chromosome or plasmids by non-homologous recombination, which is transposition. Integrons (dark green) use similar mechanisms to exchange single gene cassettes (brown). This figure was replicated from (*54*).

1.4.2.Drug specific enzymatic inactivation

Bacteria encode substrate-specific enzymes that hydrolyse and inactivate drugs thus reducing the intracellular concentration of the active compound. Drugs known to be enzymatically inactivated in *A. baumannii* include ß-lactams, aminoglycosides, rifampin, and chloramphenicol (Table 1.1 and 1.2).

1.4.2.1 ß-lactamases

As carbapenems are the first line therapy for the treatment of *A. baumannii* infections, ß-lactamases are the most intensively studied antibiotic hydrolytic enzymes in this organism. These enzymes inactivate substrates through hydrolysing the four-membered ß-lactam ring of ß-lactam antibiotics. The ß-lactam ring is the key structure of ß-lactam antibiotics required for binding to the active site of penicillin binding proteins (PBP) to interfere peptidoglycan biosynthesis and initiate cell lysis (*37*).

A. baumannii has various conserved ß-lactamases (Table 1.1), such as the AmpC-type cephalosporinase that can catalyse ß-lactam ring hydrolysis of broad spectrum cephalosporins and carbapenems, but only when overexpressed. In clinical strains, overexpression of *ampC* is typically caused by the upstream insertion of an IS*Aba1* element (*61*). Notably, more than 20 different carbapenemases have been reported in *A. baumannii* (Table 1.1). Among them, enzymes belonging to OXA subclass are exclusively found only in *Acinetobacter* spp. (on the chromosome and/or in MGEs), and the others were often reported from various other bacterial hosts and constantly identified in MGEs (*62*). OXA-23 is the most widespread carbapenemase in *Acinetobacter* spp. Although relatively poorly effective in ß-lactam ring hydrolysis, its overexpression induced by the upstream IS*Aba1* is usually associated with significant carbapenem resistance in many MDR *A. baumannii* strains, including those responsible for hospital outbreaks worldwide (*63*).

Table 1.1. Carbapenem-hydrolyzing ß-lactamases identified in *A. baumannii*

ß-lactamase	Genetic location	Regulation	References
KPC-2	NI	NI [a]	(*64*)
KPC-3	NI	NI	(*64*)
KPC-4	NI	NI	(*64*)
KPC-10	NI	NI	(*64*)
GES-14	Integron, plasmid	NI	(*65*)
IMP-1	Integron, plasmid	PcS promoter	(*66*)
IMP-2	Integron, plasmid	NI	(*66, 67*)
IMP-4	NI	NI	(*68*)
IMP-5	Integron	In76 promoter	(*66, 69*)
IMP-10	Integron	PcS promoter	(*70*)
NDM-1	Chromosome	NI	(*71*)
NDM-2	Chromosome	IS*Aba125*	(*72*)
VIM-1	Integron	NI	(*73*)
SIM-1	Integron	NI	(*74*)
OXA-23	Tn*2006*, Tn*2007*, Tn*2008*, plasmid	IS*Aba1*	(*63*)
OXA-24	Chromosome	NI	(*75*)
OXA-25	Chromosome	NI	(*76*)
OXA-26	Chromosome	NI	(*76*)
OXA-51-like	Chromosome	*ISAba1*	(*55*)
OXA-58	Plasmid, Chromosome	NI	(*77*)
OXA-97	Plasmid, Chromosome	IS*Aba8*	(*78*)

a. NI means not identified.

1.4.2.2Aminoglycoside modifying enzymes

In the treatment of *A. baumannii* infections, aminoglycosides are usually used in combination with ß-lactams. The killing mechanism initiates from aminoglycosides binding to 16S rRNA of the 30S subunit of the ribosome, which leads to protein mistranslation and eventually arrested cell growth (*79*). Unfortunately, aminoglycoside resistant *A. baumannii* isolates are widespread around the world. The prevalent resistance mechanisms are acetyltransferase (AAC), nucleotidyltransferase (ANT) and phophotransferase (APH) aminoglycoside modifying enzymes (Table 1.2) (*80, 81*). Through modifying aminoglycosides, they can reduce drug-target binding affinity and hence increase the resistance level of the host. AACs belong to the ubiquitous GCN5-related N-acetyltransferase (GNAT) superfamily, and can catalyse the acetylation of the amine groups of the 2-deoxystreptamine nucleus of the drug by using acetyl coenzyme A as the donor substrate. *aacA4* confers resistance to

amikacin and several other aminoglycosides, and is the most intensively studied acetyltransferase in both Gram-positive and Gram-negative bacteria (*80, 82*). *aacC* genes have only been identified in Gram-negative bacteria so far, conferring resistance to gentamicin, sisomicin and fortimicin (*80, 82*). ANTs inactivate aminoglycosides through catalysing the transfer of an AMP group from the donor substrate ATP to a hydroxyl group in the aminoglycoside molecule. Two of the subclasses, *aadB* and *aadA1* have been identified in *A. baumannii*, mediating relatively narrow aminoglycoside resistance (Table 1.2) (*83*). APHs catalyse the transfer of a phosphate group to the drug molecule. The two APHs identified in *A. baumannii* (Table 1.2) are widely distributed mainly among Gram-negative bacteria (*83, 84*). The aminoglycoside-modifying enzymes found in *A. baumannii* so far are encoded on transferable plasmids, transposons and particularly on class I integrons (Table 1.2).

1.4.2.3 Rifampin and chloramphenicol modifying enzymes

Rifampin (or rifampicin) has been used in combination with colistin and carbapenems for the treatment of MDR *A. baumannii* infections (*85*). It belongs to the rifamycin group of antibiotics. The bactericidal effect of rifamycin antibiotics is initiated from inhibiting the *ß*-subunit of bacterial DNA-dependent RNA polymerase by obstructing the exit tunnel for the nascent RNA (*86*). A rifampin ADP-ribosyltransferase (*87*) that can catalyze ADP-ribosylation of rifamycins has been identified on integrons from various Gram-negative bacteria (*88*), including *A. baumannii* (*27*) (Table 1.2).

Chloramphenicol targets the 23S rRNA within the ribosomal 50S subunit, and blocks peptidyl transferase activity by hampering the binding of the aminoacyl-tRNA to the A site (*89-91*). This antibiotic is not currently used as a therapeutic option for *A. baumannii* infections. This is partly due to the prevalence of chloramphenicol resistance mediated via chloramphenicol acetyltransferase (CAT) and MDR efflux pumps (*92, 93*). The CAT enzyme belongs to type A CATs (*catA1*) and catalyzes drug modification through acetylation of the C-3 –OH group on chloramphenicol (*94*). The *cat* gene is typically carried within AbaR-type RIs in *A. baumannii* clinical strains (Table 1.2) (*27, 56*).

Table 1.2. Resistance mechanisms mediated via drug inactivation or target alteration in *A. baumannii*

Resistance gene	Resistance mechanisms	Substrates	Genetic location(s) [a]	Reference
aadB, aadA1	Aminoglycoside nucleotidyltransferase (ANT), drug enzymatic inactivation.	***aadB***: kanamycin, gentamicin, sisomicin, and tobramycin. ***aadA1***: spectinomycin and streptomycin.	pRAY plasmid, class I integron	(*80-82*)
aphA1, aphA6	Aminoglycoside phosphotransferase (APH) , drug enzymatic inactivation.	***aphA1***: kanamycin, neomycin, paromomycin, ribostamycin, lividomycin. ***aphA6***: neomycin, paromomycin and ribostamycin.	AbaR1, class I integron	(*80, 82*)
aacC1, aacC2, aacA4	Aminoglycoside acetyltransferase (AAC), drug enzymatic inactivation.	***aacA4***: amikacin and several other aminoglycosides. ***aacC***: gentamicin, sisomicin and fortimicin.	AbaR1, class I integron	(*80, 82*)
armA	16S rRNA methylation, target alteration.	Amikacin, tobramycin, gentamycin	Tn1548/ plasmid	(*95, 96*)
arr-2	ADP-ribosylating transferase, drug enzymatic inactivation.	Rifampin	Class I integron	(*27, 87*)
catA1,	Chloramphenicol acetyltransferase, drug enzymatic inactivation.	Chloramphenicol	AbaR3 RI	(*56, 94*)
dfr genes	Dihydrofolate reductase is the drug target. Target bypass.	Trimethoprim, cotrimoxazole	AbaR1, Tn6020	(*27, 56, 97*)
gyrA	Mutations in DNA topoisomerase II, target alteration.	Fluoroquinolones	Core	(*98*)
parC	Mutations in DNA topoisomerase IV, target alteration.	Fluoroquinolones	Core	(*98*)
lpxA, *lpxC*, *lpxD*	Mutations in lipid A biosynthesis genes, loss of drug target.	Colistin	Core	(*99*)
pmrCAB	Lipid A modification, target byass	Polymyxin B	Core	(*100*)
penA	PBP profile alteration, potential target bypass.	ß-lactams	Core	(*101*)
sul1 and *sul2*	Mutations on dihydropteroate synthase, target alteration.	Sulphonamide	Mobile element CR2	(*27, 56, 97*)

a. Core means "core genome of *A. baumannii*"

1.4.3. Antibiotic target alteration or bypass

An alternate bacterial drug resistance mechanism is through alteration of the drug cellular target through mutation(s), to interfere with the drug binding to the target. Another similar mechanism is target bypass where the target enzyme is replaced by an alternate enzyme that is not affected by the antimicrobial. Both these resistance mechanisms are also substrate specific.

Quite a few actinomycetes are aminoglycoside producers and known to be intrinsically resistant to this class of antibiotics (*102*). The resistance mechanisms include the modifying enzymes introduced previously and protection of the drug target 16S rRNA within the 30S ribosome subunit through production of 16S rRNA methylase (*80, 96*). Five related enzymes have been discovered in various Gram-negative pathogens, which are designated ArmA, RmtA, RmtB, RmtC and RmtD (*103*). The *armA* gene has been increasingly detected in clinical MDR *A. baumannii* isolates (*95, 104, 105*).

Fluoroquinolones target topoisomerase II and IV to interfere with DNA replication. Multiple mutations on the genes encoding GyrA (a subunit of topoisomerase II) and ParC (a subunit of topoisomerase IV) were found to increase *A. baumannii* resistance levels to fluoroquinolones (*98*). In one study, all the fluoroquinolone resistant *A. baumannii* isolates tested were found to have *gyrA* mutations, with some isolates also carrying mutations in *parC*, indicating *parC* might be a secondary target for quinolones (*106*). In addition to mutations in *gyrA* and *parC*, other resistance determinants also play crucial roles in fluoroquinolone resistance in *A. baumannii*, such as the multidrug efflux pump AdeABC (see below).

Colistin, a peptide antibiotic belonging to the polymyxin class, is used as a drug of last resort for *A. baumannii* treatment. It is often used in combination with other drugs, such as carbapenems and aminoglycosides. Unfortunately, strains that are resistant to both polymyxins and carbapenems are emerging and increasingly reported (*34*). The bactericidal effect of polymyxins is proposed to be via the positively charged peptide drug binding to the negatively charged lipid A, which leads to

destabilization of the outer membrane (*107*). Lipid A is the endotoxic component of lipopolysaccharide (LPS). Colistin resistant mutants have been generated *in vitro*, with mutations in the lipid A biosynthesis genes, *lpxA*, *lpxC* and *lpxD*, resulting in loss of lipid A production (*108*). In clinical isolates, polymyxin B resistance has been observed associated with in the *pmrCAB* genes, which encode a lipid A phosphoethanolamine transferase, and a two-component signal transduction system, which are involved in lipidA modification through addition of phosphoethanolamine to lipidA (*100*). A transmissible plasmid-borne colistin resistant gene *mcr-1* was lately identified in colistin resistant *E. coli* strains isolated from a pig (*109*). *mcr-1* encodes an enzyme that belongs to the phosphoethanolamine transferase family, and the colistin resistance is also mediated by addition of phosphoethanolamine to lipid A (*109*). Subsequently pathogenic bacterial isolates carrying this gene were also reported from Denmark, Germany and other European countries and United States (*110-112*). Heterologous expression of *mcr-1* in *A. baumannii* has been shown in the lab to result in both phosphoethanolamine modification of lipid A and reduced colistin susceptibility (*113*).

Trimethoprim and sulfamethoxazole target two essential enzymes in bacteria, i.e. dihydrofolate reductase and dihydropteroate synthase respectively (*97*), which are essential in tetrahydrofolate biosynthesis. They are used as combination therapy with colistin for the treatment of *A. baumannii* (*8*). Typically, trimethoprim resistance is mediated via expression of alternative *dfr* genes that encode a drug-insensitive dihydrofolate reductase. Similarly, sulphonamide resistance is mediated by the *sul1* or *sul2* genes that encode drug-insensitive dihydropteroate synthases (*97*). *dfr* genes and *sul1/sul2* are now widespread in *A. baumannii*, usually localised within RIs (*27, 56, 114*), and trimethoprim-sulfamethoxazole resistant *A. baumannii* clinical isolates are common (*115*).

In addition to ß-lactamases, there are a few reports showing that the alteration or reduced expression of PBPs, the target of ß-lactams, might also play a role in carbapenem resistance in *A. baumannii* (*101, 116*). This is based on the comparisons of PBP expression between resistant and susceptible strains, and the effect on carbapenem resistance has not yet been shown in isogenic strains.

1.4.4.Reduced drug uptake

Antibacterials that have intracellular targets in Gram-negative bacteria need to penetrate through two cellular membranes to mediate their antimicrobial effects. The OM of the Gram-negative cell wall provides a selectively permeable barrier in addition to the cytoplasmic membrane that reduces the uptake of many antimicrobial compounds. The OM is an asymmetric lipid bilayer with an outer leaflet composed primarily of lipopolysaccharides (LPS). This LPS layer acts as a strong impermeability barrier for various molecules, especially for hydrophilic solutes, including antibiotics such as some ß-lactams, fluoroquinolones, tetracyclines, chloramphenicol and aminoglycosides (*117*). The OM is one of the reasons why Gram-negative bacteria are intrinsically resistant to large lipophilic antibiotics, such as macrolides, rifamycins, novobiocin, and fusidic acid, which are normally considered to be effective only against Gram-positive bacteria (*118-120*).

Various outer membrane proteins (OMP) facilitate the transduction of biological and chemical signals or the transport of diverse substances, e.g. bio-molecules, metabolites, nutrients and antibiotics across the outer membrane. Water filled channels, one type of OMPs, allow the entry of small hydrophilic compounds (Figure 1.2) (*117*). These proteins are normally composed of two domains. The N-terminal domain is a transmembrane ß-barrel with 12 to 18 strands, and the C-terminal domain is a periplasmic globular domain. Two types of channels are classified based on their protein-substrate interaction pattern. Those containing substrate-binding sites in the ß-barrel are classified as substrate-specific channels, which can selectively transport specific sets of compounds. The other type of channel is non-specific. These channels, also called porins, do not have measurable binding affinities with solutes. The existence of such channels has been suggested to allow the intake of antibacterials in Gram-negative bacteria. Consistently, reduced expression of these proteins or deletion of the respective genes has been reported to result in an increase in drug resistance in various pathogenic bacteria, such as *Escherichia coli*, *Pseudomonas aeruginosa* and *A. baumannii* (*121-123*).

The permeability of the whole *A. baumannii* OM for antibiotics (e.g. cephalothin and cephaloridine)

is relatively low compared to other organisms, such as *E. coli*. This is probably because the major OMP in *A. baumannii* $OmpA_{Ab}$, a homolog of *E. coli* OmpA and *P. aeruginosa* OprF, is a slow porin allowing very slow nonspecific diffusion of solutes (*124*). *ompA* deletion mutants in a relatively drug-susceptible *A. baumannii* strain ATCC 17978, were shown to have increased resistance levels to chloramphenicol, nalidixic acid, and some ß-lactams (*124, 125*). The second most abundant OMP identified in *A. baumannii* is the carbapenem-associated outer membrane protein (CarO) (*122*). Previous studies have shown that CarO probably allows carbapenem uptake, due to observations of decreased expression levels or disruption of *carO* by insertion elements in various carbapenem-resistant clinical strains (*122, 126, 127*). The X-ray crystal structure however showed that CarO is a small eight-stranded barrel membrane channel (not a porin) with only one open end (Figure 1.2) (*128*). Small OMPs are not known to transport large molecules like carbapenem antibiotics. Therefore, the relationship between CarO and carbapenem uptake needs further investigation. Other uncharacterized putative porin-like OMPs were also found to have diminished expression levels in imipenem-resistant *A. baumannii* isolates, indicating that more than one channel in this organism may facilitate carbapenem entry (*129*).

1.4.5. Drug efflux and decreased intracellular concentration

When antimicrobials cross the outer or inner membrane(s) and reach the periplasmic or cytoplasmic space, bacteria can employ efflux mechanisms to export the compounds using membrane transport proteins expressed in the inner (or cytoplasmic) membrane (IM), namely efflux pumps (*130*) (Figure 1.2). These efflux pumps mediate export against the compound concentration gradient using the energy of the transmembrane electrochemical proton, sodium or potassium gradients (secondary transporters) (*131, 132*), or ATP hydrolysis (primary transporters) (*133*). For effective reduction of antimicrobial cellular concentration the drug efflux rate should be fast enough to compensate for the rate of accumulation. This means a balance between the OM permeability and efflux pump efficiency is crucial for a significant drug resistance phenotype (*130, 134*).

Efflux systems are ubiquitous from bacteria to higher eukaryotes and are involved in the transport of various substrates (e.g. ions, metabolites, and toxins). Based on phylogenetic classification, there have been seven families of transport proteins that include drug efflux systems identified in bacteria: the ATP-binding cassette (ABC) superfamily (*135*), the multidrug and toxic-compound extrusion (MATE) family (*136*), the small multidrug resistance (SMR) family (*137*), the resistance nodulation division (RND) family (*138*) and the Proteobacterial antimicrobial compound efflux (PACE) family (*139*) and the antimetabolite transporter (AbgT) family (*140*). According to their respective energy source, ABC superfamily proteins are primary transporters, whereas the other five families belong to secondary transporters. Depending on the strain, the *A. baumannii* chromosome typically encodes seven RND, more than thirty MFS, and several MATE, SMR, ABC and PACE efflux systems (*141*). Some drug efflux pumps are thought to have very narrow substrate specificities, for example the Tet efflux pumps principally export tetracycline drugs (*142*). However, some drug efflux pumps are known to transport a broad range of structurally unrelated substrates, namely MDR efflux pumps (Table 1.3) (*130*).

Drug efflux pumps can cause clinically significant multiple drug resistance in pathogenic bacteria (*143*). Some of them are known to be inducible through transcriptional regulation, i.e. their expression is up regulated upon challenging the host with antibacterial substrate(s), whereas others are constitutively expressed, or induced by alternative factors (*144*). Clinical MDR pathogenic bacterial strains, such as *Enterobacteriaceae*, *P. aeruginosa* and *A. baumannii*, which constitutively overexpress drug efflux pumps relative to other drug susceptible strains, have also been isolated from patients (*145-147*).

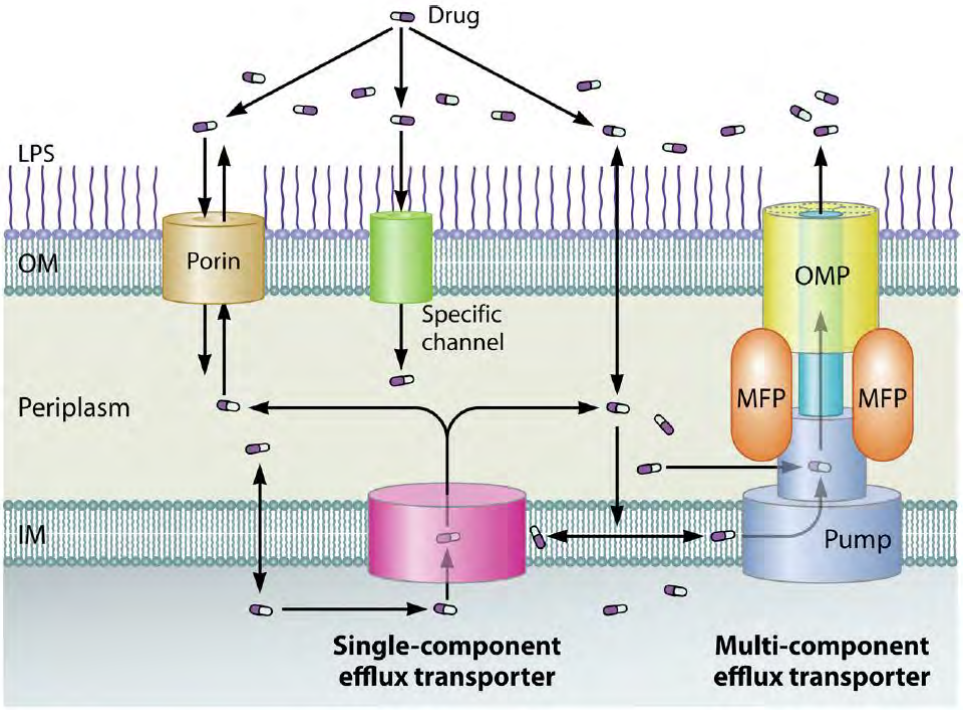

Figure 1.2. Drug influx and efflux in Gram-negative bacteria. Three pathways are know to enable the influx of drugs (shown as pills) through the OM: porin channels (e.g., OmpA for broad substrates), specific protein channels (e.g., CarO for carbapenems), and the LPS-containing asymmetric lipid bilayer region. After their entry into the periplasmic space, the drug molecules can further penetrate the IM via diffusion. However, these drugs can be extruded out of the cell by IM transporters, which exist as either single-component pumps (CraA) or multicomponent pumps (AdeABC). While the singlet pumps may take up the drug from the cytosol and the periplasm and function with porins or other types of protein channels to make the efflux process effective. The multicomponent exporters capture their substrates from the periplasm and the IM and directly pump them into the medium. The competition between the influx and efflux processes ultimately determines the steady state of drug molecules in bacterial cells. This figure is adapted from (*130*).

1.4.5.1. Multiple-component efflux systems

In MDR Gram-negative bacteria, some RND efflux pumps confer resistance to a wide spectrum of antimicrobials at clinically significant levels. These pumps can be constitutively highly expressed

and the deletion mutants can cause the host to become much more susceptible to multiple antibacterials (*148-152*). As seen in the most intensively studied RND prototype AcrB in *E. coli*, these pumps have very large periplasmic homo-trimeric domains and form tripartite complexes with outer membrane channels (*E. coli* prototype TolC) and membrane fusion proteins (*E. coli* prototype AcrA) (Figure 1.2) (*153*). AcrAB-TolC homologs and other RND drug efflux pumps are normally encoded in the core genome, and are the major intrinsic MDR efflux systems in Gram-negative organisms. However, in an opportunistic human pathogen *Citrobacter freundii*, a RND pump was found to be carried on a IncHI1 plasmid, which indicates that this system may be transmissible among bacteria (*154*).

As shown by the related studies of *E. coli* AcrB, RND transporters are proposed to capture substrates from both cytoplasmic membrane and periplasmic spaces, and then pump them out to the extracellular medium through a TolC channel. Co-crystallization of *E. coli* AcrB in complex with drug substrates and crystallization of other singlet efflux pumps (see below) revealed large hydrophobic substrate-binding pockets within their transmembrane domain, which can accommodate substrates with different sizes and properties (*155-159*). A common property of the substrates is that they all have hydrophobic moieties. These observations might explain the broad substrate specificities of MDR efflux systems. The hypothesis is that the hydrophobic binding pockets could capture and bind the compounds through hydrophobic interactions and electrostatic attraction, in contrast to classical stringent enzyme-ligand binding (*160*). Studies of efflux substrate competition (*161, 162*) and site-directed mutagenesis within the binding pockets of AcrB (*163, 164*) have also shown that some amino acid residues in the pocket are critical for substrate binding and efflux, emphasizing the importance of hydrophobic substrate binding pockets in an active drug efflux pump.

The outer membrane factor (OMF) TolC is essential for a functional AcrB efflux pump, as the drug resistance levels of *tolC* deletion mutants (even in the presence of AcrB) were substantially lower

than the wild type strains, and even lower than an *acrB* mono-mutant (*152, 165*). However, TolC also interacts with other RND pumps and transporters from the MFS and ABC superfamilies to form tripartite efflux systems. For instance, the ABC transport system MacAB-TolC confers macrolide resistance (*166*), and MFS system EmrAB-TolC confers resistance to some macrolides, josamycin and tylosin (*167, 168*). A TolC crystal structure has been resolved at 2.1 Å resolution, which showed that it is a trimeric 'channel-tunnel' over 140 Å long that spans both the outer membrane and periplasmic space (*169*). Recently, a structure of the complete tripartite system of *E. coli* AcrAB-TolC has been resolved at 16 Å, suggesting that the stoichiometry of the pump (AcrB/AcrA/TolC) is 3/6/3 (*153*).

The membrane fusion protein (MFP) AcrA is a lipoprotein located in the periplasmic space. It is a crucial component for AcrAB-TolC drug-export function, and contributes to the *E. coli* MDR phenotype (*170, 171*). The cross-membrane substrate efflux process between AcrB and TolC is coordinated via AcrA. The crystal structure showed that AcrA composes three domains, β-barrel, lipoyl and α-helical hairpin (*172*). The β-barrel domain of AcrA connects to AcrB (*173*). The conformational flexibility of the α-helical hairpin of AcrA was shown to be required for the coupling between AcrA conformation changes with the opened or closed TolC channel (*172, 173*). Like outer membrane factors, membrane fusion proteins are not only required for RND systems, they can also couple with other efflux pumps such as ABC and MFS transporters to export substrates across two membranes (*174, 175*). However, each inner-membrane efflux pump has a specific cognate MFP.

1.4.5.2. Other efflux systems

Other efflux pumps are expressed in the IM as single component pumps, including transporters from the ABC, MFS, SMR, MATE and PACE families. In theory the majority of these pumps can only transport drugs from the cytosol, the membrane or the inner-leaflet of the IM to the periplasm, as they do not form complexes with AcrA and TolC-like proteins known to facilitate expulsion of their substrates across the OM (Figure 1.2). This is probably why very few of these efflux systems were

found to be the predominant efflux pumps in Gram-negative bacterial drug resistance. However, the first identified drug efflux protein was a single-component MFS pump TetA, which confers significant levels of tetracycline resistance (*176*). TetA can be encoded on plasmids or on transposons in the chromosome of Gram-positive and Gram-negative organisms. Another well characterized MFS transporter is MdfA from *E. coli*. Although this pump has been shown to confer resistance to a broad spectrum of substrates when overexpressed, the clinical relevance of this pump is still unknown (*177*).

Evidence for synergistic transport between single component inner-membrane efflux pumps and tripartite systems has been generated by studying the major *E. coli* multidrug efflux pumps. Single deletions of *mdfA* or *emrE* in *E. coli* did not significantly increase host sensitivity to drugs. However double deletions of these pumps resulted in the host being even more sensitive to cationic agents than an *acrB* mutant (*178*). These findings, along with the fact that AcrB and single-component pumps have overlapping substrate specificities, support the contention of the two-stage antibacterial removing strategy: 1) singlet efflux pumps in the IM remove cytosolic compounds to the periplasm, and 2) tripartite pumps such as AcrAB-TolC then expel the compounds from periplasmic space to the extracellular environment (*178*).

1.4.5.3. Drug efflux systems and their transcriptional regulations in *A. baumannii*

Increased expression of chromosomal or plasmid-borne genes encoding efflux pumps plays a significant role in multidrug resistance of clinical MDR *A. baumannii* isolates. Three RND, seven MFS, three MATE, two SMR, one PACE and one ABC proteins have been implicated in drug efflux in *A. baumannii* (Table 1.3), among which the three RND efflux systems are the major drug efflux pumps conferring clinically significant levels of multiple antibacterial resistance (*179*).

Table 1.3. Identified drug efflux systems in *A. baumannii*

Efflux system	Drug substrates	Transcriptional regulators [b]	Physiological functions [b]	Genetic location [c]	References
RND family					
AdeABC [a]	Aminoglycosides, fluoroquinolones, ß-lactams, macrolides, tetracyclines, tigecycline chloramphenicol, triclosan, chlorhexidine	AdeRS, IS*Aba1*, BaeSR	Biofilm formation, plasmid transmission	Core	(*179-182*)
AdeIJK [a]	Fluoroquinolones, ß-lactams, macrolides, tetracyclines, tigecycline chloramphenicol, triclosan, fusidic acid, trimethoprim, sulfadoxine	AdeN	Biofilm formation, plasmid transmission	Core	(*179, 182, 183*)
AdeFGH [a]	Sulfamethoxazole, chloramphenicol, trimethoprim, clindamycin, fluoroquinolones, tetracycline-tigecycline	AdeL	Biofilm formation	Core	(*179, 184*)
MFS family					
AmvA	Amikacin, ciprofloxacin, erythromycin, norfloxacin, novobiocin, benzalkonium chloride, chlorhexidine,	AmvR	NI	Core	(*185, 186*)
CraA [a]	Chloramphenicol	NI	NI	Core	(*187*)
AedC	Chloramphenicol, tetracycline	NI	NI	Core	(*188*)
CmlA	Chloramphenicol	NI	NI	AbaR1	(*27, 189*)
FloR	Florfenicol/chloramphenicol	NI	NI	AbaR1	(*27, 190*)
TetA/B [a]	Tetracyclines	TetR	NI	Plasmid, AbaR1, transposon	(*27, 191, 192*)
MATE family					
AbeM	Kanamycin, gentamicin, norfloxacin, ofloxacin, ciprofloxacin, erythromycin, triclosan, trimethoprim	NI	NI	Core	(*193*)
AbeM2	Ciprofloxacin	NI	NI	Core	(*194*)
AbeM4	Ciprofloxacin	NI	NI	Core	(*194*)
SMR family					
AbeS	Chloramphenicol, amikacin, ciprofloxacin, erythromycin, norfloxacin, novobiocin, benzalkonium chloride, chlorhexidine	NI	NI	Core	(*195*)
QacE	Quaternary ammonium	NI	NI	AbaR1, integron	(*27, 182, 196*)
PACE family					
AceI	Chlorhexidine	AceR	NI	Core	(*132, 139*)
ABC family					
MsI	Macrolides	NI	NI	Plasmid	(*197*)

a. Efflux pumps confer clinical significant resistance
b. NI means "not identified"
c. Core means "core genome of *A. baumannii*

1.4.5.3.1 RND drug efflux systems in *A. baumannii*

The three major RND efflux systems in MDR *A. baumannii* isolates include AdeABC, AdeFGH and AdeIJK. AdeA, AdeF and AdeI are the membrane fusion proteins, AdeB, AdeG and AdeJ are the RND efflux pumps, and AdeC, AdeH and AdeK are the outer membrane factors of these respective tripartite efflux pumps. AdeABC and AdeIJK share similar substrates, including most ß-lactams and aminoglycosides, fluoroquinolones, tetracyclines, tigecycline, macrolides-lincosamides, chloramphenicol and several biocides such as triclosan and chlorhexidine (*179*). The constitutive overexpression of *adeABC* was found to be correlated with the MDR phenotypes of some clinical *A. baumannii* isolates (*198*). Correspondingly, the deletion mutants of *adeA* and *adeB* were also shown to have decreased resistance levels to the same antibacterials (*151*). In contrast, deletion of *adeC* didn't change the host drug resistance levels as much as deletion of *adeA* or *adeB* (*181*). The genomes of quite a few *A. baumannii* MDR strains encode *adeAB* but without *adeC* (*199*). Altogether this suggested that AdeC might not be essential for the drug efflux function of AdeAB, and there might be alternative outer membrane factor(s) that could facilitate AdeAB drug transportation. Although *adeABC* presents in both MDR and drug susceptible strains, the expression levels in the susceptible strains are much lower than in the MDR strains (*181*). No drug resistance phenotype change was observed for an *adeB* deletion mutant in a drug-susceptible strain (*200*). These observations suggest that basal expression level of *adeB* is not mediating observable drug resistance levels, and its transcription is tightly controlled in drug sensitive *A. baumannii*.

A two-component transcriptional regulation system AdeRS that is encoded divergently from and immediately up-stream of *adeABC*, plays a key role in activating the overexpression of this efflux system (*181*). AdeS is a sensor kinase and AdeR is the cognate response regulator. The constitutive overexpression of AdeABC in the MDR *A. baumannii* strains has been shown to be associated with several specific amino acid mutations on the N-terminal of AdeR, which are speculated to enhance the C-terminal DNA binding affinity, even though without phosphorylation (*181, 201*). AdeABC

constitutive overexpression was also observed in the MDR strains that have mutations in *adeS* (*147, 198, 202, 203*). These specific mutations were not found in strains that do not overexpress AdeABC (*198*). A second two-component transcriptional system BaeSR was also found to serve as an activator of *adeAB* expression, and a *baeR* knockout mutant resulted in increased tigecycline susceptibility (*180*).

Expression of *adeAB* is induced by various drugs including chlorhexidine (*132*). The *adeAB* system may play important physiological roles in the cell outside of drug resistance. For example, *A. baumannii adeRS* and *adeB* deletion mutants are both impaired for biofilm formation on both abiotic surfaces and in a porcine mucosal model (*204*). Furthermore, mutants constitutively overexpressing AdeAB show decreased expression of biofilm-related proteins (*179*).

AdeIJK is highly expressed in both drug sensitive and resistant *A. baumannii* isolates, and expression can be further increased by treatment with biocides such as benzalkonium or chlorhexidine (*182, 198*). A TetR-type regulator AdeN has been identified as a transcriptional repressor for *adeIJK* (*183*), however it is still unclear whether the overexpression induced by the biocide is associated with AdeN regulation.

A third *A. baumannii* RND efflux system is encoded by *adeFGH,* and spontaneous mutants which overexpress this system can confer increased antimicrobial resistance (*179, 184*). However, clinical strains that constitutively overexpress this pump have not been frequently reported (*184*). A divergently transcribed LysR-type transcriptional regulator is encoded by *adeL*, and mutations in this gene have been shown to be correlated with *adeFGH* overexpression (*184*).

1.4.5.3.2 Other drug efflux systems in *A. baumannii*

Most known or putative drug efflux pumps identified in *A. baumannii* belong to the MFS family. For example, *craA* (*187*) and *tetA* or *tetB* (*191*) confer clinically-significant drug resistance to chloramphenicol and tetracyclines, respectively. CraA is conserved in all *A. baumannii* genomes

published so far, and the expression is inducible by chloramphenicol (Penesyan, Hassan, Paulsen, unpublished data), indicating it is an adaptive chloramphenicol resistance determinant in addition to AdeIJK. *tetA*, a plasmid borne efflux gene first identified in *E. coli*, now has been detected on MGEs such as transposons from various Gram-positive and negative bacteria (*205*). Many tetracycline resistant *A. baumannii* MDR clinical strains, were found to carry *tetA/B* genes on Tn5- or Tn10-like transposons, plasmids or RI (such as AbaR1). The *tetA* or *tetB* genes are typically encoded adjacent to *tetR* encoding a transcriptional repressor (*27*). Tetracycline drugs such as tetracycline and minocycline can bind to TetR to induce TetR dissociation from the promoter region of *tetA/B* genes, resulting in increased expression of the pumps and consequently increased tetracycline resistance (*206*). Two other chloramphenicol efflux genes *cmlA* and *floR* were identified on the AbaR1 island (*27*). Although not phenotypically characterized in *A. baumannii*, like *tetA*, they are circulating among different bacterial species (*189, 190*). AmvA is a conserved chromosomally encoded pump, and a deletion mutant showed marginally reduced resistance to several antimicrobials (Table 1.3) (*185*). When heterologously expressed in *E. coli*, another *A. baumannii* conserved MFS pump AedC increased host resistance levels to chloramphenicol and tetracycline (*188*).

Several chromosomally encoded drug efflux pumps belonging to the MATE, SMR and PACE families, also contribute to the MDR phenotypes of *A. baumannii* (Table 1.3). Apart from the MATE family protein AbeM (*193*) and the SMR family protein AbeS (*195*) that confer multidrug resistance, the rest of these pumps have only been described to export a single drug substrate, e.g., chlorhexidine is a substrate for the PACE family protein AceI (*132, 207*), and the MATE family members AbeM2 and AbeM4 confer resistance to ciprofloxacin (*188*). The remaining two drug transporters identified in this organism, namely ABC macrolide exporter Mel (*197*) and SMR quaternary ammonium transporter QacEΔ1 (*196*), are transferable, and carried on a plasmid and AbaR1 respectively. As shown in Table 1.3, one drug can be the substrate of multiple efflux pumps. For instance, AdeABC, AdeIJK, AdeFGH, CraA, AedC, CmlA, FloR and AbeS all confer resistance to chloramphenicol, suggesting that they may work synergistically in a two-stage chloramphenicol removal strategy as

introduced previously (Figure 1.2).

1.5. Scope of Thesis

The work described in this thesis corporated three novel high throughput technologies for functional characterization of putative drug efflux systems, and discovery of novel resistance determinants in *A. baumannii* against tigecycline and ten structurally different biocides. Drug efflux systems play a significant role in *A. baumannii* MDR. Tigecycline is one of the last resorts for the treatment of *A. baumannii* infections, and worryingly clinical resistant isolates have been increasingly reported. The ten biocides are either listed as essential medicine by the WHO or are commonly used in hospital for infection control and in household hygiene products. The indiscriminate use of biocides in both hospital and household areas has raised a concern that residual biocides in these environments might contribute to pathogenic bacteria MDR development.

Although seventeen drug efflux systems have been functionally characterized in this organism, numerous genes on *A. baumannii* genomes are annotated as putative efflux pumps, which are not functionally characterized yet. To investigate whether these putative drug efflux pumps have any role in drug resistance, a novel approach was developed that combined Biolog Phenotype Microarrays (*208*) and quantitative PCR to screen multiple *A. baumannii* putative efflux pumps heterologously expressed in *E. coli* against 240 compounds simultaneously (Figure 1.3A).

Tigecycline targets bacterial 16S rRNA which results in protein translation perturbation. RNA sequencing (RNA-Seq) (*209*) was used for a global scale identification of potential novel tigecycline resistance determinants, and for exploration of the global physiological response of *A. baumannii* upon tigecycline treatment (Figure 1.3B). To complement the transcriptomic analysis, a saturated transposon mutant library of a MDR *A. baumannii* clinical strain was screened against tigecycline using Transposon Directed Insertion-site Sequencing (TraDIS). TraDIS is a novel high throughput sequencing technology, which can detect dynamic population changes within a saturated transposon mutant library upon specific environmental stimuli challenge (Figure 1.3C) (*210*).

Despite their importance in clinical infection control, little is known about the mode of action of biocides or biocide resistance mechanisms in bacteria. TraDIS was used to screen an *A. baumannii* transposon mutant library against ten structurally different biocides (Figure 1.3C), with the aims to identify important factors in biocide resistance and the cellular pathways that are targeted by the biocides.

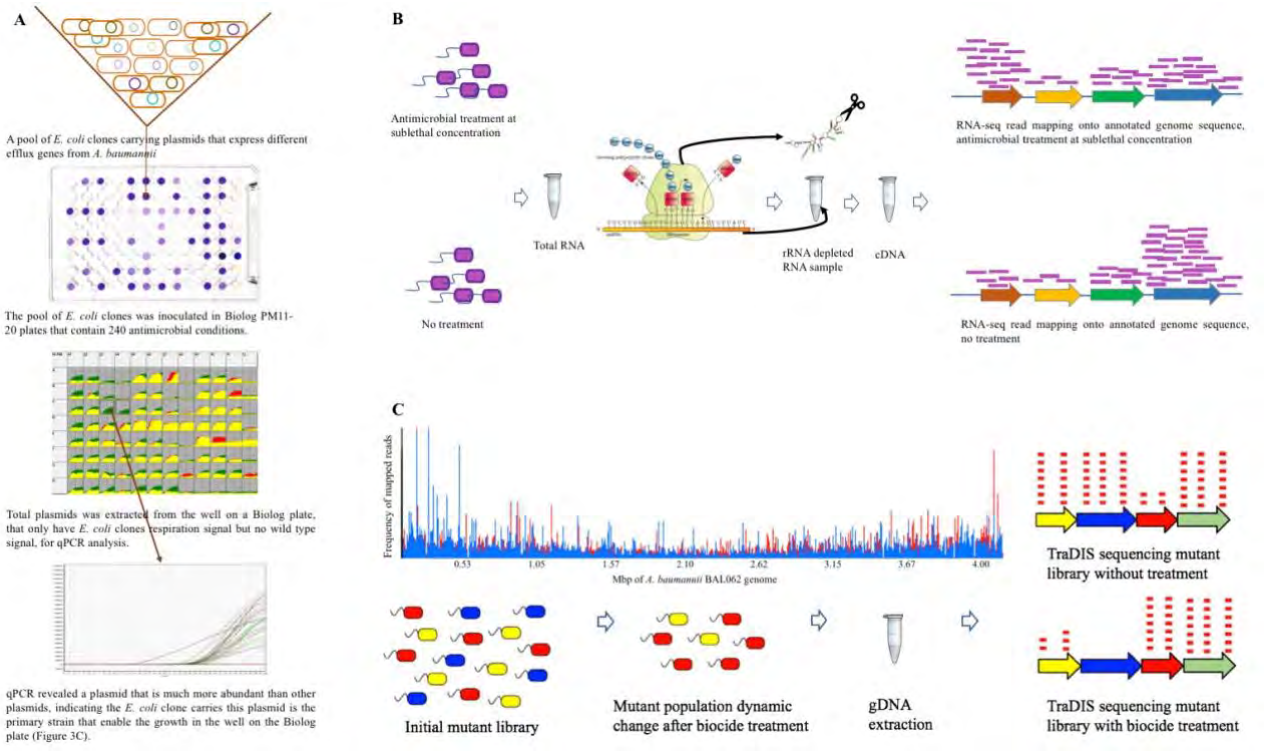

Figure 1.3. Schematic illustration of the three high throughput technologies used in this thesis. **A)** Biolog phenotype microarray and qPCR for high throughput phenotype identification of putative bacterial drug efflux pump (Chapter 2). Multiple *E. coli* clones carrying plasmids that express different heterologous putative drug efflux genes are pooled together. The mixed cell culture is then inoculated into each well on Biolog PM11-20 plates. Cells that can survive antimicrobial treatment will have active respiration, which will reduce and transform tetrazolium dye in Biolog plate from colorless to purple. The kinetic response curves is an example of the respiration results of an overlay of multiplexed *E. coli clones* and wild type control of same Biolog PM plate. Green and red curves represent cell respiration signals for multiplexed E. coli strains and control respectively. Yellow is

overlap signal. *E. coli* clone mixture has stronger respiration signal (green) than the wild type strain are extracted for qPCR analysis, to determine the clone that is responsible for the growth difference. **B)** RNA-seq for deciphering bacterial transcriptomic response to environmental stimuli. mRNA is the target of interest in our tigecycline transcriptomics study (chapter 5). After total RNA extraction from bacterial samples, ribosomal RNAs (rRNA), the major RNA species in a cell, are removed, and the remaining RNA samples are reverse-transcribed to complementary DNA (cDNA) for sequencing. Reads from DNA sequencing are then mapped to the respective annotated genome sequence. mRNA read differences between cells grown with environmental stimuli and control are informative for deciphering how the environmental stimuli affect gene expression in majority of the bacterial population. **C)** Direct transposon insertion site sequencing (TraDIS) for simultaneous assessment of the contribution of each redundant gene to host fitness in a specific environment. For example, after antimicrobial treatment, the population dynamic of a saturated transposon mutant library (in chapter 6, our *A. baumannii* library contains more than 100,000 unique individual mutants, with an average of one insertion every 38 bp) will change. The mutants that have higher fitness level in the antimicrobial supplemented media will grow better than the ones less fit. TraDIS uses an outward-facing transposon specific primer to sequence the mutant genomic DNA adjacent to transposon, which is to determine transposon insertion site (equivalent to the barcode of an individual mutant) and can also determine the copy numbers of each mutant in the bacterial population. Transposon insertion read differences between mutant libraries before and after antimicrobial treatment will provide insights of the cellular pathways that are affected by the compound and the resistance mechanisms.

1.6. References

1. M. L. Cohen, Changing patterns of infectious disease. *Nature* **406**, 762-767 (2000).
2. WHO, *The selection and use of essential medicines: report of the 19th WHO Expert Committee*. (World Health Organization, Geneva, Switzerland, 2013).
3. C. G. Giske, D. L. Monnet, O. Cars, Y. Carmeli, R. ReAct-Action on Antibiotic, Clinical and economic impact of common multidrug-resistant gram-negative bacilli. *Antimicrob Agents Chemother* **52**, 813-821 (2008).
4. S. Baker, Infectious disease. A return to the pre-antimicrobial era? *Science* **347**, 1064-1066 (2015).
5. L. B. Rice, Federal funding for the study of antimicrobial resistance in nosocomial pathogens: No ESKAPE. *Journal of Infectious Diseases* **197**, 1079-1081 (2008).
6. H. W. Boucher *et al.*, Bad bugs, no drugs: no ESKAPE! An update from the infectious diseases society of America. *Clin. Infect. Dis.* **48**, 1-12 (2009).
7. G. D. Wright, Antibiotics: A New Hope. *Chemistry & Biology* **19**, 3-10 (2012).
8. A. Y. Peleg, H. Seifert, D. L. Paterson, *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin. Microbial. Rev.* **21**, 538-582 (2008).
9. J. P. Euzeby, List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet. *Int J Syst Bacteriol* **47**, 590-592 (1997).
10. E. L. Carr, P. Kämpfer, B. K. Patel, V. Gurtler, R. J. Seviour, Seven novel species of *Acinetobacter* isolated from activated sludge. *Int J Syst Evol Microbiol* **53**, 953-963 (2003).
11. I. Vaz-Moreira *et al.*, *Acinetobacter rudis* sp. nov., isolated from raw milk and raw wastewater. *Int. J. Syst. Evol. Microbiol.* **61**, 2837-2843 (2011).
12. Y. S. Kang, J. Jung, C. O. Jeon, W. Park, *Acinetobacter* oleivorans sp. nov. is capable of adhering to and growing on diesel-oil. *J Microbiol* **49**, 29-34 (2011).
13. M. Lee, S. G. Woo, L. N. Ten, Characterization of novel diesel-degrading strains *Acinetobacter haemolyticus* MJ01 and Acinetobacter johnsonii MJ4 isolated from oil-contaminated soil. *World J Microbiol Biotechnol* **28**, 2057-2067 (2012).
14. A. Nemec *et al.*, Acinetobacter bereziniae sp. nov. and *Acinetobacter guillouiae* sp. nov., to accommodate *Acinetobacter* genomic species 10 and 11, respectively. *Int J Syst Evol Microbiol* **60**, 896-903 (2010).
15. A. Nemec *et al.*, *Acinetobacter seifertii* sp. nov., a member of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex isolated from human clinical specimens. *Int J Syst Evol Microbiol* **65**, 934-942 (2015).
16. N. G. Regalado, G. Martin, S. J. Antony, *Acinetobacter lwoffii*: bacteremia associated with acute gastroenteritis. *Travel Med Infect Dis* **7**, 316-317 (2009).
17. T. Gaillard *et al.*, *Acinetobacter parvus* bacteraemia community-acquired. *Int J Med Microbiol* **302**, 327-329 (2012).
18. P. Visca, H. Seifert, K. J. Towner, *Acinetobacter* infection--an emerging threat to human health. *IUBMB Life* **63**, 1048-1054 (2011).
19. J. A. X. Corbella *et al.*, Epidemiological significance of cutaneous, pharyngeal, and digestive tract colonization by multiresistant *Acinetobacter baumannii* in ICU patients. *J. Hosp. Infect.* **37**, 287-295 (1997).
20. H. Wisplinghoff, R. Schmitt, A. Wöhrmann, D. Stefanik, H. Seifert, Resistance to disinfectants in epidemiologically defined clinical isolates of *Acinetobacter baumannii*. *J Hosp Infect* **66**, 174-181 (2007).
21. A. Kramer, I. Schwebke, G. Kampf, How long do nosocomial pathogens persist on inanimate surface? A systematic review. *BMC Infect. Dis.* **6**, 130 (2006).
22. N. M. Anstey *et al.*, Community-acquired bactermic *Acinetobacter* pneumonia in tropical Australia is caused by diverse strains of *Acinetobacter baumannii*, with carriage in the throat in at-risk groups. *J. Clin. Microbioal.* **40**, 685-686 (2002).
23. E. T. S. Houang *et al.*, Epidemiology and infection control implications of *Acinetobacter spp*. in Hong Kong. *J. Clin. Microbioal.* **39**, 228-234 (2001).
24. C. W. M. Ong *et al.*, Severe community-acquired *Acinetobacter baumannii* pneumonia: an emerging highly lethal infectious disease in the Asia-Pacific. *Respirology* **14**, 1200-1205 (2009).
25. M. Eveillard, M. Kempf, O. Belmonte, H. Pailhoriès, M. L. Joly-Guillou, Reservoirs of *Acinetobacter baumannii* outside the hospital and potential involvement in emerging human community-acquired infections. *Int J Infect Dis* **17**, e802-805 (2013).

26. D. N. Farrugia *et al.*, The complete genome and phenome of a community-acquired *Acinetobacter baumannii*. *PLoS One* **8**, e58628 (2013).
27. P. E. Fournier *et al.*, Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genet.* **2**, 0062-0072 (2006).
28. D. Landman *et al.*, Citywide clonal outbreak of multoresistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in Brooklyn, NY. *Arch. Intern. Med.* **162**, 1515-1520 (2002).
29. P. Scott *et al.*, An outbreak of multidrug-resistant *Acinetobacter baumannii-calcoaceticus* complex infection in the US military health care system associated with military operations in Iraq. *Clin Infect Dis* **44**, 1577-1584 (2007).
30. A. Y. Peleg, C. Franklin, J. M. Bell, D. W. Spelman, Emergence of carbapenem resistance in *Acinetobacter baumannii* recovered from blood cultures in Australia. *Infect Control Hosp Epidemiol* **27**, 759-761 (2006).
31. C. L. Jones *et al.*, Fatal outbreak of an emerging clone of extensively drug-resistant *Acinetobacter baumannii* with enhanced virulence. *Clin Infect Dis* **61**, 145-154 (2015).
32. J. Fishbain, A. Y. Peleg, Treatment of Acinetobacter infections. *Clin. Infect. Dis.* **51**, 79-84 (2010).
33. R. M. Mera, L. A. Miller, H. Amrine-Madsen, D. F. Sahm, *Acinetobacter baumannii* 2002-2008: increase of carbapenem-associated multiclass resistance in the United States. *Microb Drug Resist* **16**, 209-215 (2010).
34. Z. A. Qureshi *et al.*, Colistin-resistant *Acinetobacter baumannii*: beyond carbapenem resistance. *Clin Infect Dis* **60**, 1295-1303 (2015).
35. S. Navon-Venezia, A. Leavitt, Y. Carmeli, High tigecycline resistance in multidrug-resistant *Acinetobacter baumannii*. *J Antimicrob Chemother* **59**, 772-774 (2007).
36. M. A. Kohanski, D. J. Dwyer, J. J. Collins, How antibiotics kill bacteria: from targets to networks. *Nat. Rev. Microbiol.* **8**, 423-435 (2010).
37. T. D. H. Bugg, C. T. Walsh, Intracellular steps of bacterial cell wall peptidoglycan biosynthesis: enzymology, antibiotics, and antibiotic resistance. *Nat. Prod. Rep.* **9**, 199-215 (1992).
38. D. Kahne, C. Leimkuhler, W. Lu, C. Walsh, Glycopeptide and lipoglycopeptide antibiotics. *Chem Rev* **105**, 425-448 (2005).
39. A. Tomasz, A. Albino, E. Zanati, Multiple antibiotic resistance in a bacterium with suppressed autolytic system. *Nature* **227**, 138-140 (1970).
40. L. Katz, G. W. Ashley, Translation and protein synthesis: macrolides. *Chem. Rev.* **105**, 499-527 (2005).
41. I. Chopra, M. Roberts, Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology bacterial resisance. *Microbiol Mol Biol Rev* **65**, 232-260 (2001).
42. D. N. Wilson, The A-Z of bacterial translation inhibitors. *Crit Rev Biochem Mol Biol* **44**, 393-433 (2009).
43. J. Lin, D. Zhou, T. A. Steitz, Y. S. Polikanov, M. G. Gagnon, Ribosome-targeting antibiotics: modes of Action, mechanisms of resistance, and implications for drug design. *Annu Rev Biochem* **87**, 451-478 (2018).
44. B. D. Davis, Mechanism of bactericidal action of aminoglycosides. *Microbiol Rev* **51**, 341-350 (1987).
45. A. Sugino, C. L. Peebles, K. N. Kreuzer, N. R. Cozzarelli, Mechanism of action of nalidixic acid: purification of *Escherichia coli* nalA gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme. *Proc Natl Acad Sci U S A* **74**, 4767-4771 (1977).
46. J. Courcelle, P. C. Hanawalt, RecA-dependent recovery of arrested DNA replication forks. *Annu Rev Genet* **37**, 611-646 (2003).
47. J. W. Beaber, B. Hochhut, M. K. Waldor, SOS response promotes horizontal dissemination of antibiotic resistance genes. *Nature* **427**, 72-74 (2004).
48. I. Matic, C. Rayssiguier, M. Radman, Interspecies gene exchange in bacteria: the role of SOS and mismatch repair systems in evolution of species. *Cell* **80**, 507-515 (1995).
49. R. T. Cirz *et al.*, Inhibition of mutation and combating the evolution of antibiotic resistance. *PLoS Biol* **3**, e176 (2005).
50. M. A. Kohanski, D. J. Dwyer, B. Hayete, C. A. Lawrence, J. J. Collins, A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* **130**, 797-810 (2007).
51. C. Miller *et al.*, SOS response induction by beta-lactams and bacterial defense against antibiotic lethality. *Science* **305**, 1629-1631 (2004).
52. J. P. Gogarten, J. P. Townsend, Horizontal gene transfer, genome innovation and evolution. *Nat Rev Microbiol* **3**, 679-687 (2005).

53. L. Fernández, E. B. Breidenstein, R. E. Hancock, Creeping baselines and adaptive resistance to antibiotics. *Drug Resist Updat* **14**, 1-21 (2011).
54. L. S. Frost, R. Leplae, A. O. Summers, A. Toussaint, Mobile genetic elements: the agents of open source evolution. *Nat Rev Microbiol* **3**, 722-732 (2005).
55. M. S. Wright *et al.*, New insights into dissemination and variation of the health care-associated pathogen *Acinetobacter baumannii* from genomic analysis. *mBio* **5**, e00963-00913 (2014).
56. V. Post, P. A. White, R. M. Hall, Evolution of AbaR-type genomic resistance islands in multiply antibiotic-resistance *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* **65**, 1162-1170 (2010).
57. P. D. Mugnier, L. Poirel, P. Nordmann, Functional analysis of insertion sequence ISAba1, responsible for genomic plasticity of *Acinetobacter baumannii*. *J Bacteriol* **191**, 2414-2418 (2009).
58. L. Diancourt, V. Passet, A. Nemec, L. Dijkshoorn, S. Brisse, The population structure of *Acinetobacter baumannii*: expanding multiresistant clones from an ancestral susceptible genetic pool. *PLoS ONE* **5**, e10034 (2010).
59. H. van Dessel *et al.*, Identification of a new geographically widespread multiresistant *Acinetobacter baumannii* clone from European hospitals. *Res Microbiol* **155**, 105-112 (2004).
60. E. S. Snitkin *et al.*, Genome-wide recombination drives diversification of epidemic strains of *Acinetobacter baumannii*. *Proc Natl Acad Sci U S A* **108**, 13758-13763 (2011).
61. S. Corvec *et al.*, AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. *J Antimicrob Chemother* **52**, 629-635 (2003).
62. L. Poirel, R. A. Bonnin, P. Nordmann, Genetic basis of antibiotic resistance in pathogenic *Acinetobacter baumannii*. *IUBMB Life* **63**, 1061-1067 (2011).
63. P. D. Mugnier, L. Poirel, T. Naas, P. Nordmann, Worldwide dissemination of the *blaOXA-23* carbapenemase gene of *Acinetobacter baumannii*. *Emerg Infect Dis* **16**, 35-40 (2010).
64. I. E. Robledo *et al.*, Detection of KPC in *Acinetobacter spp*. in Puerto Rico. *Antimirob. Agents Chemother.* **54**, 1354-1357 (2010).
65. R. A. Bonnin *et al.*, Carbapenem-hydrolyzing GES-type extended-spectrum beta-lactamase in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **55**, 349-354 (2011).
66. L. Poirel, P. Nordmann, Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clin Microbiol Infect* **12**, 826-836 (2006).
67. M. L. Riccio *et al.*, Characterization of the metallo-beta-lactamase determinant of *Acinetobacter baumannii* AC-54/97 reveals the existence of blaIMP allelic variants carried by gene cassettes of different phylogeny. *Antimirob. Agents Chemother.* **44**, 1229-1235 (2000).
68. R. A. Bonnin, T. Naas, L. Poirel, P. Nordmann, Phenotypic, biochemical, and molecular techiques for detection of metallo-beta-lactamase NDM in *Acinetobacter baumannii*. *J. Clin. Microbiol.* **50**, 1419-1421 (2012).
69. G. J. D. Silva *et al.*, Molecular characterization of *blaIMP-5*, a new integron-borne metallo-beta-lactamase gene from an *Acinetobacter baumannii* nosocomial isolate in Portugal. *FEMS Microbiol. Lett.* **215**, 33-39 (2002).
70. R. Cayâ *et al.*, Identification of a new integron harboring *blaIMP-10* in carbapenem-resistant *Acinetobacter baumannii* clinical isolates. *Antimirob. Agents Chemother.* **59**, 3687-3689 (2015).
71. K. Karthikeyan, M. A. Thirunarayan, P. Krishnan, Coexistence of *blaOXA-23* with *blaNDM-1* and *armA* in clinical isolates of *Acinetobacter baumannii* from India. *J Antimicrob Chemother* **65**, 2253-2254 (2010).
72. M. Kaase *et al.*, NDM-2 carbapenemase in *Acinetobacter baumannii* from Egypt. *J Antimicrob Chemother* **66**, 1260-1262 (2011).
73. A. Tsakris *et al.*, VIM-1 metalo-beta-lactamase in *Acinetobacter baumannii*. *Emerg. Infect. Dis.* **12**, 981-983 (2006).
74. K. Lee *et al.*, Novel acquired metallo-beta-lactamase gene, *blaSMI-1*, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. *Antimirob. Agents Chemother.* **49**, 4485-4491 (2005).
75. G. Bou, A. Oliver, J. Martínez-Beltrán, OXA-24, a novel class D beta-lactamase with carbapenemase activity in an *Acinetobacter baumannii* clinical strain. *Antimicrob Agents Chemother* **44**, 1556-1561 (2000).
76. M. Afzal-Shan, N. Woodford, D. M. Livermore, Characterization of OXA-25, OXA-26, and OXA-27, molecular class D beta-lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii*. *Antimirob. Agents Chemother.* **45**, 583-588 (2001).

77. C. Héritier, A. Dubouix, L. Poirel, N. Marty, P. Nordmann, A nosocomial outbreak of *Acinetobacter baumannii* isolates expressing the carbapenem-hydrolysing oxacillinase OXA-58. *J Antimicrob Chemother* **55**, 115-118 (2005).
78. L. Poirel, W. Mansour, O. Bouallegue, P. Nordmann, Carbapenem-resistant *Acinetobacter baumannii* isolates from Tunisia producing the OXA-58-like carbapenem-hydrolyzing oxacillinase OXA-97. *Antimicrob Agents Chemother* **52**, 1613-1617 (2008).
79. L. P. Kotra, J. Haddad, S. Mobashery, Aminoglycosides: perspective on mechanisms of action and resistance and strategies to counter resistance. *Antimicrob. Agents Chemother.* **44**, 3249-3256 (2000).
80. M. S. Ramirez, M. E. Tolmasky, Aminoglycoside modifying enzymes. *Drug Resist. Updat.* **13**, 151-171 (2010).
81. F. H. Cameron, D. J. G. Obbink, V. P. Ackerman, R. M. Hall, Nucleotide sequence of the AAD(2") aminoglycoside adenylyltransferase determinant *aadB*. Evolutionary relationship of this region with those surrounding *aadA* in R538-1 and *dhfrII* in R388. *Nucleic Acids Res.* **14**, 8625-8635 (1986).
82. S. J. Nigro, V. Post, R. M. Hall, Aminoglycoside resistance in multiply antibiotic-resistant *Acinetobacter baumannii* belonging to global clone 2 from Australia hospitals. *J. Antimicrob. Chemother.* **66**, 1504-1509 (2011).
83. A. Nemec, L. Dolzani, S. Brisse, P. Broek, L. Dijkshoorn, Diversity of aminoglycoside-resistance genes and their association with class 1 integrons among strains of pan-European *Acinetobacter baumannii* clones. *J. Med. Microbiol.* **53**, 1233-1240 (2004).
84. S. J. Nigro, R. M. Hall, Amikacin resistance plasmids in extensively antibiotic-resistant GC2 *Acinetobacter baumannii* from two Australian hospitals. *J Antimicrob Chemother* **69**, 3435-3437 (2014).
85. N. Petrosillo *et al.*, Combined colistin and rifampicin therapy for carbapenem-resistant *Acinetobacter baumannii* infections: clinical outcome and adverse events. *Clin Microbiol Infect* **11**, 682-683 (2005).
86. E. A. Campbell *et al.*, Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell* **104**, 901-912 (2001).
87. J. Baysarowich] *et al.*, Rifamycin antibiotic resistance by ADP-ribosylation: structure and diversity of Arr. *Proc. Natl. Acad. Sci.* **105**, 4886-4891 (2007).
88. C. Tribuddharat, M. Fennewald, Integron mediated rifampin resistance in *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **43**, 960-962 (1999).
89. F. Schlünzen *et al.*, Structural basis for the interaction of antibiotics with the peptidyl transferase centre in eubacteria. *Nature* **413**, 814-821 (2001).
90. D. Moazed, H. F. Noller, Chloramphenicol, erythromycin, carbomycin and vernamycin B protect overlapping sites in the peptidyl transferase region of 23S ribosomal RNA. *Biochimie* **69**, 879-884 (1987).
91. C. Rodriguez-Fonseca, R. Amils, R. A. Garrett, Fine structure of the peptidyl transferase centre on 23 S-like rRNAs deduced from chemical probing of antibiotic-ribosome complexes. *J Mol Biol* **247**, 224-235 (1995).
92. J. Vila *et al.*, In vitro antimicrobial production of beta-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferase by and susceptibility of clinical isolates of *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **37**, 138-141 (1993).
93. H. Seifert, R. Baginski, A. Schulze, G. Pulverer, Antimicrobial susceptibility of *Acinetobacter* species. *Antimicrob Agents Chemother* **37**, 750-753 (1993).
94. S. Schwarz, C. Kehrenberg, B. Doublet, A. Cloeckaert, Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS Microbiol Rev* **28**, 519-542 (2004).
95. Y. Doi, J. M. Adams, K. Yamane, D. L. Paterson, Identification of 16S rRNA methylase-producing *Acinetobacter baumannii* clinical strains in North Amercia. *Antimicrob. Agents Chemother.* **51**, 4209-4210 (2007).
96. Doi Y, Y. Arakawa, 16S rRNA methylation: emerging resistance mechanism against aminoglycosides. *Clin. Infect. Dis.* **45**, 88-94 (2007).
97. O. Skold, Resistance to trimethoprim and sulfonamides. *Vet Res* **32**, 261-273 (2001).
98. A. Hamouda, S. G. B. Amyes, Novel *gyrA* and *parC* point mutations in two strains of *Acinetobacter baumannii* resistant to ciprofloxacin. *J. Antimicrob. Chemother.* **54**, 695-696 (2004).
99. R. Henry *et al.*, Colistin-resistant, lipopolysaccharide-deficient *Acinetobacter baumannii* responds to lipopolysaccharide loss through increased expression of genes involved in the synthesis and transport of lipoproteins, phospholipids, and poly-beta-1,6-N-acetylglycosamine. *Antimicrob. Agents Chemother.* **56**, 59-69 (2011).

100. L. A. Arroyo *et al.*, The *pmrCAB* operon mediates polymyxin resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. *Antimicrob Agents Chemother* **55**, 3743-3751 (2011).
101. F. Fernández-Cuenca *et al.*, Relationship between beta-lactamase production, outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates of *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* **51**, 565-574 (2003).
102. E. Cundliffe, How antibiotic-producing organisms avoid suicide. *Annual Review of Microbiology* **43**, 207-233 (1989).
103. K. Yokoyama *et al.*, Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. *Lancet* **362**, 1888-1893 (2003).
104. Karthikeyan K, Thirunarayan MA, P. Krishnan, Coexistence of *blaOXA-23* with *blaNDM-1* and *armA* in clinical isolates of *Acinetobacter baumannii* from India. *J. Antimicrob. Chemother.* **65**, 2253-2254 (2010).
105. J. M. Adams-Haduch *et al.*, Genetic basis of multidrug resistance in *Acinetobacter baumannii* clinical isolates at a tertiary medical center in Pennsylvania. *Antimicrob. Agents Chemother.* **52**, 3837-3843 (2008).
106. H. Wisplinghoff *et al.*, Mutations in *gyrA* and *parC* associated with resistance to fluoroquinolones in epidemiologically defined clinical strains of *Acinetobacter baumannii*. *J Antimicrob Chemother* **51**, 177-180 (2003).
107. M. Zasloff, Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389-395 (2002).
108. J. H. Moffatt *et al.*, Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob. Agents Chemother.* **54**, 4971-4977 (2010).
109. Y. Y. Liu *et al.*, Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* **16**, 161-168 (2016).
110. L. Falgenhauer *et al.*, Colistin resistance gene *mcr-1* in extended-spectrum beta-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany. *Lancet Infect Dis* **16**, 282-283 (2016).
111. J. R. Mediavilla *et al.*, Colistin- and carbapenem-resistant *Escherichia coli* harboring *mcr-1* and *blaNDM-5*, causing a complicated urinary tract infection in a patient from the United States. *mBio* **7**, e01191-01116 (2016).
112. N. Stoesser, A. J. Mathers, C. E. Moore, N. P. Day, D. W. Crook, Colistin resistance gene *mcr-1* and pHNSHP45 plasmid in human isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *Lancet Infect Dis* **16**, 285-286 (2016).
113. Y. Y. Liu *et al.*, Structural modification of lipopolysaccharide conferred by *mcr-1* in Gram-negative ESKAPE pathogens. *Antimicrob Agents Chemother* **61**, (2017).
114. S. J. Nigro, V. Post, R. M. Hall, The multiresistant *Acinetobacter baumannii* European clone I type strain RUH875 (A297) carries a genomic antibiotic resistance island AbaR21, plasmid pRAY and a cluster containing ISAba1-*sul2*-CR2-*strB-strA*. *J Antimicrob Chemother* **66**, 1928-1930 (2011).
115. N. Martins, R. C. Picão, S. Adams-Sapper, L. W. Riley, B. M. Moreira, Association of class 1 and 2 integrons with multidrug-resistanct *Acinetobacter baumannii* international cloens and *Acinetobacter nosocomialis* isolates. *Antimicrob. Agents Chemother.* **59**, 698-701 (2015).
116. C. Urban, E. Go, N. Mariano, J. J. Rahal, Interaction of sulbactam, clavulanic acid and tazobactam with penicillin-binding proteins of imipenem-resistant and -susceptible *Acinetobacter baumannii*. *FEMS Microbil. Lett.* **125**, 193-197 (1995).
117. H. Nikaido, Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* **67**, 593-656 (2003).
118. M. Vaara, Agents that increase the permeability of the outer-membrane. *Microbiological Reviews* **56**, 395-411 (1992).
119. M. Vaara, Antibiotic-supersusceptible mutants of *Escherichia coli* and *Salmonella typhimurium*. *Antimicrob Agents Chemother* **37**, 2255-2260 (1993).
120. X. Z. Li, L. Zhang, K. Poole, Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of *Pseudomonas aeruginosa*. *J. Antimicrob. Chemother.* **45**, 433-436 (2000).
121. L. Fernández, R. E. Hancock, Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clin Microbiol Rev* **25**, 661-681 (2012).

122. M. A. Mussi, A. S. Limansky, A. M. Viale, Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of *Acinetobacter baumannii*: natural insertional inactivation of a gene encoding a member of a novel family of B-barrel outer membrane proteins. *Antimirob. Agents Chemother.* **49**, 1432-1440 (2005).
123. F. Yoshimura, H. Nikaido, Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. *J. Bacteriol.* **152**, 636-642 (1982).
124. E. Sugawara, H. Nikaido, OmpA is the principle nonspecific slow porin of *Acinetobacter baumannii*. *J. Bacteriol.* **194**, 4089-4096 (2012).
125. Y. Smani *et al.*, Role of OmpA in the multidrug resistance phenotype of *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **58**, 1806-1808 (2014).
126. Lee Y *et al.*, A novel insertion sequence, ISAba10, inserted into ISAba1 adjacent to the *blaOXA-23* gene and disrupting the outer membrane protein gene *carO* in *Acinetobacter baumannii*. *Antimirob. Agents Chemother.* **55**, 361-363 (2011).
127. G. Bou, G. Cerveró, M. A. Domínguez, C. Quereda, J. Martínez-Beltrán, Characterization of a nosocomial outbreak caused by a multiresistant *Acinetobacter baumannii* strain with a carbapenem-hydrolyzing enzyme: high-level carbapenem resistance in *A. baumannii* is not due to solely to the presence of β-lactamases. *J. Clin. Microbiol.* **38**, 3299-3305 (2000).
128. M. Zahn *et al.*, Small-molecule transport by CarO, an abundant eight-stranded β-barrel outer membrane protein from *Acinetobacter baumannii*. *J. Mol. Biol.* **427**, 2329-2339 (2015).
129. J. Quale, S. Bratu, D. Landman, R. Heddurshetti, Molecular epidemiology and mechanisms of carbapenem resistance in *Acinetobacter baumannii* endemic in New York City. *Clin Infect Dis* **37**, 214-220 (2003).
130. X. Z. Li, P. PlésiatM, H. Nikaido, The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin. Microbiol. Rev.* **28**, 337-418 (2015).
131. I. T. Paulsen, M. H. Brown, R. A. Skurray, Proton-dependent multidrug efflux systems. *Microbiol. Rev.* **60**, 575-608 (1996).
132. K. A. Hassan *et al.*, Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad Sci U S A* **110**, 20254-20259 (2013).
133. A. L. Davison, E. Dassa, C. Orelle, J. Chen, Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol. Mol. Biol. Rev.* **72**, 317-364 (2008).
134. H. Nikaido, Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **264**, 382-388 (1994).
135. A. L. Davidson, E. Dassa, C. Orelle, J. Chen, Structure, function, and evolution of bacterial ATP-binding cassette systems. *Microbiol Mol Biol Rev* **72**, 317-364, table of contents (2008).
136. T. Kuroda, T. Tsuchiya, Multidrug efflux transporters in the MATE family. *Biochim Biophys Acta* **1794**, 763-768 (2009).
137. D. C. Bay, K. L. Rommens, R. J. Turner, Small multidrug resistance proteins: A multidrug transporter family that continues to grow. *Bba-Biomembranes* **1778**, 1814-1838 (2008).
138. H. Nikaido, Y. Takatsuka, Mechanisms of RND multidrug efflux pumps. *Biochim Biophys Acta* **1794**, 769-781 (2009).
139. K. A. Hassan, Q. Liu, P. J. F. Henderson, I. T. Paulsen, Homologs of the *Acinetobacter baumannii* AceI transporter represent a new family of bacterial multidrug efflux systems. *mBio* **6**, e01982-01914 (2015).
140. J. A. Delmar, E. W. Yu, The AbgT family: A novel class of antimetabolite transporters. *Protein Sci* **25**, 322-337 (2016).
141. S. Coyne, P. Courvalin, B. Périchon, Efflux-mediated antibiotic resistance in *Acinetobacter* spp. *Antimicrob Agents Chemother* **55**, 947-953 (2011).
142. A. C. Fluit, A. Florijn, J. Verhoef, D. Milatovic, Presence of tetracycline resistance determinants and susceptibility to tigecycline and minocycline. *Antimicrob Agents Chemother* **49**, 1636-1638 (2005).
143. L. J. V. Piddock, Cliniccally relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin. Microbiol. Rev.* **19**, 382-402 (2006).
144. S. Grkovic, M. H. Brown, R. A. Skurray, Regulation of bacterial drug export systems. *Microbiol Mol Biol Rev* **66**, 671-701, table of contents (2002).
145. M. J. Everett, Y. F. Jin, V. Ricci, L. J. V. Piddock, Contributions of individual mechanisms to fluoroquinolone resistance in 36 *Escherichia coli* strains isolated from humans and animals. *Antimicrob Agents Chemother* **40**, 2380-2386 (1996).

146. L. Pumbwe, L. J. Piddock, Two efflux systems expressed simultaneously in multidrug-resistant *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **44**, 2861-2864 (2000).
147. A. Y. Peleg, J. Adams, D. L. Paterson, Tigecycline efflux as a mechanism for nonsusceptibility in *Acinetobacter baumannii*. *Antimirob. Agents Chemother.* **51**, 2065-2069 (2007).
148. K. Nishino, T. Latifi, E. A. Groisman, Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol* **59**, 126-141 (2006).
149. A. Schumacher *et al.*, Intracellular accumulation of linezolid in *Eschericia coli*, *Citrobacter freundii* and *Enterobacter aeroagenes*: role of enhanced efflux pump activity and inactivation. *J. Antimicrob. Chemother.* **59**, 1261-1264 (2007).
150. X. Z. Li, H. Nikaido, K. Poole, Role of mexA-mexB-oprM in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **39**, 1948-1953 (1995).
151. S. Magnet, P. Courvalin, T. Lambert, Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrob Agents Chemother* **45**, 3375-3380 (2001).
152. M. C. Sulavik *et al.*, Antibiotic susceptibility profiles of *Escherichia coli* strains lacking multidrug efflux pump genes. *Antimirob. Agents Chemother.* **45**, 1126-1136 (2001).
153. D. Du *et al.*, Structure of the AcrAB-TolC multidrug efflux pump. *Nature* **509**, 512-515 (2014).
154. M. Dolejska, L. Villa, L. Poirel, P. Nordmann, A. Carattoli, Complete sequencing of an IncHI1 plasmid encoding the carbapenemase NDM-1, the ArmA 16S RNA methylase and a resistance-nodulation-cell division/multidrug efflux pump. *J Antimicrob Chemother* **68**, 34-39 (2013).
155. E. W. Yu, G. MacDermott, H. I. Zgurskaya, H. Nikaido, D. E. Koshland Jr, Structural basis of multiple drug-binding capacity of the AcrB multidrug efflux pump. *Science* **300**, 976-980 (2003).
156. R. Nakashima, K. Sakurai, S. Yamasaki, K. Nishino, A. Yamaguchi, Structures of the multidrug exporter AcrB reveal a proximal multisite drug-binding pocket. *Nature* **480**, 565-569 (2011).
157. Y. Yin, X. He, P. Szewczyk, T. Nguyen, G. Chang, Structure of the multidrug transporter EmrD from *Escherichia coli*. *Science* **312**, 741-744 (2006).
158. Y. Tanaka *et al.*, Structural basis for the drug extrusion mechanism by a MATE multidrug transporter. *Nature* **496**, 247-251 (2013).
159. Y. J. Chen *et al.*, X-ray structure of EmrE supports dual topology model. *Proc Natl Acad Sci U S A* **104**, 18999-19004 (2007).
160. A. A. Neyfakh, Mystery of multidrug transporters: the answer can be simple. *Molecular Microbiology* **44**, 1123-1130 (2002).
161. V. Dastidar, W. Mao, O. Lomovskaya, H. I. Zgurskaya, Drug-induced conformational changes in multidrug efflux transporter AcrB from *Haemophilus influenzae*. *J Bacteriol* **189**, 5550-5558 (2007).
162. J. A. Bohnert, B. Karamian, H. Nikaido, Optimized nile red efflux assay of AcrAB-TolC multidrug efflux system shows competition between substrates. *Antimirob. Agents Chemother.* **54**, 3770-3775 (2010).
163. J. A. Bohnert *et al.*, Site-directed mutagenesis reveals putative substrate binding residues in the *Escherichia coli* RND efflux pump AcrB. *J Bacteriol* **190**, 8225-8229 (2008).
164. E. W. Yu, J. R. Aires, G. McDermott, H. Nikaido, A periplasmic drug-binding site of the AcrB multidrug efflux pump: a crystallographic and site-directed mutagenesis study. *J. Bacteriol.* **187**, 6804-6815 (2005).
165. J. A. Fralick, Evidence that TolC is required for functioning of the Mar/AcrAB efflux pump of *Escherichia coli*. *Journal of Bacteriology* **178**, 5803-5805 (1996).
166. N. Kobayashi, K. Nishino, A. Yamaguchi, Novel macrolide-specific ABC-type efflux transporter in *Escherichia coli*. *J Bacteriol* **183**, 5639-5644 (2001).
167. M. Tanabe *et al.*, The multidrug resistance efflux complex, EmrAB from Escherichia coli forms a dimer in vitro. *Biochem Biophys Res Commun* **380**, 338-342 (2009).
168. O. Lomovskaya, K. Lewis, Emr, an *Escherichia coli* locus for multidrug resistance. *Proc Natl Acad Sci U S A* **89**, 8938-8942 (1992).
169. V. Koronakis, A. Sharff, E. Koronakis, B. Luisi, C. Hughes, Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405**, 914-919 (2000).
170. D. Ma *et al.*, Molecular-Cloning and Characterization of Acra and Acre Genes of *Escherichia-Coli*. *Journal of Bacteriology* **175**, 6299-6313 (1993).
171. T. Dinh, I. T. Paulsen, M. H. Saier, Jr., A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of gram-negative bacteria. *J Bacteriol* **176**, 3825-3831 (1994).

172. J. Mikolosko, K. Bobyk, H. I. Zgurskaya, P. Ghosh, Conformational flexibility in the multidrug efflux system protein AcrA. *Structure* **14**, 577-587 (2006).
173. Z. Wang *et al.*, An allosteric transport mechanism for the AcrAB-TolC multidrug efflux pump. *Elife* **6**, (2017).
174. M. Putman, H. W. van Veen, W. N. Konings, Molecular properties of bacterial multidrug transporters. *Microbiol Mol Biol Rev* **64**, 672-693 (2000).
175. H. I. Zgurskaya, Y. Yamada, E. B. Tikhonova, Q. Ge, G. Krishnamoorthy, Structural and functional diversity of bacterial membrane fusion proteins. *Biochim Biophys Acta* **1794**, 794-807 (2009).
176. L. McMurry, R. E. Petrucci Jr, S. B. Levy, Active efflux of tetracycline encoded by four genetically different tetracycline resistance determiants in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A* **77**, 3974-3977 (1980).
177. R. Edgar, E. Bibi, MdfA, an *Escherichi coli* multidrug resistance protein with an extraordinary broad spectrum of drug recognition. *J. Bacteriol.* **179**, 2274-2280 (1997).
178. N. Tal, S. Schuldiner, A coordinated network of transporters with overlapping specificities provides a robust survival strategy. *Proc Natl Acad Sci U S A* **106**, 9051-9056 (2009).
179. E. J. Yoon *et al.*, Contribution of resistance-nodulation-cell division efflux systems to antibiotic resistance and biofilm formation in *Acinetobacter baumannii*. *MBio* **6**, e00309-00315 (2015).
180. M. F. Lin, Y. Y. Lin, H. W. Yeh, C. Y. Lan, Role of the BaeSR two-component system in the regulation of *Acinetobacter baumannii adeAB* genes and its correlation with tigecycline susceptibility. *BMC Microbiol* **14**, 119 (2014).
181. I. Marchand, L. Damier-Piolle, P. Courvalin, T. Lambert, Expression of the RND-type efflux pumps AdeACB in *Acinetobacter baumannii* is regulated by AdeRS two-component system. *Antimirob. Agents Chemother.* **48**, 3298-3304 (2004).
182. Rajamohan G, Srinivasan VB, W. A. Gebreyes, Novel role of *Acinetobacter baumannii* RND efflux transporters in mediating decreased susceptibility to biocides. *J. Antimicrob. Chemother.* **65**, 228-232 (2010).
183. N. Rosenfeld, C. Bouchier, P. Courvalin, B. Périchon, Expression of the resistance-nodulation-cell division pump AdeIJK in *Acinetobacter baumannii* is regulated by AdeN, a TetR-type regulator. *Antimirob. Agents Chemother.* **56**, 2504-2510 (2012).
184. S. Coyne, N. Rosenfeld, T. Lambert, P. Courvalin, B. Périchon, Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*. *Antimirob. Agents Chemother.* **54**, 4389-4393 (2010).
185. G. Rajamohan, V. B. Srinivasan, W. A. Gebreyes, Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant resistance in *Acinetobacter baumannii*. *J Antimicrob Chemother* **65**, 1919-1925 (2010).
186. K. A. Hassan *et al.*, Fluorescence-Based Flow sorting in parallel with transposon insertion site sequencing identifies multidrug efflux systems in *Acinetobacter baumannii*. *MBio* **7**, e01200-01216 (2016).
187. I. Roca *et al.*, CraA, a major facilitator superfamily efflux pump associated with chloramphenicol resistance in *Acinetobacter baumannii*. *Antimirob. Agents Chemother.* **53**, 4013-4014 (2009).
188. K. A. Hassan *et al.*, Roles of DHA2 family transporters in drug resistance and iron homeostasis in *Acinetobacter* spp. *J Mol Microbiol Biotechnol* **20**, 116-124 (2011).
189. L. Bissonnette, S. Champetier, J. P. Buisson, P. H. Roy, Characterization of the nonenzymatic chloramphenicol resistance (*cmlA*) gene of the In4 integron of Tn1696: similarity of the product to transmembrane transport proteins. *J Bacteriol* **173**, 4493-4502 (1991).
190. S. Coyne, P. Courvalin, M. Galimand, Acquisition of multidrug resistance transposon Tn6061 and IS6100-mediated large chromosomal inversions in *Pseudomonas aeruginosa* clinical isolates. *Microbiology* **156**, 1448-1458 (2010).
191. A. Ribera, I. Roca, J. Ruiz, I. Gibert, J. Vila, Partial characterization of a transposon containing the *tet(A)* determinant in a clinical isolate of *Acinetobacter baumannii*. *J Antimicrob Chemother* **52**, 477-480 (2003).
192. V. B. Srinivasan *et al.*, Genetic relatedness and molecular characterization of multidrug rsistant *Acinetobacter baumannii* isolated in central Ohio, USA. *Annu. Clin. Microbiol. Antimicro.* **8**, (2009).
193. X. Z. Su, J. Chen, T. Mizushima, T. Kuroda, T. Tsuchiya, AbeM, an H+-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. *Antimicrob Agents Chemother* **49**, 4362-4364 (2005).

194. B. A. Eijkelkamp, K. A. Hassan, I. T. Paulsen, M. H. Brown, Development of a high-throughput cloning strategy for characterization of *Acinetobacter baumannii* drug transporter proteins. *J Mol Microbiol Biotechnol* **20**, 211-219 (2011).
195. V. B. Srinivasan, G. Rajamohan, W. A. Gebreyes, Role of AbeS, a novel efflux pump of the SMR family of transporters, in resistance to antimicrobial agents in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* **53**, 5312-5316 (2009).
196. M. C. Ploy, F. Denis, P. Courvalin, T. Lambert, Molecular characterization of integrons in *Acinetobacter baumannii*: description of a hybrid class 2 integron. *Antimicrob Agents Chemother* **44**, 2684-2688 (2000).
197. H. Zhou *et al.*, Genomic analysis of the multidrug-resistance *Acinetobacter baumannii* strain MDR-ZJ06 widely spread in China. *Antimicrob. Agents Chemother.* **55**, 4506-4512 (2011).
198. E. J. Yoon, P. Courvalin, C. Grillot-Courvalin, RND-type efflux pumps in multidrug-resistant clinical isolates of *Acinetobacter baumannii*: major role for AdeABC overexpression and AdeRS mutations. *Antimicrob Agents Chemother* **57**, 2989-2995 (2013).
199. A. Nemec, M. Maixnerová, T. J. K. Reijden, P. Broek, L. Dijkshoorn, Relationship between the AdeABC efflux system gene content, netilmicin susceptibility and multidrug resistance in a genotypically diverse collection of *Acinetobacter baumannii* strains. *J. Antimicrob. Chemother.* **60**, 483-489 (2007).
200. M. Hornsey *et al.*, AdeABC-mediated efflux and tigecycline MICs for epidemic clones of *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* **65**, 1589-1593 (2010).
201. Y. R. Wen *et al.*, Mechanistic insight into how multidrug resistant *Acinetobacter baumannii* response regulator AdeR recognizes an intercistronic region. *Nucleic Acids Res* **45**, 9773-9787 (2017).
202. J. R. Sun *et al.*, AdeRS combination codes differentiate the response to efflux pump inhibitors in tigecycline-resistant isolates of extensively drug-resistant *Acinetobacter baumannii*. *Eur J Clin Microbiol Infect Dis* **33**, 2141-2147 (2014).
203. S. Coyne, G. Guigon, P. Courvalin, B. Périchon, Screening and quantification of the expression of antibiotic resistance genes in *Acinetobacter baumannii* with a microarray. *Antimicrob. Agents Chemother.* **54**, 333-340 (2010).
204. G. E. Richmond *et al.*, The *Acinetobacter baumannii* two-component system AdeRS regulates genes required for multidrug efflux, biofilm formation, and virulence in a strain-specific manner. *mBio* **7**, e00430-00416 (2016).
205. M. Thaker, P. Spanogiannopoulos, G. D. Wright, The tetracycline resistome. *Cell Mol Life Sci* **67**, 419-431 (2010).
206. M. C. Roberts, Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiol Rev* **19**, 1-24 (1996).
207. A. T. Tucker *et al.*, Defining gene-phenotype relationships in *Acinetobacter baumannii* through one-step chromosomal gene inactivation. *mBio* **5**, e01313-01314 (2014).
208. A. M. Mackie, K. A. Hassan, I. T. Paulsen, *Biolog phenotype microarrays for phenotypic characterization of microbial cells*. Paulsen IT, A. J. Holmes, Eds., Environmental Microbiology (Humana Press, 2014).
209. F. Ozsolak, P. M. Milos, RNA sequencing: advances, challenges and opportunities. *Nat Rev Genet* **12**, 87-98 (2011).
210. G. C. Langridge *et al.*, Simultaneous assay of every *Salmonella typhi* gene using one million transposon mutants. *Genome Res.* **19**, 2308-2316 (2009).

Chapter 2 Rapid multiplexed phenotypic screening identifies drug resistance functions for three novel efflux pumps in *Acinetobacter baumannii*

Li, L., Hassan, K. A., Brown, M. H., & Paulsen, I. T. (2016). Rapid multiplexed phenotypic screening identifies drug resistance functions for three novel efflux pumps in *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy*, 71(5), 1223-1232.

DOI: https://doi.org/10.1093/jac/dkv460

Chapter 3 An ace up their sleeve: a transcriptomic approach exposes the AceI efflux protein of *Acinetobacter baumannii* and reveals the drug efflux potential hidden in many microbial pathogens

frontiers in Microbiology

PERSPECTIVE
published: 22 April 2015
doi: 10.3389/fmicb.2015.00333

An ace up their sleeve: a transcriptomic approach exposes the AceI efflux protein of *Acinetobacter baumannii* and reveals the drug efflux potential hidden in many microbial pathogens

Karl A. Hassan[1]*, Liam D. H. Elbourne[1], Liping Li[1], Hasinika K. A. H. Gamage[1], Qi Liu[1], Scott M. Jackson[2†], David Sharples[2], Anne-Brit Kolstø[3], Peter J. F. Henderson[2] and Ian T. Paulsen[1]*

[1] Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia, [2] Astbury Centre for Structural Molecular Biology, School of Biomedical Sciences, University of Leeds, Leeds, UK, [3] Laboratory for Microbial Dynamics, Department of Pharmaceutical Biosciences, School of Pharmacy, University of Oslo, Oslo, Norway

The era of antibiotics as a cure-all for bacterial infections appears to be coming to an end. The emergence of multidrug resistance in many hospital-associated pathogens has resulted in "superbugs" that are effectively untreatable. Multidrug efflux pumps are well known mediators of bacterial drug resistance. Genome sequencing efforts have highlighted an abundance of putative efflux pump genes in bacteria. However, it is not clear how many of these pumps play a role in antimicrobial resistance. Efflux pump genes that participate in drug resistance can be under tight regulatory control and expressed only in response to substrates. Consequently, changes in gene expression following antimicrobial shock may be used to identify efflux pumps that mediate antimicrobial resistance. Using this approach we have characterized several novel efflux pumps in bacteria. In one example we recently identified the *Acinetobacter* chlorhexidine efflux protein (AceI) efflux pump in *Acinetobacter*. AceI is a prototype for a novel family of multidrug efflux pumps conserved in many proteobacterial lineages. The discovery of this family raises the possibility that additional undiscovered intrinsic resistance proteins may be encoded in the core genomes of pathogenic bacteria.

Keywords: multidrug efflux systems, bacterial transmembrane pair, adaptive resistance, bacterial drug resistance, transcriptomics

OPEN ACCESS

Edited by:
Keith Poole,
Queen's University, Canada

Reviewed by:
Govindan Rajamohan,
Council of Scientific and Industrial Research – Institute of Microbial Technology, India
Ayush Kumar,
University of Manitoba, Canada

****Correspondence:***
Ian T. Paulsen and Karl A. Hassan, Department of Chemistry and Biomolecular Sciences, Macquarie University, Eastern Road, Sydney, NSW 2109, Australia
ian.paulsen@mq.edu.au; karl.hassan@mq.edu.au

†Present address:
Scott M. Jackson, Institute of Molecular Biology and Biophysics, ETH Zurich, Zurich, Switzerland

Specialty section:
This article was submitted to Antimicrobials, Resistance and Chemotherapy, a section of the journal Frontiers in Microbiology

Received: *12 February 2015*
Accepted: *02 April 2015*
Published: *22 April 2015*

Citation:
Hassan KA, Elbourne LDH, Li L, Gamage HKAH, Liu Q, Jackson SM, Sharples D, Kolstø A-B, Henderson PJF and Paulsen IT (2015) An ace up their sleeve: a transcriptomic approach exposes the AceI efflux protein of Acinetobacter baumannii and reveals the drug efflux potential hidden in many microbial pathogens. Front. Microbiol. 6:333. doi: 10.3389/fmicb.2015.00333

Introduction

Multidrug efflux pumps are a significant obstacle preventing the control of infections caused by pathogenic bacteria. Genes encoding these transporters have been found in all bacterial genomes sequenced, and the overexpression of just one can lead to the reduced efficacy of a range of structurally and mechanistically unrelated antimicrobials (Ren and Paulsen, 2007; Brzoska et al., 2013). Five families of transporters that include multidrug efflux systems have been studied extensively, and include representative proteins that have

been characterized biochemically and by tertiary structural analyses. These include the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the resistance/nodulation/division (RND) superfamily, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family (**Figure 1**).

Significant longstanding difficulties surround identifying the physiological functions of these multidrug efflux transport proteins and determining which of the many pumps encoded by bacterial strains actually contribute to antimicrobial resistance (Piddock, 2006; Schindler et al., 2015). Studies have shown that these efflux pumps often have overlapping substrate recognition profiles (Tal and Schuldiner, 2009). Furthermore, it is not uncommon for a bacterial genome to encode a large number of efflux pumps that have predicted drug substrates, e.g., strains of *Bacillus cereus* encode more than 100 of these pumps accounting for more than 2% or their predicted protein coding potential (Ren and Paulsen, 2007; Simm et al., 2012). It is unlikely that all these pumps share the primary function of protection against toxic compounds, highlighting a need for higher throughput approaches to assess the physiological roles of individual proteins, be they in drug resistance, native housekeeping functions, or other cellular roles.

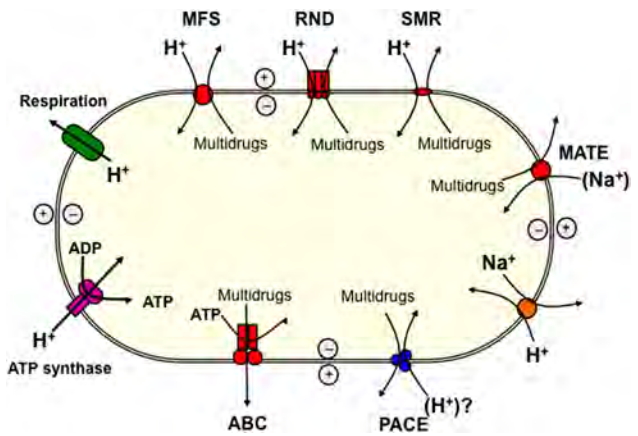

FIGURE 1 | Schematic diagram showing the basis for the energisation of multidrug efflux pumps operating in bacteria. The large oval represents the bacterial cell. An electrochemical H^+ gradient (proton-motive-force) across the cytoplasmic membrane is generated as a result of respiration (green). Energy from the proton-motive-force is used to power transport in secondary active transport systems, such as those within the major facilitator superfamily (MFS), resistance/nodulation/division (RND), and small multidrug resistance (SMR) (super) families. Sodium/proton antiporters (orange) harness the proton-motive-force to generate a Na^+ gradient that powers transport by other multidrug efflux pumps, including those in the multidrug and toxic compound extrusion (MATE) family. ATP production by ATP-synthase (pink) is also powered by the proton-motive-force, and ATP is used to power transport by the primary active transporters of the ATP-binding cassette (ABC) superfamily. Previously characterized efflux pump (super) families are shown in red, whereas the proteobacterial antimicrobial compound efflux (PACE) family is shown in blue. The PACE family transport proteins are likely to be powered by the H^+ gradient.

Efflux Pumps Participate in Intrinsic, Adaptive, and Acquired Resistance

Bacterial drug resistance can be divided into three general categories, intrinsic, adaptive, and acquired (Fernandez and Hancock, 2012). Depending on their mode(s) of regulation and their local genetic context, bacterial multidrug efflux pumps can be geared to participate in any of these three resistance categories. Intrinsic resistance stems from inherent properties of a bacterial cell and can occur as a result of high constitutive expression and activity of some multidrug efflux pumps. Adaptive resistance is related to physiological alterations that are induced by environmental changes and can occur when multidrug efflux pumps are expressed in response to antimicrobial substrates. Finally, acquired resistance can result from mutations promoting constitutive expression of an ordinarily tightly controlled endogenous multidrug efflux system, or when efflux pump genes are acquired on a mobile genetic element, such as a plasmid or phage.

Adaptive Resistance Responses Identify Efflux Pumps that Mediate Drug Resistance

High-level expression of efflux pumps can have a negative impact on cell growth (Brzoska et al., 2013), resulting in a need to control the timing of efflux system expression to coincide with specific physiological requirements. As such, efflux pumps with physiological resistance functions may be characteristically expressed in response to drug substrates. These pumps may be part of an adaptive drug resistance response or part of general stress response regulons. For example, expression of the *adeAB* and *adeIJK* efflux pump genes in *Acinetobacter baumannii* (Hassan et al., 2013; our unpublished data), the *acrAB* and *acrF* genes in *Escherichia coli* (Shaw et al., 2003; Bailey et al., 2009), the *mexXY* and *mexCD* genes in *Pseudomonas aeruginosa* (Morita et al., 2014), the *norA* gene in *Staphylococcus aureus* (Kaatz and Seo, 2004), and the *bmr* gene in *Bacillus subtilis* (Ahmed et al., 1994), is induced in response to antimicrobial shock treatments.

The mode of regulation and regulatory cues of most efflux pumps are typically only investigated after their functional characterisation. However, global gene expression profiles that show heightened expression of putative efflux pump genes following drug or toxin shocks have provided the impetus to assess the drug resistance functions of these pumps. For example, members of our team recognized that an uncharacterised MFS exporter, BC4707, was expressed in response to bile salt shock in the human food-poisoning associated pathogen *Bacillus cereus*, and went on to characterize its role in drug resistance (Kristoffersen et al., 2007). The gene encoding BC4707 is conserved in the core genome of *B. cereus* and its deletion from *B. cereus* ATCC 14579 resulted in increased susceptibility to norfloxacin (Simm et al., 2012). Overexpression of BC4707 in *E. coli* BL21 Δ*acrAB* resulted in increased resistance to norfloxacin, ciprofloxacin, and kanamycin and fluorescence

transport assays showed that accumulation of norfloxacin is reduced by BC4707 in an energy dependent manner (Simm et al., 2012).

Adaptive Resistance Responses Identify a New Class of Drug Efflux Pump

Extending from this work, we have exploited adaptive resistance responses to identify efflux pumps that may mediate drug resistance in hospital-acquired bacterial pathogens, with a focus on biocide resistance. For example, in recent work we conducted a transcriptomic study to examine the regulatory response of *A. baumannii* to a shock treatment with the synthetic biocide chlorhexidine (Hassan et al., 2013). Chlorhexidine is commonly applied in antibacterial soaps, mouthwashes and antiseptics, and is listed as an "Essential Medicine" by the World Health Organization. Chlorhexidine is a membrane active biocide and as such, multidrug efflux pumps are commonly associated with reduced levels of susceptibility (Russell, 1986; McDonnell and Russell, 1999). In line with the discussion above, the most highly overexpressed genes in our chlorhexidine shock treatment encoded AdeAB, components of a major tripartite RND multidrug efflux system in *A. baumannii* (Hassan et al., 2013). This efflux system has previously been shown to mediate resistance to a very broad range of antimicrobials and biocides, including chlorhexidine (Rajamohan et al., 2009). The overexpression of genes encoding AdeAB in response to chlorhexidine confirmed the role of this efflux system in adaptive resistance to chlorhexidine in *A. baumannii*. Apart from the genes encoding AdeAB, only one gene was highly (>10-fold) overexpressed in response to chlorhexidine. This gene was originally annotated as encoding a hypothetical membrane protein. Using biochemical approaches we showed that this protein is in fact a chlorhexidine resistance protein that functions via an active efflux mechanism (Hassan et al., 2013). We named this protein the *Acinetobacter* chlorhexidine efflux protein I (AceI).

The AceI Transporter is a Prototype for a New Family of Bacterial Multidrug Efflux Systems

The AceI transport protein contains two tandem "Bacterial Transmembrane Pair" (BTP) protein domains defined within the Pfam database (Finn et al., 2014). There are more than 750 protein sequences containing this domain architecture listed in the Pfam database (version 27.0). Genes encoding these proteins are particularly common among proteobacterial lineages, but can also be found in the genomes of unrelated bacterial genera, including the Firmicutes and Actinobacteria. We have not yet identified these genes in the genomes of any archaeal or eukaryotic organisms.

We have recently characterized more than 20 homologs of the AceI transporter by heterologous expression in *E. coli* (Hassan et al., 2015). These studies have demonstrated that many AceI homologs are able to provide resistance to an array of biocides in addition to chlorhexidine. For example, the VP1155 protein from *Vibrio parahaemolyticus* and Bcen2424_2356 protein from *Burkholderia cenocepacia* each conferred increased resistance to chlorhexidine, benzalkonium, acriflavine, and proflavine, when expressed in *E. coli* (Hassan et al., 2015). Fluorescence transport assays conducted on cells expressing these and other AceI homologs that conferred resistance to acriflavine and proflavine, demonstrated that these compounds are actively exported from the cell by these transporters (Hassan et al., 2015). These results corroborate our earlier findings that chlorhexidine is actively transported by AceI (Hassan et al., 2013), and indicate that efflux is the mechanism of resistance operating in this group of proteins. Taken together all the observations suggest that these proteins comprise a new family of multidrug efflux pumps common amongst Proteobacterial lineages. We have named this family the Proteobacterial Antimicrobial Compound Efflux (PACE) family (**Figure 1**; Hassan et al., 2015).

PACE Family Proteins are Encoded Within the Core Genome

Given that the PACE family represents a new class of resistance determinants, we were interested in gathering basic information regarding the mode of inheritance of these genes in bacteria. To this end, we examined their level of conservation within representative bacterial lineages following the basic premise that highly conserved genes within core bacterial genomes are expected to have been inherited vertically, whereas those in the accessory genome are likely to have been horizontally acquired.

We examined PACE family protein conservation in four γ-Proteobacterial species (*A. baumannii*, *P. aeruginosa*, *V. parahaemolyticus,* and *E. coli*) a β-Proteobacterial species (*B. cenocepacia*) and a member of the Firmicutes (*Veillonella parvula*). Annotated protein sequences from all complete and draft genomes of these species were downloaded from the NCBI Genbank database (October, 2014) and were queried using the BTP PfamHMM (Finn et al., 2014) in HMMER3 searches (Eddy, 2011). These searches determined that PACE family proteins are encoded in the pan-genomes of all six species examined. To determine the number of distinct orthologous groups of PACE family proteins in each species we performed a clustering analysis based on sequence identity (cluster stringency >90%) using cd-hit v4.6.1 (Fu et al., 2012). This analysis demonstrated that *A. baumannii* had three clusters (100, 96.7, and 0.3% conservation in 623 strains); *P. aeruginosa* had three clusters (99.5, 99.5, and 0.5% conservation in 197 strains); *V. parahaemolyticus* had one cluster (90.1% conservation in 101 strains); *E. coli* had 4 clusters (0.2, 0.1, 0.1, and 0.1% conservation in 1986 strains); *B. cenocepacia* had three clusters (100, 88.9, and 88.9% conservation in nine strains); and *V. parvula* had one cluster (100% conservation in four strains).

These data demonstrate that the pattern of PACE family protein conservation is variable between the species. For example, both *A. baumannii* and *P. aeruginosa* each encode two highly

conserved PACE family proteins present in virtually all sequenced strains, and one additional PACE protein encoded in one or two specific strains. Whereas, *V. parahaemolyticus* and *V. parvula* each encode only one highly conserved PACE protein, and *B. cenocepacia* encodes three highly conserved PACE proteins. Most *E. coli* strains do not encode a PACE family protein, although a small handful of strains encode one of four PACE protein variants. The highly conserved PACE family proteins encoded by *A. baumannii*, *P. aeruginosa*, *V. parahaemolyticus*, *B. cenocepacia,* and *V. parvula* are likely to constitute part of the core genome in these species and to have been inherited vertically rather than on mobile genetic elements. The almost complete lack of genes encoding PACE family proteins in *E. coli* strains suggests that these genes were lost early in the development of the *E. coli* lineage, but after its divergence from other γ-proteobacteria. In the few cases where *E. coli* strains were found to encode a PACE family protein, it was sometimes associated with mobile genetic elements suggesting that it had been acquired by horizontal gene transfer. The paucity of PACE genes in *E. coli* strains confirms our previous conclusion that *E. coli* is an excellent host to study the function of these proteins (Hassan et al., 2013).

Physiological Substrates for PACE Family Transporters

To date, the substrates identified for PACE family transport proteins include synthetic biocides only, such as chlorhexidine, dequalinium, benzalkonium, proflavine, and acriflavine. The presence of these toxic biocides in the environments occupied by Proteobacteria is likely to have been negligible across evolutionary time, until perhaps the last 50–100 years when these compounds were applied in various industries. Given that the organisms encoding PACE family genes are likely to have diverged long before the development of this potential selective pressure, it is seems unlikely that biocides are the native physiological substrates of PACE efflux pumps. Nonetheless, these genes are transcriptionally responsive to at least one biocide, chlorhexidine in four species, *A. baumannii*, *A. baylyi*, *P. aeruginosa,* and *B. cenocepacia* (Nde et al., 2009; Coenye et al., 2011; Hassan et al., 2013), suggesting that chlorhexidine can serve as a mimic of their natural physiological substrate for inducing efflux pump expression.

Regulatory Proteins Acting on PACE Efflux Pumps

In addition to antimicrobial resistance, the promiscuous substrate recognition profiles of multidrug efflux pumps allow them to participate in diverse physiological processes. For example, efflux systems in Gram-negative bacteria function in cell adherence, invasion, biofilm formation, virulence in animals and plants, and resistance to host encoded factors (Piddock, 2006). Consequently, the regulation of bacterial drug efflux systems can be highly complex and responsive to a range of cellular and extracellular conditions. Complex regulation may be particularly apparent in efflux pumps, such as AceI and its homologs, which are encoded within core bacterial genomes. These genes are likely to have been present in these species for significant periods of evolutionary time, allowing fine-tuning of their expression in response to a range of environmental cues. A case in point, as summarized within the EcoCyc database (Keseler et al., 2013), transcription of the *acrAB* efflux system genes, within the core genome of *E. coli*, is controlled by at least seven distinct regulatory proteins, which are themselves subject to a range of regulatory pressures. These regulatory proteins are likely to integrate efflux pumps into the adaptive resistance responses observed in bacteria, as well as other pathways controlling their alternative physiological functions.

Regulators mediating the most direct control of genes encoding efflux pumps are often encoded locally – adjacent to and divergently transcribed from the gene(s) encoding the efflux system. These regulators, either activators or repressors, typically bind a similar spectrum of compounds to their cognate efflux pump with high affinity as a signal for transcriptional activation or relief of transcriptional repression. Some well characterized examples include AcrR, which controls transcription of the *E. coli acrAB* efflux pump genes (Li et al., 2007), and QacR, which controls *qacA*/*qacB* expression in *S. aureus* (Grkovic et al., 1998; Schumacher et al., 2001).

The PACE family transporters that we have studied to date are each encoded adjacent to a divergently transcribed LysR family regulator. To determine whether these regulators control the expression of their cognate PACE family gene, we used our established methods (Brzoska et al., 2013) to construct a

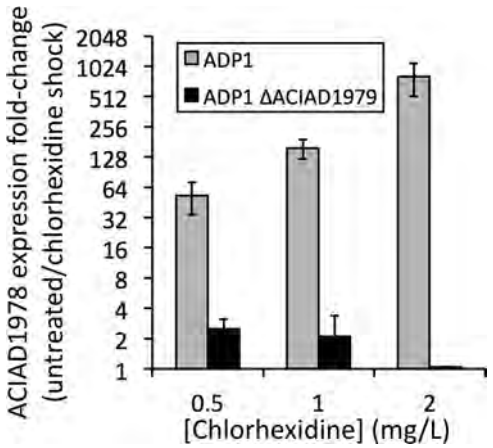

FIGURE 2 | Expression of the PACE family gene, ACIAD1978, in wild-type and mutant (ΔACIAD1979) *A. baylyi* ADP1 in response to chlorhexidine shock treatments. Cells were grown in LB broth to $OD_{600} = 0.6$, then split, with one half of each culture treated with chlorhexidine and the other half receiving no treatment. RNA was isolated and assessed by qRT-PCR following our established protocols (Brzoska and Hassan, 2014) to determine relative expression levels of ACIAD17978 in chlorhexidine treated and untreated samples. Error bars show the SEM. Changes in expression of ACIAD1978 were negligible in the ACIAD1979 mutant treated with 2 mg/L chlorhexidine, and thus the bar is difficult to see.

deletion mutant of the regulator gene ACIAD1979 in *A. baylyi* ADP1, which is encoded adjacent to the PACE family chlorhexidine resistance gene ACIAD1978. We examined the expression of ACIAD1978 in both the wild-type and the ΔACIAD1979 regulatory mutant in response to chlorhexidine shock treatments using quantitative real-time PCR analysis (Brzoska and Hassan, 2014). In the absence of chlorhexidine the expression of ACIAD1978 was similar in both strains. However, whereas increasing concentrations of chlorhexidine induced ACIAD1978 gene expression in the wild-type strain, chlorhexidine addition failed to induce ACIAD1978 expression in the ΔACIAD1979 mutant (**Figure 2**). These results suggest that the ACIAD1979 LysR family regulator functions as an activator of the PACE family gene ACIAD1978. We are currently investigating the role of LysR family proteins in controlling expression of PACE family pumps in other species and are determining whether the spectrum of ligands recognized by these regulators is closely linked to the substrate recognition profile of their cognate PACE family pump. It also remains to be determined whether the PACE-associated regulators control expression of other genes, or if there are distally encoded regulators that also modulate expression of PACE transporter genes.

Conclusion and Future Directions

Transcriptomic analyses of antimicrobial shock treatments are valuable in identifying the potential resistance mechanisms operating in bacteria, including multidrug efflux pumps participating in the adaptive resistance response. Using transcriptomic analyses, we have defined roles for new efflux pumps and identified the PACE family of multidrug transport proteins, the first new family of drug efflux proteins discovered in over a decade.

Transporters within the PACE family are currently enigmas. We have identified drug substrates, such as chlorhexidine that are common to many of these pumps. Furthermore, PACE family gene expression is induced by chlorhexidine, a response that is mediated via locally encoded regulators. This highlights a close relationship between the function of these pumps and their regulatory control. Since PACE family genes are encoded in the core genomes of bacterial lineages that diverged long ago, this functional-regulatory relationship is likely to have arisen early in the evolution of these proteins. However, the substrates/inducers that have been identified for PACE proteins are synthetic biocides that are likely to have been absent from the environment until the last 50–100 years. Therefore, it is unlikely that these biocides would have provided the selective pressure required to drive the functional or regulatory evolution of PACE family pumps. Consequently, a deeper understanding of these novel resistance proteins requires future investigations aimed at identifying their physiological substrate(s) and primary functional roles in bacteria.

The discovery of the PACE family opens up the possibility that there may be more novel efflux proteins waiting to be discovered. There are many hypothetical membrane proteins of unknown function encoded in all bacterial genomes. For example, even in the best-studied bacterial genome, *E. coli* K12, there are 409 membrane proteins of unknown function. At least some of these may represent entirely novel types of efflux pumps.

Acknowledgments

This work was supported by National Health and Medical Research Council (Australia) Project Grant (1060895) to IP, KH, and PH and a Macquarie University Research Development Grant (9201401563) to KH and LE. Collaboration between the Australian and UK laboratories is supported by an EU International Research Staff Exchange Scheme BacMT 247634 (AK, PH, and IP). Additional travel support was provided by the Australian Academy of Science (IP). Work in the PH laboratory was supported by EU European Drug Initiative for Channels and Transporters Grant 201924, and also by a Leverhulme Trust Emeritus Award to PH.

References

Ahmed, M., Borsch, C. M., Taylor, S. S., Vázquez-Laslop, N., and Neyfakh, A. A. (1994). A protein that activates expression of a multidrug efflux transporter upon binding the transporter substrates. *J. Biol. Chem.* 269, 28506–28513.

Bailey, A. M., Constantinidou, C., Ivens, A., Garvey, M. I., Webber, M. A., Coldham, N., et al. (2009). Exposure of *Escherichia coli* and *Salmonella enterica* serovar typhimurium to triclosan induces a species-specific response, including drug detoxification. *J. Antimicrob. Chemother.* 64, 973–985. doi: 10.1093/jac/dkp320

Brzoska, A. J., and Hassan, K. A. (2014). Quantitative PCR for detection of mRNA and gDNA in environmental isolates. *Methods Mol. Biol.* 1096, 25–42. doi: 10.1007/978-1-62703-712-9_3

Brzoska, A. J., Hassan, K. A., De Leon, E. J., Paulsen, I. T., and Lewis, P. J. (2013). Single-step selection of drug resistant *Acinetobacter baylyi* ADP1 mutants reveals a functional redundancy in the recruitment of multidrug efflux systems. *PLoS ONE* 8:e56090. doi: 10.1371/journal.pone.0056090

Coenye, T., Van Acker, H., Peeters, E., Sass, A., Buroni, S., Riccardi, G., et al. (2011). Molecular mechanisms of chlorhexidine tolerance in *Burkholderia cenocepacia* biofilms. *Antimicrob. Agents Chemother.* 55, 1912–1919. doi: 10.1128/AAC.01571-10

Eddy, S. R. (2011). Accelerated profile HMM searches. *PLoS Comput. Biol.* 7:e1002195. doi: 10.1371/journal.pcbi.1002195

Fernandez, L., and Hancock, R. E. (2012). Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. *Clin. Microbiol. Rev.* 25, 661–681. doi: 10.1128/CMR.00043-12

Finn, R. D., Bateman, A., Clements, J., Coggill, P., Eberhardt, R. Y., Eddy, S. R., et al. (2014). Pfam: the protein families database. *Nucleic. Acids Res.* 42, D222–D230. doi: 10.1093/nar/gkt1223

Fu, L., Niu, B., Zhu, Z., Wu, S., and Li, W. (2012). CD-HIT: accelerated for clustering the next-generation sequencing data. *Bioinformatics* 28, 3150–3152. doi: 10.1093/bioinformatics/bts565

Grkovic, S., Brown, M. H., Roberts, N. J., Paulsen, I. T., and Skurray, R. A. (1998). QacR is a repressor protein that regulates expression of the *Staphylococcus aureus* multidrug efflux pump QacA. *J. Biol. Chem.* 273, 18665–18673. doi: 10.1074/jbc.273.29.18665

Hassan, K. A., Jackson, S. M., Penesyan, A., Patching, S. G., Tetu, S. G., Eijkelkamp, B. A., et al. (2013). Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc. Natl. Acad. Sci. U.S.A.* 110, 20254–20259. doi: 10.1073/pnas.1317052110

Hassan, K. A., Li, Q., Henderson, P. J. F., and Paulsen, I. T. (2015). Homologs of the *Acinetobacter baumannii* aceI transporter represent a new family of bacterial multidrug efflux systems. *mBio* 6, e01982–e01914. doi: 10.1128/mBio.01982-14

Kaatz, G. W., and Seo, S. M. (2004). Effect of substrate exposure and other growth condition manipulations on *norA* expression. *J. Antimicrob. Chemother.* 54, 364–369. doi: 10.1093/jac/dkh341

Keseler, I. M., Mackie, A., Peralta-Gil, M., Santos-Zavaleta, A., Gama-Castro, S., Bonavides-Martinez, C., et al. (2013). EcoCyc: fusing model organism databases with systems biology. *Nucleic. Acids Res.* 41, D605–D612. doi: 10.1093/nar/gks1027

Kristoffersen, S. M., Ravnum, S., Tourasse, N. J., Okstad, O. A., Kolsto, A. B., and Davies, W. (2007). Low concentrations of bile salts induce stress responses and reduce motility in *Bacillus cereus* ATCC 14579. *J. Bacteriol.* 189, 5302–5313. doi: 10.1128/JB.00239-07

Li, M., Gu, R., Su, C. C., Routh, M. D., Harris, K. C., Jewell, E. S., et al. (2007). Crystal structure of the transcriptional regulator AcrR from *Escherichia coli*. *J. Mol. Biol.* 374, 591–603. doi: 10.1016/j.jmb.2007.09.064

McDonnell, G., and Russell, A. D. (1999). Anitseptics and disinfectants: activity, action and resistance. *Clin. Microbiol. Rev.* 12, 147–179.

Morita, Y., Tomida, J., and Kawamura, Y. (2014). Responses of *Pseudomonas aeruginosa* to antimicrobials. *Front. Microbiol.* 4:422. doi: 10.3389/fmicb.2013.00422

Nde, C. W., Jang, H. J., Toghrol, F., and Bentley, W. E. (2009). Global transcriptomic response of *Pseudomonas aeruginosa* to chlorhexidine diacetate. *Environ. Sci. Technol.* 43, 8406–8415. doi: 10.1021/es9015475

Piddock, L. J. (2006). Multidrug-resistance efflux pumps - not just for resistance. *Nat. Rev. Microbiol.* 4, 629–636. doi: 10.1038/nrmicro1464

Rajamohan, G., Srinivasan, V. B., and Gebreyes, W. A. (2009). Novel role of *Acinetobacter baumannii* RND efflux transporters in mediating decreased susceptibility to biocides. *J. Antimicrob. Chemother.* 65, 228–232. doi: 10.1093/jac/dkp427

Ren, Q., and Paulsen, I. T. (2007). Large-scale comparative genomic analyses of cytoplasmic membrane transport systems in prokaryotes. *J. Mol. Microbiol. Biotechnol.* 12, 165–179. doi: 10.1159/000099639

Russell, A. D. (1986). Chlorhexidine: antibacterial action and bacterial resistance. *Infection* 14, 212–215. doi: 10.1007/BF01644264

Schindler, B. D., Frempong-Manso, E., Demarco, C. E., Kosmidis, C., Matta, V., Seo, S. M., et al. (2015). Analyses of multidrug efflux pump-like proteins encoded on the *Staphylococcus aureus* chromosome. *Antimicrob. Agents Chemother.* 59, 747–748. doi: 10.1128/AAC.04678-14

Schumacher, M. A., Miller, M. C., Grkovic, S., Brown, M. H., Skurray, R. A., and Brennan, R. G. (2001). Structural mechanisms of QacR induction and multidrug recognition. *Science* 294, 2158–2163. doi: 10.1126/science.1066020

Shaw, K. J., Miller, N., Liu, X., Lerner, D., Wan, J., Bittner, A., et al. (2003). Comparison of the changes in global gene expression of *Escherichia coli* induced by four bactericidal agents. *J. Mol. Microbiol. Biotechnol.* 5, 105–122. doi: 10.1159/000069981

Simm, R., Voros, A., Ekman, J. V., Sodring, M., Nes, I., Kroeger, J. K., et al. (2012). BC4707 is a major facilitator superfamily multidrug resistance transport protein from *Bacillus cereus* implicated in fluoroquinolone tolerance. *PLoS ONE* 7:e36720. doi: 10.1371/journal.pone.0036720

Tal, N., and Schuldiner, S. (2009). A coordinated network of transporters with overlapping specificities provides a robust survival strategy. *Proc. Natl. Acad. Sci. U.S.A.* 106, 9051–9056. doi: 10.1073/pnas.0902400106

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Chapter 4 Fluorescence-based flow sorting in parallel with transposon insertion site sequencing identifies multidrug efflux systems in *Acinetobacter baumannii*

AMERICAN SOCIETY FOR MICROBIOLOGY | mBio®

OBSERVATION

Fluorescence-Based Flow Sorting in Parallel with Transposon Insertion Site Sequencing Identifies Multidrug Efflux Systems in *Acinetobacter baumannii*

Karl A. Hassan,[a] Amy K. Cain,[b,c] TaoTao Huang,[a] Qi Liu,[a] Liam D. H. Elbourne,[a] Christine J. Boinett,[b] Anthony J. Brzoska,[a] Liping Li,[a] Martin Ostrowski,[a] Nguyen Thi Khanh Nhu,[d,e] Tran Do Hoang Nhu,[d] Stephen Baker,[d,f] Julian Parkhill,[b] Ian T. Paulsen[a]

Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia[a]; Wellcome Trust Sanger Institute, Hinxton, Cambridge, United Kingdom[b]; Liverpool School of Tropical Medicine, Malawi-Liverpool-Wellcome Trust Clinical Research Programme, Blantyre, Malawi[c]; The Hospital for Tropical Diseases, Wellcome Trust Major Overseas Programme, Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam[d]; School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Queensland, Australia[e]; Centre for Tropical Medicine, Nuffield Department of Clinical Medicine, Oxford University, Oxford, United Kingdom[f]

ABSTRACT **Multidrug efflux pumps provide clinically significant levels of drug resistance in a number of Gram-negative hospital-acquired pathogens. These pathogens frequently carry dozens of genes encoding putative multidrug efflux pumps. However, it can be difficult to determine how many of these pumps actually mediate antimicrobial efflux, and it can be even more challenging to identify the regulatory proteins that control expression of these pumps. In this study, we developed an innovative high-throughput screening method, combining transposon insertion sequencing and cell sorting methods (TraDISort), to identify the genes encoding major multidrug efflux pumps, regulators, and other factors that may affect the permeation of antimicrobials, using the nosocomial pathogen *Acinetobacter baumannii*. A dense library of more than 100,000 unique transposon insertion mutants was treated with ethidium bromide, a common substrate of multidrug efflux pumps that is differentially fluorescent inside and outside the bacterial cytoplasm. Populations of cells displaying aberrant accumulations of ethidium were physically enriched using fluorescence-activated cell sorting, and the genomic locations of transposon insertions within these strains were determined using transposon-directed insertion sequencing. The relative abundance of mutants in the input pool compared to the selected mutant pools indicated that the AdeABC, AdeIJK, and AmvA efflux pumps are the major ethidium efflux systems in *A. baumannii*. Furthermore, the method identified a new transcriptional regulator that controls expression of *amvA*. In addition to the identification of efflux pumps and their regulators, TraDISort identified genes that are likely to control cell division, cell morphology, or aggregation in *A. baumannii*.**

IMPORTANCE **Transposon-directed insertion sequencing (TraDIS) and related technologies have emerged as powerful methods to identify genes required for bacterial survival or competitive fitness under various selective conditions. We applied fluorescence-activated cell sorting (FACS) to physically enrich for phenotypes of interest within a mutant population prior to TraDIS. To our knowledge, this is the first time that a physical selection method has been applied in parallel with TraDIS rather than a fitness-induced selection. The results demonstrate the feasibility of this combined approach to generate significant results and highlight the major multidrug efflux pumps encoded in an important pathogen. This FACS-based approach, TraDISort, could have a range of future applications, including the characterization of efflux pump inhibitors, the identification of regulatory factors controlling gene or protein expression using fluorescent reporters, and the identification of genes involved in cell replication, morphology, and aggregation.**

Received 16 July 2016 **Accepted** 12 August 2016 **Published** 6 September 2016

Citation Hassan KA, Cain AK, Huang T, Liu Q, Elbourne LDH, Boinett CJ, Brzoska AJ, Li L, Ostrowski M, Nhu NTK, Nhu TDH, Baker S, Parkhill J, Paulsen IT. 2016. Fluorescence-based flow sorting in parallel with transposon insertion site sequencing identifies multidrug efflux systems in *Acinetobacter baumannii*. mBio 7(5):e01200-16. doi:10.1128/mBio.01200-16.

Editor Claire M. Fraser, University of Maryland School of Medicine

Address correspondence to Karl A. Hassan, karl.hassan@mq.edu.au.

To be effective in killing or stalling the growth of bacterial cells, antimicrobials must reach their cellular targets. For the majority of antimicrobials, these targets are in the cytoplasm, meaning that they must cross the cell envelope to induce their effects. The cell envelope is a particularly important factor for antimicrobial resistance in Gram-negative bacteria, since it includes two membrane permeability barriers with different surface chemistries, presenting significant potential to limit the accumulation of chemically diverse antimicrobial compounds (1).

In addition to preventing accumulation of antimicrobials, all bacteria employ sets of efflux pumps that mediate the active expulsion of these compounds should they cross a biological membrane (2). Many antimicrobial efflux pumps in bacteria have multidrug recognition profiles. Therefore, the increased expression of a single pump can result in resistance to a broad spectrum of antimicrobial classes. In Gram-negative bacteria, efflux pump overexpression has been shown to promote clinically significant levels of antimicrobial resistance (3). Genes encoding efflux

pumps have been identified in all bacterial genomes sequenced to date and can be found in large numbers (4). For example, strains of the opportunistic human pathogen *Acinetobacter baumannii* typically encode more than 50 putative efflux pumps, accounting for approximately 1.5% of their protein coding potential (5).

Despite their abundance, only a few transporters resembling drug efflux pumps have been experimentally characterized in most bacterial species. It can be difficult to discern which, if any, of the uncharacterized pumps could play an active role in protecting the cell against cytotoxic compounds without conducting labor-intensive experimental investigations. Furthermore, it can be even more challenging to identify the regulatory proteins that control expression of active multidrug efflux pumps. In this study, we sought to identify these proteins in *A. baumannii* by directly assessing drug accumulation within a population of more than 100,000 random transposon mutants. To this end, we applied fluorescence-activated cell sorting (FACS) in parallel with transposon-directed insertion sequencing (TraDIS) (6, 7). This novel approach, which we have named "TraDISort," was able to identify genes in *A. baumannii* that are associated with increased or decreased accumulation of ethidium bromide, a cationic quaternary ammonium derivative and a common substrate of multidrug efflux pumps.

Fluorescence-activated cell sorting to enrich for mutants displaying aberrant accumulation of ethidium. Ethidium readily intercalates into nucleic acids, whereupon its fluorescence intensity increases significantly. Consequently, ethidium is differentially fluorescent inside and outside cells, and cellular fluorescence can be used as a proxy for its cytoplasmic concentration (8). We hypothesized that when cells are treated with a subinhibitory concentration of ethidium, the ethidium concentrations in the cytoplasm of cells with defective multidrug efflux machinery should be higher than the concentration in wild-type cells at equilibrium, and conversely, the concentration in cells with overactive efflux machinery should be below that in wild-type cells To test this hypothesis, we examined populations of three isogenic strains of *A. baumannii* AB5057-UW (9) that differentially expressed AdeIJK, a major multidrug efflux pump in *A. baumannii*, which recognizes ethidium as a substrate (10, 11): (i) wild-type AB5075-UW, (ii) a mutant containing a transposon insertion in *adeJ*, and (iii) a mutant containing a transposon insertion in *adeN*, which encodes a negative regulator of *adeIJK* expression (9). When examined by flow cytometry, populations of the different cell types displayed distinct but partially overlapping fluorescence profiles that were in agreement with our predictions, i.e., the average fluorescence of the *adeJ* and *adeN* mutant populations was above and below that of the wild-type population, respectively (see Fig. S1A in the supplemental material). We repeated this experiment, using equivalent isogenic strains of *Acinetobacter baylyi* ADP1 (5), and made the same observations (see Fig. S1B). Based on these experiments, we predicted that it would be possible to use FACS to enrich cells from a large mutant pool that display differential ethidium accumulation or efflux based on their fluorescence intensity.

A mutant library containing more than 100,000 unique insertion mutants of *A. baumannii* BAL062 was generated using a Tn*5*-based custom transposon, and the insertion sites in the mutant pool were mapped by TraDIS (7). This library was treated with 40 μM ethidium bromide (1/16× MIC of the parental strain) and subjected to FACS to collect cells containing the highest concentrations of ethidium (i.e., the 2% most fluorescent cells) and cells containing the lowest concentrations of ethidium (i.e., the 2% least fluorescent cells). DNA was isolated from the selected pools of cells, and TraDIS was used to identify the chromosomal locations of the Tn*5* insertion sites in these cells (7). Transposon insertions were significantly (>2-fold change; *Q* value, <0.05) less abundant in 162 genes and more abundant in 24 genes in the low-fluorescence population and less abundant in 159 genes and more abundant in 24 genes in the high-fluorescence population compared to the input pool (see Data Set S1 in the supplemental material).

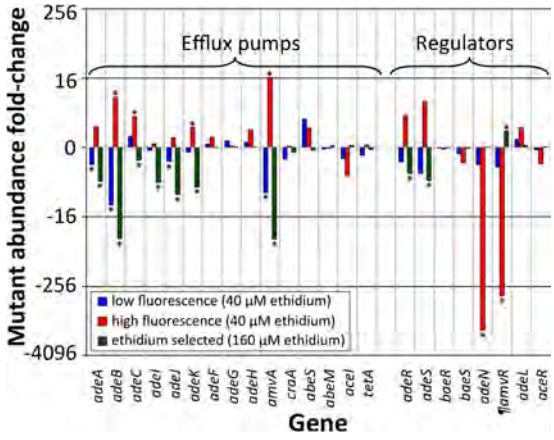

FIG 1 Selection of *A. baumannii* mutants carrying insertions in genes encoding the characterized efflux pumps AdeABC (12), AdeIJK (10), AdeFGH (20), AmvA (13, 14), CraA (21), AbeS (22), AbeM (23), and AceI (24, 25) and regulators AdeRS and BaeRS, which control expression of *adeABC* (15, 26); AdeN, which controls *adeIJK* (16); AdeL, which controls *adeFGH* (20); and AceR, which controls *aceI* (27). Bars represent the fold change in mutant abundance in cells selected for low ethidium fluorescence (blue), high ethidium fluorescence (red), or growth in 62.5 μg/ml (approximately 158 μM) ethidium bromide (hatched green; 1/4× MIC) compared to the starting mutant pool. Positive values indicate higher mutant abundance in the selected pool, whereas negative values indicate lower abundance. Asterisks indicate values supported by a *Q* value of 0.05 or below. ¶, the gene named here as *amvR* encodes a TetR family regulator that represses *amvA* gene expression (see text for details).

FACS in parallel with TraDIS identifies the active ethidium efflux pumps encoded by *A. baumannii* and core efflux pump regulators. Following the experiments with targeted mutants, we hypothesized that many cells containing the highest concentrations of ethidium would have transposon insertions in genes encoding efflux pumps or activators of efflux pumps, and conversely, cells containing the lowest concentrations of ethidium would have insertions in genes encoding negative regulators of efflux pumps. Comparisons of the insertion sites in the mutant input pool with those in the high- and low-fluorescence pools supported this proposal (Fig. 1; see also Data Set S1 in the supplemental material). Mutants carrying insertions in genes encoding several multidrug efflux pumps, particularly *adeABC* (12), *adeIJK* (10), and *amvA* (13, 14), and genes encoding the *adeABC* activator, *adeRS* (15), were overrepresented in the highly fluorescent populations (Fig. 1). Inactivation of these genes is likely to reduce the rate of efflux and thus result in a higher cytosolic concentration of ethidium. In contrast, inactivated mutants of these genes were less abundant in the low-fluorescence populations (Fig. 1), since the efflux pumps encoded or regulated by these genes help to lower the concentration of ethidium in the cell. We used the

Transporter Automated Annotation Pipeline (http://www.membranetransport.org/) to search for genes encoding novel efflux pumps in the *A. baumannii* BAL062 genome. We identified 56 genes that are likely to encode novel efflux pumps, or components of novel efflux pumps, based on their primary sequence characteristics (see Table S1 in the supplemental material). These efflux pumps are likely to recognize small-molecule substrates, but our data did not suggest that any of these efflux pumps have a significant *in vivo* role in ethidium efflux, since none were significantly differentially selected by our fluorescence-based selection (see Table S1).

Some of the most highly differentially selected genes in the flow-sorted samples were genes that encode transcriptional repressors known or predicted to control expression of multidrug efflux systems. For example, mutants carrying insertions in *adeN*, which controls expression of *adeIJK* (16), were 1,469-fold less abundant in the highly fluorescent output pool compared to the input pool (Fig. 1). Additionally, mutants carrying insertions in BAL062_01495, which encodes a TetR family regulator, were 371-fold less abundant in the highly fluorescent output pool compared to the input pool (Fig. 1). BAL062_01495 is adjacent to and divergently transcribed from *amvA* in the BAL062 chromosome. To test whether the TetR family protein encoded by BAL062_01495 was able to regulate expression of *amvA*, we compared *amvA* expression levels in the *A. baumannii* AB5075-UW parental strain and a strain harboring a transposon insertion in the gene orthologous to BAL062_01495. The level of *amvA* expression measured by reverse transcription-quantitative PCR (qRT-PCR) (5) in the mutant strain was 5.7- ± 1.9-fold higher than that in the parental strain during late exponential phase, indicating that the TetR family regulator controls expression of *amvA*. Consequently, we have tentatively named this novel regulator AmvR.

To confirm the specific involvement of different multidrug efflux pumps and their regulators in controlling the accumulation of ethidium in *A. baumannii*, we conducted flow cytometry on targeted mutants of *adeB*, *adeR*, *adeJ*, *adeN*, *adeG*, *adeL*, *amvA*, *amvR*, *craA*, *abeS*, and *abeM*. These mutant strains were loaded with 40 μM ethidium bromide, and the fluorescence of 10,000 cells was determined by flow cytometry (Fig. 2). The TraDISort method identified the AdeABC, AdeIJK, and AmvA efflux systems and their regulators, AdeRS, AdeN, and AmvR, as playing a role in ethidium accumulation. The fluorescence profiles of the specific mutant populations closely reflected these findings. As seen in our preliminary experiments (see Fig. S1 in the supplemental material), the average fluorescence of the *adeJ* and *adeN* mutant populations was above and below that of the parental cell population, respectively (Fig. 2B). The *amvA* and *amvR* mutant cells showed fluorescence profiles very similar to those of *adeJ* and *adeN* mutants, respectively (Fig. 2D), in line with the function of AmvR as a repressor of *amvA* expression. The average fluorescence of the *adeB* and *adeR* mutant cell populations was a similar degree higher than that of the parental population, highlighting the role of AdeB in ethidium efflux and of AdeR in controlling the expression of *adeABC* (Fig. 2A). The fluorescence profiles of mutant populations of other multidrug efflux systems, which were not identified using the TraDISort approach, were very similar to that of the parental strain (Fig. 2).

TraDIS following fitness-induced selection using ethidium bromide. In addition to FACS to enrich for cells displaying aberrant accumulation of ethidium, we cultured the mutant library in the presence of ethidium bromide. This experiment used a higher concentration of ethidium bromide (1/4× MIC of the parental strain) than that used in the FACS analyses to impose a chemical selection that would allow us to identify mutants with a fitness advantage or defect in the presence of ethidium by TraDIS. In the ethidium bromide-selected mutant pools, transposon insertions were less abundant in 63 genes and more abundant in eight genes compared with the input control pools. This suggests that gene loss generally results in a fitness defect, rather than advantage, under ethidium selection, which is in keeping with general evolutionary theory. Mutants containing transposon insertions in efflux pump genes and their regulators were the most highly differentially selected by ethidium bromide. The pattern of selection among these mutants overlapped with the selection pattern in the low-ethidium-fluorescence FACS experiment (Fig. 1). For example, mutants carrying transposon insertions in the *adeABC*, *adeIJK*, *amvA*, and *adeRS* genes were less abundant in the ethidium-

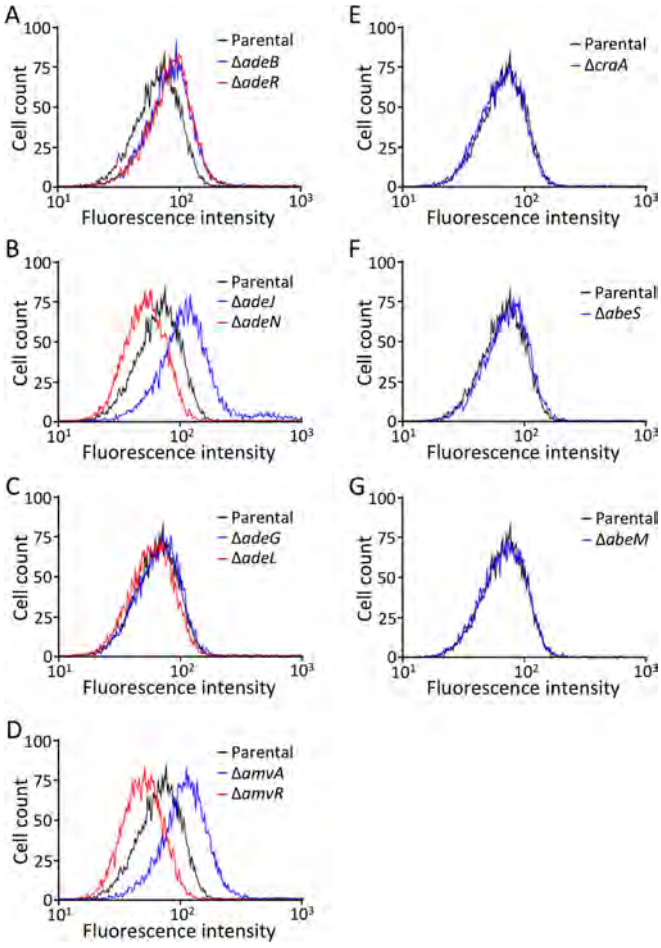

FIG 2 Flow cytometric analysis of *Acinetobacter baumannii* AB5075-UW parental strain (black), inactivated efflux pump mutants (blue), and inactivated efflux regulatory mutants (red). The fluorescence profile of the parental population is shown in all panels and is overlaid with the profiles of Δ*adeB* and Δ*adeR* (A), Δ*adeJ* and Δ*adeN* (B), Δ*adeG* and Δ*adeL* (C), Δ*amvA* and Δ*amvR* (D), Δ*craA* (E), Δ*abeS* (F), and Δ*abeM* (G). Cell populations were exposed to 40 μM ethidium bromide, and each curve shows the fluorescence intensity for 10,000 cells. The cell populations show distinct fluorescence profiles based on the concentration of ethidium in the cell cytoplasm.

selected output pool (Fig. 1), confirming the role of these multidrug efflux pumps and regulators in resistance to ethidium.

Similarly to efflux pump genes and their regulators, mutations in the DNA modification methylase gene, BAL062_03687, were significantly negatively selected by ethidium bromide and less abundant in the low-fluorescence samples compared to the input pool. Methylation mediated by BAL062_03687 could protect DNA from ethidium intercalation and thereby reduce fluorescence and provide resistance to ethidium-induced mutation.

Several genes controlling the composition of the cell membranes, cell wall, or capsule were also negatively selected by ethidium bromide (BAL062_00585, BAL062_00596, BAL062_01038, BAL062_03374, BAL062_03418, BAL062_03480, BAL062_03481, BAL062_03674, and BAL062_03869 [see Data Set S1 in the supplemental material]). These genes may help to reduce uptake of ethidium. Some of these genes were significantly negatively selected in both the low- and high-fluorescence FACS-selected samples and could thus play a role in controlling cell morphology or size (see below). In contrast, several capsule biosynthesis genes (BAL062_03853, BAL062_03857, and BAL062_03858) were positively selected by the ethidium treatment. This highlights the influence that the sugar composition of the capsule could play in regulating the accumulation of amphipathic small molecules into the cell.

FACS in parallel with TraDIS identifies genes involved in cell division and aggregation. In conducting FACS to enrich for mutants displaying aberrant accumulation of ethidium in *A. baumannii*, we gated to target cells with uniform forward and side scatter and limited the collection of dead or aggregated cells that may complicate downstream analyses (see Fig. S2 in the supplemental material). As a consequence of this gating, we identified a number of mutants that are likely to have cell division defects or enhanced aggregation properties. These mutants were negatively selected in both the low- and high-fluorescence FACS-selected pools relative to the input pool, and included 80 (49.4 to 50.3%) of the significantly selected genes in these pools. For example, mutants carrying insertions in the *mreBCD* gene cluster (BAL062_00713 to BAL062_00715), *rlpA* (BAL062_01224), *rodA* (BAL062_01226), and *ftsI* (BAL062_02811), which are likely to function in cell division, were in very low abundance in each of the flow-sorted mutant pools relative to the input pool (see Fig. S3). Mutants carrying insertions in biotin biosynthesis genes were also significantly less abundant in the FACS-selected pools than in the input pool and, to a lesser extent, in the ethidium-selected pools. The role of biotin in ethidium resistance, cell structure, or aggregation is at present unknown but may be related to its function as a cofactor in fatty acid synthesis. Two capsular polysaccharide biosynthetic genes were significantly less abundant in both of the flow-sorted mutant pools than in the input pool. These mutants may have a higher tendency toward aggregation or different cell morphologies or may display light-scattering properties different from those of other mutant cells (see Fig. S3). Approximately 20% of the inactivated genes in mutants negatively selected by FACS were annotated as hypothetical proteins, and many more had been assigned only putative functions. These genes could be targeted in future investigations exploring cell division and aggregation/biofilm formation in *A. baumannii*.

While insertions in genes implicated in cell replication and increased aggregation were negatively selected by the flow sorting, there appeared to be enrichment for mutants that are less likely to aggregate in culture. The majority of these mutants harbored transposon insertions in the *csu* type I pilus biosynthesis and regulatory gene cluster (BAL062_01328 to BAL062_01334 [see Fig. S4 in the supplemental material]). These genes are likely to function in biotic or abiotic cell adherence/aggregation and biofilm formation (17). Therefore, we suspect that the strains carrying mutations in these genes are less likely to aggregate, leading to their enrichment in our flow-sorted samples.

Conclusions. In this study, we identified the genes that control accumulation of the antimicrobial dye ethidium into the Gram-negative hospital-associated pathogen *A. baumannii*. We exploited the differential fluorescence of ethidium inside and outside the cell to enrich for mutants showing aberrant accumulation of ethidium by FACS and used TraDIS to identify the transposon insertion sites within the enriched mutants. This work highlighted the importance of three multidrug efflux systems, AdeABC, AdeIJK, and AmvA, in reducing ethidium accumulation and promoting resistance. We also confirmed the importance of two regulatory systems, AdeRS and AdeN, that control expression of two of these pumps and identified the first known regulator for the AmvA efflux pump, which we have called AmvR. These results demonstrate the utility of the TraDISort method in identifying bacterial multidrug resistance efflux pumps and will be particularly useful when studying bacterial species for which little is known with respect to the major efflux systems. In addition to the core efflux pumps, the TraDISort method identified a large number of novel genes that are likely to be involved in cell division and/or aggregation. This application considerably expands the scope of utility for this method.

To our knowledge, this study represents the first time that FACS or any other physical selection method has been applied in parallel with TraDIS to physically enrich for phenotypes of interest in mutant populations prior to sequencing. The results demonstrate the feasibility of this combined approach to generate statistically significant results and avoid potential false positives that can arise in traditional fluorescent screening approaches, where individual strains are isolated and studied. In addition to those applications described above, we anticipate that FACS applied in parallel with TraDIS could have a range of additional applications in microbiological research: for example, to rapidly screen saturation mutant libraries carrying fluorescent reporters for genes involved in regulation, to identify the efflux pumps inhibited by novel efflux inhibitors, and to inform *in vitro* evolution studies with fluorescent reporters to identify mutants with improved metabolic productivity (18).

Ethidium accumulation in isogenic *Acinetobacter* mutants measured by flow cytometry. *Acinetobacter baumannii* AB5075-UW and Tn*26* insertion mutants of *adeB* (ABUW_1975-150::T26), *adeR* (ABUW_1973-195::T26), *adeJ* (ABUW_0843-122::T26), *adeN* (ABUW_1731-148::T26) *adeG* (ABUW_1335-195::T26), *adeL* (ABUW_1338-193::T26), *amvA* (ABUW_1679-169::T26), *amvR* (ABUW_1678-136::T26), *craA* (ABUW_0337-173::T26), *abeS* (ABUW_1343-187::T101), and *abeM* (ABUW_3486-184::T26) were obtained from the Manoil lab collection (9). The strains were grown in Mueller-Hinton (MH; Oxoid) broth with shaking overnight, diluted 1:100 in fresh MH broth, grown to late exponential phase, and diluted to an optical density at 600 nm (OD_{600}) of 0.6 in MH broth containing 40 μM ethidium bromide (Sigma-Aldrich), approximately 1/16 of the MIC for the parental strain (250 μg/ml). This concentration is below the MIC for all

strains tested and provided good fluorescent resolution between cells differentially expressing an efflux pump. The cells were incubated at room temperature for 20 min and then further diluted to an OD_{600} of 0.018 in MH broth containing 40 μM ethidium bromide for flow cytometric analyses. The ethidium fluorescence of 10,000 cells from each population was examined on a BD Influx flow cytometer using a 200-mW 488-nm laser (Coherent Sapphire) equipped with a small particle forward scatter detector. Ethidium bromide fluorescence was detected using a 580/30 bandpass filter. The cells were counted from within populations gated by forward scatter versus forward scatter pulse width, to discriminate against aggregated cells, followed by forward and side scatter to ensure that only living cells of uniform size were examined (see Fig. S2 in the supplemental material). *Acinetobacter baylyi* ADP1 wild type and *adeJ* and *adeN* mutants, generated in our previous studies (5), were examined according to the same method, except that 15 μM ethidium bromide was used due to the higher susceptibility of this strain to ethidium.

Transposon mutant library generation and verification by TraDIS. A dense Tn*5* mutant library was constructed in *A. baumannii* BAL062, a global clone II isolate (ENA accession numbers LT594095 to LT594096), as previously described (6, 7). Briefly, a custom transposome that included a kanamycin resistance cassette amplified from the pUT-km1 plasmid was generated using the EZ-Tn5 custom transposome construction kit (Epicentre). The custom transposome was electroporated into BAL062, and the cells were plated on kanamycin selective medium (10 mg/liter). More than 100,000 mutants were collected and stored as glycerol stocks at −80°C. Aliquots of stock containing approximately 10^9 cells were grown overnight in MH broth. Genomic DNA was isolated from the cultures, and the transposon insertion sites were sequenced across four lanes of the Illumina HiSeq sequencing system. The insertion sites were mapped and analyzed statistically using protocols and bioinformatic tools in the TraDIS toolkit (7). The number of insertions per gene, as a factor of gene size (insertion index), was calculated for cells grown in MH broth to illustrate the evenness of transposon insertions across the genome and to show that the library was sufficiently saturated for experimental analyses. Insertions at the extreme 3′ end (last 10%) of each gene were filtered since they may not inactivate the gene. When the data were plotted against frequency, we observed a bimodal distribution of insertion indexes in the BAL062 library, with the peaks correlating with genes that tolerate or do not tolerate insertions when cultured under permissive growth conditions (see Fig. S5 in the supplemental material) (19). Using the method described in reference 20, as executed through the TraDIS toolbox (7), essential genes were identified as those with an insertion index below 0.0047 (n = 475) and were excluded from later analyses (see Fig. S5). On average, among the nonessential genes (n = 3,362) there were 35.9 unique insertions per kb of gene sequence (see Fig. S5).

FACS to enrich for *A. baumannii* mutants showing aberrant accumulation of ethidium. An aliquot of BAL062 mutant library stock containing approximately 10^9 cells was grown overnight in MH broth. The overnight culture was diluted 1:100 and grown to late exponential phase (OD_{600} of 5.5). The cells were diluted to an OD_{600} of 0.6 in MH broth containing 40 μM ethidium bromide (approximately 1/16 of the MIC of the parental strain) and then further diluted 1:100 in 40 μM ethidium bromide for FACS. This concentration of ethidium bromide was used because it provided excellent differentiation between mutants known to differentially accumulate ethidium (see Fig. S1 in the supplemental material) and was well below the MIC of these mutants, so that it would not cause changes to the mutant ratios because of cell death during the sorting procedure. Cells were sorted using a BD Influx flow cytometer on the basis of ethidium fluorescence (as described above) using the highest purity mode (1 drop single). Single cells with uniform forward and side scatter were gated, and pools of the most highly and weakly fluorescent cells (2% of total single cells) within this gate were collected in separate tubes containing fresh MH broth (150,000 to 175,000 cells across four replicates [see Table S3 in the supplemental material]). The cells collected were grown overnight, DNA was isolated, and insertion sites were mapped by TraDIS as described above. Comparisons between ratios of insertion sites in the control and experimental mutant pools were made using the statistical comparison scripts in the TraDIS toolbox (7). Genes with fewer than 10 mapped reads in any data set being compared were excluded from the analyses. Genes described as being significantly differentially selected between the control and experimental samples were those showing a greater than 2-fold change in mutant abundance with a Q value below 0.05.

For comparison to the FACS-enriched mutants, we also selected mutants based on their competitive fitness in ethidium bromide. An aliquot of BAL062 mutant library stock containing approximately 10^9 cells was grown overnight in MH broth. The overnight culture was diluted 1:100 and grown overnight in 62.5 μg/ml (158.5 μM) of ethidium bromide (equivalent to 1/4 of the MIC for the parental strain) to impose a chemical selection that would allow us to identify mutants with a fitness advantage or defect in the presence of ethidium bromide. Genomic DNA was isolated, and the insertion sites were determined by TraDIS. A replicate experiment with no ethidium was used as the reference in these experiments.

Accession number(s). The TraDIS sequence data files were deposited into the European Nucleotide Archive under accession numbers listed in Table S2 in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01200-16/-/DCSupplemental.

Figure S1, JPG file, 0.1 MB.
Figure S2, JPG file, 0.2 MB.
Figure S3, JPG file, 0.3 MB.
Figure S4, JPG file, 0.2 MB.
Figure S5, JPG file, 0.1 MB.
Data Set S1, XLSX file, 0.5 MB.
Table S1, DOCX file, 0.2 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.

FUNDING INFORMATION

This work, including the efforts of Julian Parkhill, was funded by Wellcome Trust (098051). This work, including the efforts of Stephen Baker, was funded by Wellcome Trust (100087/Z/12/Z). This work, including the efforts of Amy K Cain and Christine Boinett, was funded by Medical Research Council (MRC) (G1100100/1). This work, including the efforts of Stephen Baker, was funded by Royal Society (100087/Z/12/Z). This work, including the efforts of Karl A Hassan and Ian T Paulsen, was funded by Department of Health | National Health and Medical Research

Council (NHMRC) (1060895). This work, including the efforts of Karl A Hassan and Liam D.H. Elbourne, was funded by Macquarie University (9201401563).

This work was supported by National Health and Medical Research Council (Australia) Project Grant (1060895) to ITP and KAH, a Macquarie University Research Development Grant (9201401563) to KAH and LDHE, and a Wellcome Trust grant (number 098051) to JP. AKC and CJB were supported by the Medical Research Council [grant number G1100100/1]. SB is a Sir Henry Dale Fellow, jointly funded by the Wellcome Trust and the Royal Society (100087/Z/12/Z).

REFERENCES

1. **Schweizer HP**. 2012. Understanding efflux in gram-negative bacteria: opportunities for drug discovery. Expert Opin Drug Discov **7:**633–642. http://dx.doi.org/10.1517/17460441.2012.688949.
2. **Paulsen IT, Chen J, Nelson KE, Saier MH, Jr.** 2001. Comparative genomics of microbial drug efflux systems. J Mol Microbiol Biotechnol **3:**145–150.
3. **Piddock LJ**. 2006. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clin Microbiol Rev **19:**382–402. http://dx.doi.org/10.1128/CMR.19.2.382-402.2006.
4. **Ren Q, Paulsen IT**. 2007. Large-scale comparative genomic analyses of cytoplasmic membrane transport systems in prokaryotes. J Mol Microbiol Biotechnol **12:**165–179. http://dx.doi.org/10.1159/000099639.
5. **Brzoska AJ, Hassan KA, de Leon EJ, Paulsen IT, Lewis PJ**. 2013. Single-step selection of drug resistant Acinetobacter baylyi ADP1 mutants reveals a functional redundancy in the recruitment of multidrug efflux systems. PLoS One **8:**e56090. http://dx.doi.org/10.1371/journal.pone.0056090.
6. **Langridge GC, Phan MD, Turner DJ, Perkins TT, Parts L, Haase J, Charles I, Maskell DJ, Peters SE, Dougan G, Wain J, Parkhill J, Turner AK**. 2009. Simultaneous assay of every Salmonella typhi gene using one million transposon mutants. Genome Res **19:**2308–2316. http://dx.doi.org/10.1101/gr.097097.109.
7. **Barquist L, Mayho M, Cummins C, Cain AK, Boinett CJ, Page AJ, Langridge GC, Quail MA, Keane JA, Parkhill J**. 2016. The TraDIS toolkit: sequencing and analysis for dense transposon mutant libraries. Bioinformatics **32:**1109–1111. http://dx.doi.org/10.1093/bioinformatics/btw022.
8. **Lambert B, Le Pecq JB**. 1984. Effect of mutation, electric membrane potential, and metabolic inhibitors on the accessibility of nucleic acids to ethidium bromide in *Escherichia coli* cells. Biochemistry **23:**166–176. http://dx.doi.org/10.1021/bi00296a027.
9. **Gallagher LA, Ramage E, Weiss EJ, Radey M, Hayden HS, Held KG, Huse HK, Zurawski DV, Brittnacher MJ, Manoil C**. 2015. Resources for genetic and genomic analysis of emerging pathogen Acinetobacter baumannii. J Bacteriol **197:**2027–2035. http://dx.doi.org/10.1128/JB.00131-15.
10. **Damier-Piolle L, Magnet S, Brémont S, Lambert T, Courvalin P**. 2008. AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in Acinetobacter baumannii. Antimicrob Agents Chemother **52:**557–562. http://dx.doi.org/10.1128/AAC.00732-07.
11. **Rajamohan G, Srinivasan VB, Gebreyes WA**. 2010. Novel role of Acinetobacter baumannii RND efflux transporters in mediating decreased susceptibility to biocides. J Antimicrob Chemother **65:**228–232. http://dx.doi.org/10.1093/jac/dkp427.
12. **Magnet S, Courvalin P, Lambert T**. 2001. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in Acinetobacter baumannii strain BM4454. Antimicrob Agents Chemother **45:**3375–3380. http://dx.doi.org/10.1128/AAC.45.12.3375-3380.2001.
13. **Rajamohan G, Srinivasan VB, Gebreyes WA**. 2010. Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant resistance in Acinetobacter baumannii. J Antimicrob Chemother **65:**1919–1925. http://dx.doi.org/10.1093/jac/dkq195.
14. **Hassan KA, Brzoska AJ, Wilson NL, Eijkelkamp BA, Brown MH, Paulsen IT**. 2011. Roles of DHA2 family transporters in drug resistance and iron homeostasis in Acinetobacter spp. J Mol Microbiol Biotechnol **20:**116–124. http://dx.doi.org/10.1159/000325367.
15. **Marchand I, Damier-Piolle L, Courvalin P, Lambert T**. 2004. Expression of the RND-type efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two-component system. Antimicrob Agents Chemother **48:**3298–3304. http://dx.doi.org/10.1128/AAC.48.9.3298-3304.2004.
16. **Rosenfeld N, Bouchier C, Courvalin P, Périchon B**. 2012. Expression of the resistance-nodulation-cell division pump AdeIJK in Acinetobacter baumannii is regulated by AdeN, a TetR-type regulator. Antimicrob Agents Chemother **56:**2504–2510. http://dx.doi.org/10.1128/AAC.06422-11.
17. **Eijkelkamp BA, Stroeher UH, Hassan KA, Paulsen IT, Brown MH**. 2014. Comparative analysis of surface-exposed virulence factors of Acinetobacter baumannii. BMC Genomics **15:**1020. http://dx.doi.org/10.1186/1471-2164-15-1020.
18. **Williams TC, Pretorius IS, Paulsen IT**. 2016. Synthetic evolution of metabolic productivity using biosensors. Trends Biotechnol **34:**371–381. http://dx.doi.org/10.1016/j.tibtech.2016.02.002.
19. **Barquist L, Langridge GC, Turner DJ, Phan MD, Turner AK, Bateman A, Parkhill J, Wain J, Gardner PP**. 2013. A comparison of dense transposon insertion libraries in the salmonella serovars Typhi and Typhimurium. Nucleic Acids Res **41:**4549–4564. http://dx.doi.org/10.1093/nar/gkt148.
20. **Coyne S, Rosenfeld N, Lambert T, Courvalin P, Périchon B**. 2010. Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in Acinetobacter baumannii. Antimicrob Agents Chemother **54:**4389–4393. http://dx.doi.org/10.1128/AAC.00155-10.
21. **Roca I, Marti S, Espinal P, Martínez P, Gibert I, Vila J**. 2009. CraA, a major facilitator superfamily efflux pump associated with chloramphenicol resistance in Acinetobacter baumannii. Antimicrob Agents Chemother **53:**4013–4014. http://dx.doi.org/10.1128/AAC.00584-09.
22. **Srinivasan VB, Rajamohan G, Gebreyes WA**. 2009. Role of AbeS, a novel efflux pump of the SMR family of transporters, in resistance to antimicrobial agents in Acinetobacter baumannii. Antimicrob Agents Chemother **53:**5312–5316. http://dx.doi.org/10.1128/AAC.00748-09.
23. **Su XZ, Chen J, Mizushima T, Kuroda T, Tsuchiya T**. 2005. AbeM, an H^+-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. Antimicrob Agents Chemother **49:**4362–4364. http://dx.doi.org/10.1128/AAC.49.10.4362-4364.2005.
24. **Hassan KA, Jackson SM, Penesyan A, Patching SG, Tetu SG, Eijkelkamp BA, Brown MH, Henderson PJ, Paulsen IT**. 2013. Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. Proc Natl Acad Sci U S A **110:**20254–20259. http://dx.doi.org/10.1073/pnas.1317052110.
25. **Hassan KA, Liu Q, Henderson PJ, Paulsen IT**. 2015. Homologs of the *Acinetobacter baumannii* AceI transporter represent a new family of bacterial multidrug efflux systems. mBio **6:**e01982-14. http://dx.doi.org/10.1128/mBio.01982-14.
26. **Lin MF, Lin YY, Yeh HW, Lan CY**. 2014. Role of the BaeSR two-component system in the regulation of Acinetobacter baumannii adeAB genes and its correlation with tigecycline susceptibility. BMC Microbiol **14:**119. http://dx.doi.org/10.1186/1471-2180-14-119.
27. **Hassan KA, Elbourne LD, Li L, Gamage HK, Liu Q, Jackson SM, Sharples D, Kolstø AB, Henderson PJ, Paulsen IT**. 2015. An ace up their sleeve: a transcriptomic approach exposes the AceI efflux protein of Acinetobacter baumannii and reveals the drug efflux potential hidden in many microbial pathogens. Front Microbiol **6:**333. http://dx.doi.org/10.3389/fmicb.2015.00333.

Chapter 5 Tigecycline translational inhibitive effects on *Acinetobacter baumannii*, a transcriptomics view

Title:

Tigecycline translational inhibitive effects on *Acinetobacter baumannii*, a transcriptomics view

Liping Li[1], Karl A. Hassan[1], Amy K. Cain[2], Christine J. Boinett[2], Stephen Baker[3], Julian Parkhill[2], and Ian T. Paulsen*[1]

Author's affiliation

1. Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia 2109

2. The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1SA, United Kingdom

3. Centre for Tropical Medicine and Global Health, Nuffield Department of Clinical Medicine, Oxford University, Oxford, United Kingdom

***Corresponding author:** Prof. Ian T. Paulsen, ian.paulsen@mq.edu.au, Telephone: +61 2 9850 8152, Fax: +61 2 9850 8313

Running Title: *A. baumannii* physiological response to tigecycline

5.1. Abstract

Tigecycline therapy is a treatment of last resort for infections caused by the multidrug resistant (MDR) opportunistic human pathogen *Acinetobacter baumannii*. However, strains resistant to tigecycline were reported not long after its clinical introduction. We applied RNA sequencing (RNA-Seq) to depict the way *A. baumannii* may fine-tune global gene transcription to adapt to tigecycline challenge, and Transposon Directed Insertion-site Sequencing (TraDIS), to identify factors that may be important for *A. baumannii* to tolerate tigecycline. Like other antibiotics that inhibit translation, tigecycline caused a global upregulation of genes encoding for ribosomal assembly, such as ribosomal protein genes and translation initiation factors. In contrast, the expression of genes involved in downstream translational process was decreased, such as aminoacyl-tRNA synthase genes and genes encoding peptide chain elongation factors. As a bacteriostatic antibiotic, it was shown to induce reduction in the expression of the genes involved in key metabolic pathways and cell division. Although several drug efflux pumps have been characterized as tigecycline resistance determinants, such a role was not significant in this *A. baumannii* strain. Tigecycline also induced increased expression of genes involved in horizontal gene transfer (HGT) and spontaneous mutation mediated by a DNA mismatch repair enzyme MutS. This provides a potential explanation for MDR development under tigecycline selection in this organism.

5.2. Introduction

Tigecycline is a broad-spectrum antibiotic derived from minocycline and was the first glycylcycline class antibiotic approved for clinical use (*1*). Compared to tetracyclines, tigecycline has increased antibacterial potency due to its higher binding affinity with the 70S ribosomes, or more specifically with helix 31 and 34 of 16S rRNA on the head of the 30S subunit (*2*). This effect inhibits the delivery of the thermo-unstable ternary complex elongation factor (EF-Tu)·GTP·aminoacyl-tRNA to the ribosomal A (aminoacyl) site and eventually perturbs polypeptide translation (*2*). Additionally, tigecycline is not recognized by the major tetracycline resistance determinants, namely the major facilitator family (MFS) efflux pumps such as TetA/B which export tetracyclines out of the cell (*3*), and ribosome protection proteins such as TetO and TetM which which bind to tetracycline-stalled ribosome to sequester tetracycline away (*2, 4*).

Despite the features of tigecycline that make it recalcitrant to common tetracycline resistance determinants, there have been increasing numbers of tigecycline resistant bacterial pathogens reported since its introduction in 2005. In Gram-negative organisms, a majority of these cases were linked at least in part, to the constitutive overexpression of resistance-nodulation-division (RND) efflux pumps, for instance MexXY-OprM in *Pseudomonas aeruginosa*, and AdeIJK and AdeABC in *A. baumannii* (*5-8*). Similarly, some Gram-positive organisms that display reduced susceptibility to tigecycline constitutively overexpress an efflux pump, such as the MATE family efflux pump MepA in *Staphylococcus aureus* (*9*). Mutations of ribosomal proteins and 16S rRNA have also been shown to reduce the susceptibility of various organisms to tigecycline (*10-12*), probably by affecting its target-site binding affinities. The proteobacterial TetX flavin-dependent monooxygenase, capable of inactivating tetracyclines, was also found to mediate tigecycline resistance when highly expressed (*13*). However, clinical tigecycline resistant bacterial isolates that overexpress TetX have not yet been identified.

A. baumannii is an opportunistic nosocomial human pathogen, known for its high level of drug

resistance in the hospital environment (*14*). Tigecycline has been used for *A. baumannii* infections, and tigecycline resistant mutants have been frequently isolated from patients receiving this treatment. These resistant mutants often display a hypermutator phenotype (*15*), and are resistant to a wide range of antibiotic classes (*16, 17*). In this study, RNA-Seq was used to analyse the global transcriptomic response of *A. baumannii* to tigecycline. In addition, a saturated transposon mutant library of a MDR *A. baumannii* strain was used for identification of genes that affect the host fitness to tigecycline via TraDIS. TraDIS is a high throughput transposon insertion site sequencing technology, used to detect the population dynamic change of a transposon mutant pool exposed to an environmental challenge (*18*). This study revealed that in addition to disrupting protein translation, tigecycline at a sub-inhibitory concentration also induced pleiotropic physiological effects in *A. baumannii*, including differential expression of genes involved in RNA metabolism, DNA repair and the tricarboxylic acid cycle (TCA). These observations provide further insight into why *A. baumannii* may develop tigecycline resistance rapidly.

5.3. Results and discussion

5.3.1. Overview of the transcriptomics and TraDIS data.

A global clonal lineage I (GCI) *A. baumannii* strain 6772166 (*19*), with an intermediate tigecycline resistance, was shocked with 2.5 μg/ml tigecycline, equivalent to half the minimum inhibitory concentration (MIC), and the global transcriptomic response was analysed via RNA-Seq. There were more than 1600 genes with a greater than 2-fold change in gene expression following tigecycline exposure in *A. baumannii* 6772166 (Figure S2 and supplemental data set), indicating that tigecycline induces broad physiological effects. Such alteration in gene transcription may be partially explained by significant changes in the expression of 76 genes encoding putative or characterized transcriptional regulators, the up-regulation of one highly expressed σ^{70} factor homolog gene, and the altered expression of various ribonuclease genes (Figure 6.1D).

In parallel, a saturated and random Tn*5* mutant library of *A. baumannii* BAL062 was used for identification of the genes affecting host fitness upon exposure to 0.1 μg/ml tigecycline (equivalent to 1/10 x MIC for this strain) using a TraDIS approach. In this TraDIS experiment, we expect positive selection for mutants carrying Tn*5* insertions in genes detrimental to survival in the presence of tigecycline, and negative selection of mutants that carry insertions in genes required for surviving tigecycline exposure. However, the 0.1 μg/ml tigecycline concentration appears to have a very significant inhibitive effect to most of the Tn*5* insertion mutants, as there were significantly fewer total unique insertion sites represented in the tigecycline supplemented pool (5,627) than in the control pool (122,594). Because of the difference in the size of the input (control) and output (tigecycline supplemented) pools, we can only be confident that the results are significant for genes that display a higher insertion frequency in the output pool compared to the input pool (i.e., where there was positive selection for mutants in which the gene was inactivated).

5.3.2. Tigecycline slows down protein translation but boosts ribosome synthesis.

The direct effect of tigecycline interaction with the 16S rRNA is blocking the aminoacyl-tRNA from entering the ribosomal A site (*2*). In line with this mode of action, the RNA-Seq data shows clear evidence of ribosomal stalling after translational initiation. Fourteen genes encoding aminoacyl-tRNA synthetases (Figure 5.1A) and 27 genes responsible for amino acid synthesis (Figure 5.1C) were down regulated, possibly because of a reduced turnover of aminoacyl-tRNA/tRNA, and reduced rate of amino acid incorporation into protein. Correspondingly, the transcripts of two EF-Tu (*tuf*) genes and elongation factor P encoding gene (EF-P, *efp*) were decreased (Figure 5.1D). EF-P participates in peptidyl transferase activity on the ribosomal P (peptidyl) site. The transcription of the ribosome recycling factor (RRF) encoding gene *frr* showed decreased expression in response to tigecycline as well (Figure 5.1D). RRF is required for 70S ribosome separation at the end of each peptide translation cycle and ribosomal subunit recycling for the subsequent round of translation (*20*). These observations are suggestive of a slower protein translation rate, and are consistent with the known initial antibacterial action of tigecycline in protein translation perturbation (*2*). Decreased transcripts of genes involved in amino acid synthesis and tRNA aminoacylation were also found in *Streptococcus pneumoniae* when treated by translational inhibitors including tetracycline, chloramphenicol, erythromycin and puromycin (*21*). Our RNA-Seq data also showed decreased transcriptional levels of twelve genes encoding proteases, indicative of a slower protein degradation rate following tigecycline treatment.

The transcripts of genes for sixteen 50S and five 30S ribosomal proteins were increased (Figure 5.1B), consistent with previous findings in *S. pneumoniae*, *Escherichia coli* and *Haemophilus influenzae* at either a transcriptomic or proteomic level when treated by translational inhibitors including tetracycline (*21-23*). In the *H. influenzae* study, it was demonstrated that translational inhibitors increased the total RNA synthesis rate, and hence the corresponding rate of rRNA synthesis, as it is the major RNA species in the cell (*23*). rRNA synthesis is the rate-limiting step in ribosome synthesis and assembly (*24*). Our study was based on rRNA-depleted samples, so we could not directly verify whether the rRNAs were increased in abundance upon tigecycline treatment. The

genes of two translation initiation factors IF-1 (*infA*) and IF-2 (*infB*) showed increased expression as well. IF-1 and IF-2 are required for the assembly of ribosome subunits to start a new protein translation cycle and their upregulation is consistent with an increased synthesis of ribosomal proteins and rRNA.

The transcriptional changes in genes responsible for several cellular activities involved with guanosine tetraphosphate and guanosine pentaphosphate (p)ppGpp further support the notion that, like other translational inhibitor antibiotics, tigecycline could boost rRNA and ribosome biosynthesis in *A. baumannii*. (p)ppGpp is a negative effector of the *rrnB P1* promoter of rRNA operons (*25*). In adapting to amino acid starvation, overproduction of (p)ppGpp inhibits ribosome synthesis directly and mediates ribosomal protein degradation through Lon protease, via the stringent response (*26, 27*). Upon tigecycline treatment, we observed decreased expression levels of various genes responsible for (p)ppGpp synthesis and degradation (Figure 5.1D, stringent response genes). These include *relA*, encoding a (p)ppGpp synthetase, *spoT,* encoding a synthetase and hydrolase of (p)ppGpp, and *lon,* encoding Lon protease. In addition, we observed decreased expression of genes impacting cellular levels of polyphosphate (polyP) – a known cofactor of Lon-mediated free ribosomal protein degradation (*27*), i.e. *ppk*, encoding polyphosphate kinase, and *ppx,* encoding exopolyphosphotase. These results together with the observation of reduced (p)ppGpp cellular level in translational inhibitor antibiotics stressed bacterial cells (*23*) suggest that tigecycline could also induce decreased production of (p)ppGpp in *A. baumannii*. This in turn could stimulate rRNA synthesis and inhibit Lon-mediated ribosomal protein degradation.

5.3.3. Highest-fold expression increases are in RNA metabolism genes.

A plasmid-borne toxin-antitoxin (TA) system, homologous to a chromosomal TA system *brnT/brnA* first characterized in *Brucella abortus* (*28*), showed a 39-fold increase in expression in response to tigecycline (Figure 5.1D). BrnT is a type II ribonuclease toxin, which when co-expressed with the antitoxin protein BrnA forms a tetramer $BrnT_2$-$BrnA_2$, that neutralizes BrnT toxicity and functions

as a strong autorepressor of its own operon (*28*). However, a decrease in the cellular level of BrnA can de-repress expression of the TA system and lead to increased levels of BrnT, which in turn inhibits cell growth through RNA degradation, thus interfering with protein translation (*28*). Chloramphenicol was shown to stimulate the overexpression of *brnT/brnA* in *B. abortus*, but the mechanism which leads to TA overexpression or BrnA degradation is unknown (*28*). This suggests that translational inhibitors like chloramphenicol and tigecycline could stimulate the expression of *brnT/brnA*, which then could potentially further exert the antibacterial effects of the antibiotics.

Overexpression of chromosomal TA systems is a factor in persister cell formation and can be induced by increased cellular levels of (p)ppGpp (*29*). Persistence is a phenomenon in which rare isogenic cells from an antibiotic-sensitive bacterial population transiently become non-growing or slow-growing and multidrug tolerant, but when the antibiotic is removed the persistent cells can revert to a wild type phenotype.

In this study, the overexpression of *brnT/brnA* upon tigecycline treatment in *A. baumannii* is unlikely to be triggered by (p)ppGpp overproduction, as various genes involved in the (p)ppGpp-mediated stringent response showed decreased expression (Figure 5.1D).

The two genes with the highest fold expression increase (252-fold) were *rtcB*, encoding a tRNA repair enzyme, and a hypothetical gene AB57_3343 directly downstream of *rtcB* (Figure 5.1D). *rtcB* was initially characterized in *E. coli* as a stand-alone ligase in healing and sealing broken tRNA-like stem-loop structures in vitro, and later demonstrated to catalyze tRNA and mRNA repair in yeast *in vivo* (*30, 31*). We speculate that *rtcB* overexpression might correlate with *brnT/brnA* overexpression for evading the potential programmed RNA breakage mediated by this TA system, and RtcB could potentially serve as an indirect tigecycline induced stress-response determinant. Another possibility, is that blockage of tRNAs to the ribosome caused by tigecycline leads to a cellular response in tRNA repair. TraDIS data showed that the Tn*5* insertion read count in the *rtcB* gene in *A. baumannii* BAL062 was significantly reduced in the culture exposed to 1/10 tigecycline MIC (Table S5.1),

suggesting disruption of this gene may increase cellular sensitivity to tigecycline. *rtcB* was also significantly up-regulated in a previous study of the effects of tigecycline on *A. baumannii* MDR-ZJ06 (*32*), however *brnT/brnA* is not present in this strain.

5.3.4. Central metabolism and cell division.

Bacteriostatic antibiotics capable of inducing translation perturbation have been reported to have profound downstream consequences on bacterial metabolism, including the accumulation of metabolites from central metabolic and cellular respiration pathways, indicating the suppression of these pathways (*33, 34*). Our RNA-Seq data showed consistent tigecycline-induced lower levels of gene expression for enzymes in the tricarboxylic acid (TCA) cycle and cellular respiration (Figure 5.2A&B). Transcription of the genes responsible for cell wall metabolism and cell division was also down regulated by tigecycline (Figure 5.2C). The apparent effects of tigecycline on gene expression involved in translation perturbation, RNA degradation, TCA cycle and respiration, cell division and cell wall synthesis, are suggesting a reduction in cellular growth rate in *A. baumannii* 6772166, which is consistent with tigecycline's bacteriostatic effect (*35*).

5.3.5. Drug efflux and uptake

A common mechanism for resistance to tetracycline antibiotics involves reducing drug intracellular concentration via drug efflux pump expression and/or down regulation of the outer membrane proteins (OMP) responsible for drug uptake (*36*). The multidrug efflux pumps previously characterized in *A. baumannii* tigecycline resistance are AdeABC and AdeIJK (*5, 8*). The AdeABC mediated tigecycline resistant *A. baumannii* strains reported previously were normally correlated with mutations on either *adeR* or *adeS* leading to its constitutive overexpression and hence tigecycline resistant hosts (*5, 37*). However, in our RNA-Seq data, *adeA* and *adeC* were significantly down-regulated by tigecycline by more than two-fold and the *adeIJK* operon was not differentially regulated (Figure 5.3A).

Although also transcriptionally unresponsive to tigecycline (Figure 5.3A), Tn*5* insertion mutants of *adeN* were enriched in the tigecycline-treated mutant pool (Figure 5.3C), suggesting that this transcriptional regulator contributes to *A. baumannii* fitness against tigecycline. This agrees with previous findings that AdeN is a transcriptional repressor of *adeIJK* (*38*). Therefore, we speculate that AdeN may not recognize tigecycline as a ligand, leading to relief of transcriptional repression of *adeIJK*. However, in the absence of AdeN, overexpression of *adeIJK* leads to decreased tigecycline susceptibility in *A. baumannii* because tigecycline is recognised as a substrate of AdeJ.

Two genes encoding characterized drug efflux pumps and ten genes encoding hypothetical proteins with predicted transmembrane helices, were responsive to tigecycline (Figure 5.3A). The tetracycline exporter gene *tetA* and its transcriptional repressor gene *tetR* were both upregulated (Figure 5.3A), which is consistent with a previous observation that tigecycline could induce *tetA* overexpression in *E. coli* but *tetA* does not confer tigecycline resistance (*3*). CraA is an MDR efflux pump that transports chloramphenicol (*39*) and other antimicrobials (*40*). To further investigate whether CraA and the hypothetical proteins do confer tigecycline resistance, these genes were heterologously overexpressed in *E. coli*. However, no resistance phenotype was observed for any of these clones, suggesting that although they are transcriptionally responsive to tigecycline, these efflux genes do not mediate tigecycline resistance.

Tigecycline treatment also led to decreased expression of several OMP genes (Figure 5.3B) and genes involved in capsular polysaccharide biosynthesis and export (Figures 5.2E). These changes might affect bacterial outer membrane permeability and thus tigecycline uptake.

5.3.6. Impacts on DNA repair and HGT gene expressions.

Tigecycline therapy was reported to be associated with significantly increased morbidity in patients in comparison with other antibiotic therapies, due to the quick emergence of tigecycline resistant *A. baumannii* isolates (*41*). Transcriptomic data presented here suggests that tigecycline may impact

DNA repair and DNA competence and mobility. For instance, it induced the increased expression of *mutS* encoding DNA mismatch repair (MMR) enzyme and two other putative DNA repair genes AB57_2357 and AB57_1251. In contrast, decreased expression was observed of the transcription-coupled nucleotide excision repair (TCR) genes *mfd*, *uvrA*, *uvrB and uvrC* (Figure 5.4A), suggesting that TCR activity is decreased in tigecycline-stressed *A. baumannii* cells. This notion is supported by the increased expression of the transcription elongation factor gene *greB* which can interfere with TCR, and the decreased expression of (p)ppGpp synthesis genes, which are required for efficient TCR activities (Figure 5.1D) (*42, 43*).

Tigecycline-resistant hypermutator strains of *A. baumannii* have been isolated in both laboratory and clinical settings; in each case sequencing of the isolated strain revealed an insertion sequence (IS) mediated inactivation of *mutS* (*15*). The TraDIS data showed that *mutS* mutants had significantly increased Tn5 insertion reads in the tigecycline-treated mutant pool, linking the absence of MutS with tigecycline resistance (Figure 5.4B). Together with the observed increased expression of *mutS* in the transcriptomic data (Figure 5.4A), we speculate MutS might serve as a significant hypermutator and resistance determinant in *A. baumannii* under tigecycline selection.

Tigecycline treatment increased expression of various genes associated with DNA mobility, including twenty-five putative transposase genes and four homologues of the DNA mobilization gene *bmgB* (Figure S5.4). The increased rate of transposase gene expression could be linked to the IS-mediated inactivation of *mutS* previously reported (*15*). Genes involved in DNA uptake were also induced by tigecycline treatment including the cell competence genes *comM*, *cinA* and *comEA*, and conjugal transfer protein *traR* genes. These changes in expression of DNA competence and mobilization genes suggests that tigecycline may affect rates of horizontal gene transfer. An operon encoding the type VI secretion system showed increased expression following tigecycline treatment (Figure 5.2C). The T6SS in *Vibrio cholerae* was shown to be up-regulated through competence induction. This system is responsible for releasing the DNA of the surrounding nonimmune cells

through deliberate killing to make the DNA available for uptake (*44*). Expression of a lytic transglycolase gene, proposed to play a role in creating space within the peptidoglycan sacculus for the insertion of cell-envelope spanning structures such as T6SS (*45*) was also induced by tigecycline.

5.3.7. Tigecycline and β-lactams

The most highly down-regulated genes in this study were two *ampC* β-lactamase encoding genes (Figure 5.2C), whose expression was reduced by 35-fold and 24-fold. AmpC β-lactamases are active against five classes of β-lactams (*46*). β-lactams can induce a toxic malfunctioning of the cycle of peptidoglycan synthesis, hydrolysis and recycling, and induction of β-lactamase genes typically correlates with peptidoglycan recycling (*46, 47*). This opens the intriguing possibility that tigecycline and β-lactams could be used synergistically in combination therapy, as has been suggested previously (*48*).

To investigate this possibility, we conducted checkerboard assays with *A. baumannii* 6772166, tigecycline and three AmpC β-lactamase substrates ceftriaxone, cefotaxime and cefuroxime (Figure S5.5). The fractional inhibitory concentrations were between 0.5 and 2 (*49*), suggesting there was a low level of synergy, but not at a level that is likely to be useful for combination therapy. Since tigecycline slows the growth rate of bacterial cells, this would presumably limit the effectiveness of combination therapy with β-lactams (*33*).

5.4. Conclusions

Through transcriptomic analysis we revealed that, like other translational inhibitors, tigecycline decreases the expression level of genes involved in protein translation, the TCA cycle, respiration, cell wall metabolism and cell division. The significant upregulation of a TA system observed in this study, combined with a previous observation that stress induced, slowly growing bacteria are less susceptible to antibiotic challenges (*50*), raises the possibility that tigecycline might serve as a cue in inducing persister cell formation. Chloramphenicol has been reported to induce similar

physiological effects, i.e. the overexpression of the TA system and increased persister formation (*23, 51, 52*). The RNA repair enzyme encoding gene *rtcB* was massively up regulated by tigecycline, and it might act as an indirect tigecycline resistance determinant through easing translational stress caused by the TA system.

Tigecycline at 1/10 MIC poses strong inhibitive effect on the majority of transposon mutants of *A. baumannii*, except for the mutants of *adeN* and *mutS*, which have increased fitness against tigecycline. In contrast, the known resistance determinants, such as *adeIJK* was not detected by TraDIS, due to high False Discovery Rate (FDR, q-value). This suggests mutant population dynamic change induced by tigecycline at sub-inhibitive is feasible for discovery mutants of increased tolerance level, but not for resistance determinants. The reason of this phenomenon is unknown, but it might relate to tigecycline mode of action. Similarly, a saturated transposon mutant library of *E. coli* was used for identification of gain-of-function colistin resistance mutants at concentration much higher than wild type colistin MIC, resulting to the identification of the mutant of an operon *pmrCAB* that have significantly increased colistin MIC *(61)*.

We also observed that tigecycline has potential in promoting exogenous DNA uptake, mobile genetic element translocation, and DNA mismatch repair mediated by MutS. This leads to the speculation that tigecycline at sublethal concentrations can promote the translocation of mobile genetic elements such as insertion sequences which can lead to the inactivation of *mutS*, resulting in a tigecycline resistant hypermutator. Furthermore, such hypermutator strains could lead to not only tigecycline resistant but also MDR strains. This potentially explains why tigecycline resistant bacterial pathogens evolved very soon after its clinical introduction.

5.5. Methods and materials

Bacterial strains and genome sequences

A. baumannii 6772166 is a global clone I isolate from Royal Adelaide Hospital, Australia. This isolate was used for RNA-Seq experiments because of its high level of tigecycline resistance; the tigecycline minimum inhibitory concentration (MIC) was 5μg/ml. Genomic DNA (gDNA) was extracted from *A. baumannii* 6772166 using the DNeasy Blood & Tissue Kit (QIAGEN), and sequenced through TruSeq DNA PCR-Free prep kit on 150bp pair-end Illumina HiSeq platform. The sequence reads were assembled by SPAdes (St. Peterburg genome assembler) (*53*), yielding an assembly comprised of 61 contigs with length ranging from 524 bp to 597,496 bp and coverage above 23-fold. The contigs of *A. baumannii* 6772166 were aligned to the complete genome sequences of two other GCI strains, AYE and AB0057 (*54*) using the Mauve (Multiple Genome Alignment) aligner (*55*). This analysis determined that the *A. baumannii* 6772166 genome sequence shared high identity to the genome sequence of AB0057 (Figure S5.1). Therefore, the complete plasmid and chromosome sequences of *A. baumannii* AB0057 were used as the reference sequence for the subsequent RNA-Seq read alignment and statistical analyses, due to their high quality. We used the saturated Tn5 mutant library for *A. baumannii* BAL062 (ENA accession nos.: LT594095-LT594096), which was available in our laboratory, to identify genes required for *A. baumannii* survival and fitness under tigecycline selection (*56*). *E. coli* strain DH5α (α-select chemically competent cells, Bioline Australia) was used for transporter system heterologous overexpression and functional characterization.

Tigecycline transcriptomic analysis by RNA-Seq

Three independent *A. baumannii* 6772166 colonies were suspended and grown in Muller-Hinton (MH, Oxoid) broth with shaking overnight at 37°C. The overnight cultures were diluted 1:100 in fresh MH broth and grown to OD_{600}=0.6. Each of the cultures was then divided into two same aliquots, with one supplemented with tigecycline (Sigma-Aldrich, USA) of subinhibitory

concentration (2.5μg/ml) and the other without treatment, resulting in 6 independent cultures. These 6 cultures were grown further for 30 minutes with shaking at 37°C. The total RNA of each sample was extracted by miRNeasy Mini Kit (QIAGEN, Germany) following the manufacturer's instructions. Genomic DNA residue was removed by TURBO DNA-*free*™ Kit TURBO™ DNase Treatment and Removal Reagents (Ambion Inc., USA). The resulting gDNA-free total RNA samples were immediately processed for rRNA depletion. rRNA was depleted by using the Ribo-Zero Magnetic kit (bacteria) (Illumina, Inc., USA), following the manufacturer's instructions. For each sample, 500ng of rRNA depleted total RNA was used to generate double stranded cDNA library through TruSeq® Stranded Total RNA Sample Preparation kit (Illumina, Inc., USA). The samples were then sequenced through 100 bp paired end (PE) sequencing on Illumina HiSeq2500 platform. Nearly 75 million unique 101 bp reads were obtained from each of the 6 independent *A. baumannii* 6772166 cultures, representing more than 310-fold genome average coverage (*A. baumannii* 6772166 chromosome and plasmid size together is around 4 Mb). The sequence reads from the 6 RNA-Seq samples were aligned to the *A. baumannii* AB0057 reference and counted using EDGE-pro (Estimated Degree of Gene Expression in Prokaryotic Genomes) (*57*), then gene differential expression between the control and experimental samples analyzed using the DEseq R package (Supplementary data set). More than 99% of the RNA-Seq reads of each sample were mapped to the *A. baumannii* AB0057 genome sequence.

Identification of tigecycline fitness determinants through TraDIS

Four aliquots of the glycerol stock of *A. baumannii* BAL062 Tn5 mutant library, containing approximately 10^9 cells respectively, were grown overnight in MH broth. The overnight cultures were diluted 1:100 in fresh MH broth, with two samples supplemented with tigecycline at 1/10 of the MIC of that parental strain (0.1 μg/ml) and two others untreated, and then grown overnight with shaking at 37°C. The gDNA of these four overnight cultures was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, Germany) and subjected for Tn*5* insertion site sequencing (TraDIS) as previously described (*18*). For determining tigecycline fitness factors in *A. baumannii*, the insertion

site ratios between control and experimental samples were compared by using the statistical comparison scripts in the TraDIS toolkit (*58*). Genes with fewer than 10 mapped reads were excluded from the analysis. Genes showing a greater than 2-fold increase in insertion reads (representing mutant abundance) with a P-value below 0.01 were considered as significant tigecycline fitness determinants.

Functional validation of membrane transporters through heterologous overexpression

Eight genes, including two efflux pumps and six hypothetical proteins with at least three transmembrane helices predicted through using the TMHMM Server v.2.0., were cloned into pTTQ18 under the control of the *tac* promoter, and overexpressed in *E. coli* DH5α as previously described (Table S5.1) (*59*). The accuracy of the cloned gene products was confirmed through Sanger sequencing. Protein heterologous expression was confirmed by anti-histidine antibody RGS•His HRP Conjugate Kit (QIAGEN, Germany) through Western blotting. To validate whether these genes confer tigecycline resistance, the tigecycline MIC of the *E. coli* clones overexpressing the transporters or hypothetical proteins were compared with the wild type *E. coli* strain. The MIC was determined through 2-fold serial broth dilution in MH broth according to the guidelines of CLSI (Clinical and Laboratory Standards Institute). Protein expression in the MIC assays was induced by including 0.05 mM IPTG in the media. Two independent biological replicates were performed for each MIC experiment.

Tigecycline and β-lactam antibiotics synergism validation through checkerboard assay

The synergism between tigecycline and three β-lactam antibiotics, namely ceftriaxone, cefotaxime and cefuroxime (all from Sigma-Aldrich, USA) were determined through broth microdilution checkerboard assay according to the reference (*49*). Two independent biological repeats were performed for each combination set.

5.6. References

1. P. J. Petersen, N. V. Jacobus, W. J. Weiss, P. E. Sum, R. T. Testa, In vitro and in vivo antibacterial activities of a novel glycylcycline the 9-t-butylglycylamido derivative of minocycline (GAR-936). *Antimicrob. Agents Chemother* **43**, 738-744 (1999).
2. L. Jenner *et al.*, Structural basis for potent inhibitory activity of the antibiotic tigecycline during protein synthesis. *Proc. Nati. Acad. Sci. USA* **110**, 3812-3816 (2013).
3. T. Hirata, A. Saito, K. Nishino, N. Tamura, A. Yamaguchi, Effects of efflux transporter genes on susceptibility of *Escherichia coli* to tigecycline (GAR-936). *Antimicrob. Agents Chemother* **48**, 2179-2184 (2004).
4. J. Bergeron *et al.*, Glycylcyclines bind to high-affinity tetracycline ribosomal binding site and evade Tet(M)- and Tet(O)-mediated ribosomal protection. *Antimicrob. Agents Chemother* **40**, 2226-2228 (1996).
5. A. Y. Peleg, J. Adams, D. L. Paterson, Tigecycline efflux as a mechanism for nonsusceptibility in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother* **51**, 2065-2069 (2007).
6. C. R. Dean, M. A. Visalli, S. J. Projan, P. E. Sum, P. A. Bradford, Efflux-mediated resistance to tigecycline (GAR-936) in *Pseudomonas aeruginosa* PAO1. *Antimicrob. Agents Chemother* **47**, 972-978 (2003).
7. M. A. Visalli, E. Murphy, S. J. Projan, P. A. Bradford, AcrAB multidrug efflux pump is associated with reduced levels of susceptibility to tigecycline (GAR-936) in *Proteus mirabilis*. *Antimicrob. Agents Chemother* **47**, 665-669 (2003).
8. L. Damier-Piolle, S. Magnet, S. Brémont, T. Lambert, P. Courvalin, AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother* **52**, 557-562 (2008).
9. F. McAleese *et al.*, A novel MATE family efflux pump contributes to the reduced susceptibility of laboratory-derived *Staphylococcus aureus* mutants to tigecycline. *Antimicrob. Agents Chemother* **49**, 1865-1871 (2005).
10. A. Lupien, H. Gingras, P. Leprohon, M. Ouellette, Induced tigecycline resistance in *Streptococcus pneumoniae* mutants reveals mutations in ribosomal proteins and rRNA. *J. Antimicrob. Chemother.* **70**, 2973-2980 (2015).
11. M. Niebel *et al.*, Deletions in a ribosomal protein-coding gene are associated with tigecycline resistance in *Enterococcus faecium*. *Int. J. Antimicrob. Agents* **46**, 572-575 (2015).
12. K. Beabout *et al.*, The ribosomal S10 protein is a general target for decreased tigecycline susceptibility. *Antimicrob. Agents Chemother* **59**, 5561-5566 (2015).
13. I. F. Moore, D. W. Hughes, G. D. Wright, Tigecycline is modified by the flavin-dependent monooxygenase TetX. *Biochemistry* **44**, 11829-11835 (2005).
14. A. Y. Peleg, H. Seifert, D. L. Paterson, *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin. Microbiol. Rev.* **21**, 538-582 (2008).
15. T. G. Hammerstrom, K. Beabout, T. P. Clements, G. Saxer, Y. Shamoo, *Acinetobacter baumannii* repeatedly evolves a hypermutator phenotype in response to tigecycline that effectively surveys evolutionary trajectories to resistance. *PLoS ONE* **10**, e0140489 (2015).
16. M. Hornsey *et al.*, Whole-genome comparison of two *Acinetobacter baumannii* isolates from a single patient, where resistance developed during tigecycline therapy. *J. Antimicrob. Chemother.* **66**, 1499-1503 (2011).
17. M. I. Morosini *et al.*, Antibiotic coresistance in extended-spectrum-β-lactamase-producing *Enterobacteriaceae* and in vitro activity of tigecycline. *Antimicrob. Agents Chemother* **50**, 2695-2699 (2006).
18. G. C. Langridge *et al.*, Simultaneous assay of every *Salmonella* Typhi gene using one million transposon mutants. *Genome Res* **19**, 2308-2316 (2009).
19. B. A. Eijkelkamp *et al.*, Adherence and motility characteristics of clinical *Acinetobacter baumannii* isolates. *FEMS Microbiol Lett* **323**, 44-51 (2011).

20. D. N. Wilson, The A-Z of bacterial translation inhibitors. *Crit. Rev. Biochem. Mol. Biol.* **44**, 393-433 (2009).
21. W. L. Ng, K. M. Kazmierczak, G. T. Robertson, R. Glimour, M. E. Winkler, Transcriptional regulation and signature patterns revealed by microarray analyses of *Streptococcus pneumoniae* R6 challenged with sublethal concentrations of translational inhibitors. *J. Bacteriol* **185**, 359-370 (2002).
22. R. A. VanBogelen, F. C. Neidhardt, Ribosomes as sensors of heat and cold shock in *Escherichia coli*. *Proc. Nati. Acad. Sci. USA* **87**, 5589-5593 (1990).
23. S. Evers *et al.*, Mechanism-related changes in the gene transciption and protein synthesis patterns of *Haemophilus influenzae* after treatment with translational inhibitors. *Proteomics* **1**, 522-544 (2001).
24. O. Maaløe, N. O. Kjeldgaard, *Control of macromolecular synthesis: a study of DNA, RNA and protein synthesis in bacteria*. (New York: W.A. Benjamin, 1966).
25. H. D. Murray, D. A. Schneider, D. A. Schneider, Control of rRNA expression by small molecules is dynamic and nonredundant. *Mol Cell* **12**, 125-134 (2003).
26. T. M. Wendrich, G. Blaha, D. N. Wilson, M. A. Marahiel, K. H. Nierhaus, Dissection of the mechanism for the stringent factor RelA. *Mol Cell* **10**, 779-788 (2002).
27. A. Kuroda *et al.*, Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. *Science* **293**, 705-708 (2001).
28. B. E. Heaton, J. Herrou, A. E. Blackwell, V. H. Wysocki, S. Crosson, Molecular structure and function of the novel BrnT/BrnA toxin-antitoxin system of *Brucella abortus*. *J. Biol. Chem.* **287**, 12098-12110 (2012).
29. K. Gerdes, E. Maisonneuve, Bacterial persistence and toxin-antitoxin loci. *Annu. Rev. Microbiol.* **66**, 103-123 (2012).
30. N. Tanaka, S. Shuman, RtcB is the RNA ligase component of an *Escherichia coli* RNA repair operon. *J Biol Chem* **286**, 7727-7731 (2011).
31. N. Tanaka, B. Meineke, S. Shuman, RtcB, a novel RNA ligase, can catalyze tRNA splicing and HAC1 mRNA splicing in vivo. *J Biol Chem* **286**, 30253-30257 (2011).
32. X. Hua, Q. Chen, X. Li, Y. Yu, Global transcriptional response of *Acinetobacter baumannii* to a subinhibitory concentration of tigecycline. *Int J Antimicrob Agents* **44**, 337-344 (2014).
33. M. A. Lobritz *et al.*, Antibiotic efficacy is linked to bacterial cellular respiration. *Proc Natl Acad Sci U S A* **112**, 8173-8180 (2015).
34. X. Lin, L. Kang, H. Li, X. Peng, Fluctuation of multiple metabolic pathways is required for *Escherichia coli* in response to chlortetracycline stress. *Mol Biosyst* **10**, 901-908 (2014).
35. A. I. Cocozaki *et al.*, Resistance mutations generate divergent antibiotic susceptibility profiles against translation inhibitors. *Proc Natl Acad Sci U S A* **113**, 8188-8193 (2016).
36. L. J. V. Piddock, Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. *Clin. Microbiol. Rev.* **19**, 382-402 (2006).
37. J. R. Sun *et al.*, AdeRS combination codes differentiate the response to efflux pump inhibitors in tigecycline-resistant isolates of extensively drug-resistant *Acinetobacter baumannii*. *Eur J Clin Microbiol Infect Dis* **33**, 2141-2147 (2014).
38. N. Rosenfeld, C. Bouchier, P. Courvalin, B. Perichon, Expression of the resistance-nodulation-cell division pump AdeIJK in *Acinetobacter baumannii* is regulated by AdeN, a TetR-type regulator. *Antimicrob Agents Chemother* **56**, 2504-2510 (2012).
39. I. Roca *et al.*, CraA, a major facilitator superfamily efflux pump associated with chloramphenicol resistance in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **53**, 4013-4014 (2009).
40. L. Li, K. A. Hassan, M. H. Brown, I. T. Paulsen, Rapid multiplexed phenotypic screening identifies drug resistance functions for three novel efflux pumps in *Acinetobacter baumannii*. *J Antimicrob Chemother* **71**, 1223-1232 (2016).

41. J. Nemeth, G. Oesch, S. P. Kuster, Bacteriostatic versus bactericidal antibiotics for patients with serious bacterial infections: systematic review and meta-analysis. *J Antimicrob Chemother* **70**, 382-395 (2015).
42. V. Kamarthapu *et al.*, ppGpp couples transcription to DNA repair in *E. coli*. *Science* **352**, 993-996 (2016).
43. V. Epshtein *et al.*, UvrD facilitates DNA repair by pulling RNA polymerase backwards. *Nautre* **505**, (2014).
44. S. Borgeaud, L. C. Metzger, T. Scrignari, M. Blokesch, The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Science* **347**, 63-67 (2015).
45. E. Scheurwater, C. W. Reid, A. J. Clarke, Lytic transglycosylases: bacterial space-making autolysins. *Int J Biochem Cell Biol* **40**, 586-591 (2008).
46. G. A. Jacoby, AmpC β-Lactamases. *Clin. Microbiol. Rev.* **22**, 161-182 (2009).
47. L. Li *et al.*, Sensor histidine kinase is a β-lactam receptor and induces resistance to β-lactam antibiotics. *Proc Natl Acad Sci U S A* **113**, 1648-1653 (2016).
48. M. Tumbarello *et al.*, Predictors of mortality in bloodstream infections caused by *Klebsiella pneumoniae* carbapenemase-producing *K. pneumoniae*: importance of combination therapy. *Clin Infect Dis* **55**, 943-950 (2012).
49. L. S. Garcia, in *Clinical Microbiology Procedures Handbook*. (ASM Press, Washington, DC, 2010), chap. 5.12, pp. 140-162.
50. K. Poole, Bacterial stress responses as determinants of antimicrobial resistance. *J Antimicrob Chemother* **67**, 2069-2089 (2012).
51. S. M. Amato, M. A. Orman, M. P. Brynildsen, Metabolic control of persister formation in *Escherichia coli*. *Mol Cell* **50**, 475-487 (2013).
52. A. L. Svitil, M. Cashel, J. W. Zyskind, Guanosine tetraphosphate inhibits protein synthesis in vivo a possible protective mechanism for starvation stress in *Escherichia coli*. *J. Biol. Chem.* **268**, 2307-2311 (1993).
53. A. Bankevich *et al.*, SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J Comput Biol* **19**, 455-477 (2012).
54. K. M. Hujer *et al.*, Analysis of antibiotic resistance genes in multidrug-resistant *Acinetobacter* sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. *Antimicrob Agents Chemother* **50**, (2006).
55. A. E. Darling, B. Mau, N. T. Perna, progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* **5**, e11147 (2010).
56. K. A. Hassan *et al.*, Fluorescence-Based Flow Sorting in Parallel with Transposon Insertion Site Sequencing Identifies Multidrug Efflux Systems in *Acinetobacter baumannii*. *mBio* **7**, e01200-01216 (2016).
57. T. Magoc, D. Wood, S. L. Salzberg, EDGE-pro: Estimated Degree of Gene Expression in Prokaryotic Genomes. *Evol Bioinform Online* **9**, 127-136 (2013).
58. L. Barquist *et al.*, The TraDIS toolkit: sequencing and analysis for dense transposon mutant libraries. *Bioinformatics* **32**, 1109-1111 (2016).
59. K. A. Hassan *et al.*, Roles of DHA2 family transporters in drug resistance and iron homeostasis in Acinetobacter spp. *J Mol Microbiol Biotechnol* **20**, 116-124 (2011).
60. L. Overmars, S. A. van Hijum, R. J. Siezen, C. Francke, CiVi: circular genome visualization with unique features to analyze sequence elements. *Bioinformatics* **31**, 2867-2869 (2015).
61. M. Phan et al., Modification in the pmrB gene are the primary mechanism for the development of chromosomally encoded resistance to polymyxins in uropathogenic *Escherichia coli*. *J Antimicrob Chemother* **72**, 2729-2736 (2017).

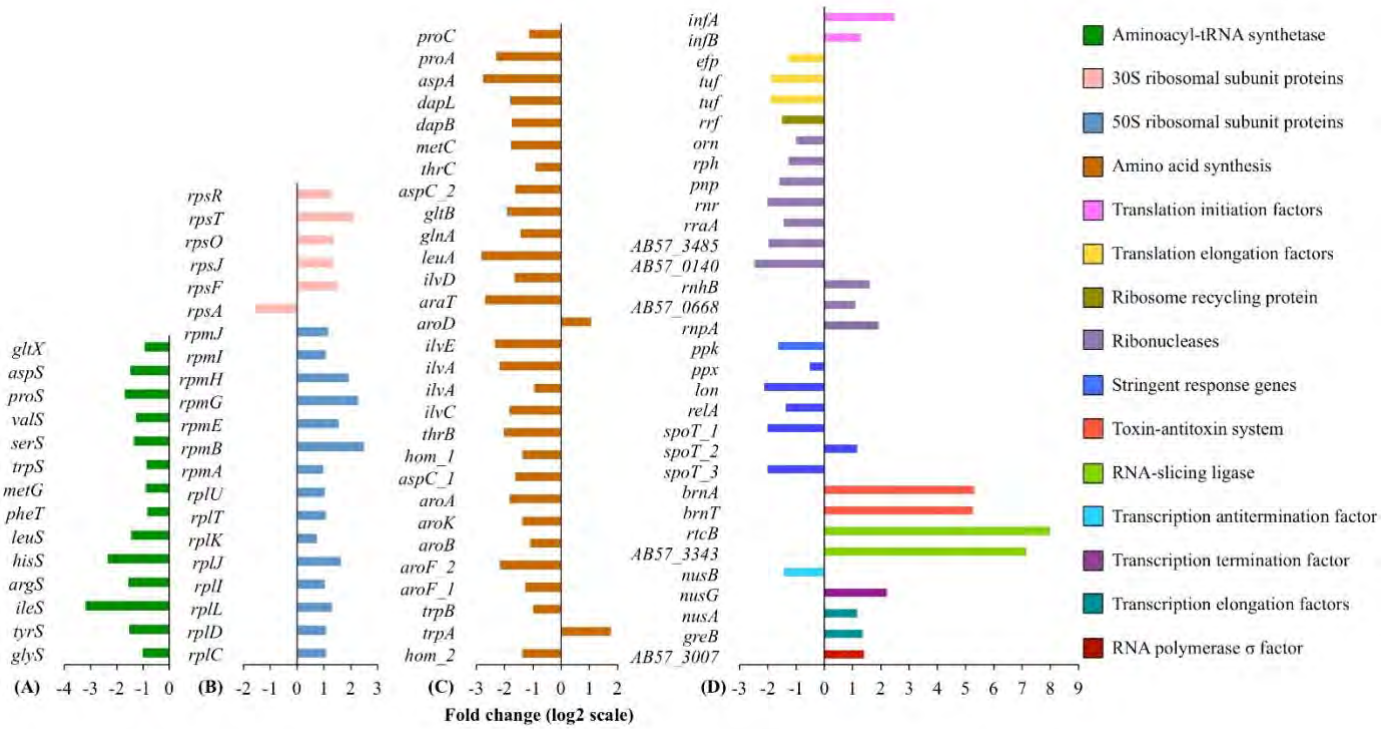

Figure 5.1. Tigecycline induced differential expression of genes involved in or affecting protein translation. Differentially regulated genes (fold change > 1 at $\log_2$ scale, adjusted p value < 0.01) are grouped by colour according to biological function or cellular pathway. Please see Supplementary data 5.1 for gene function annotation.

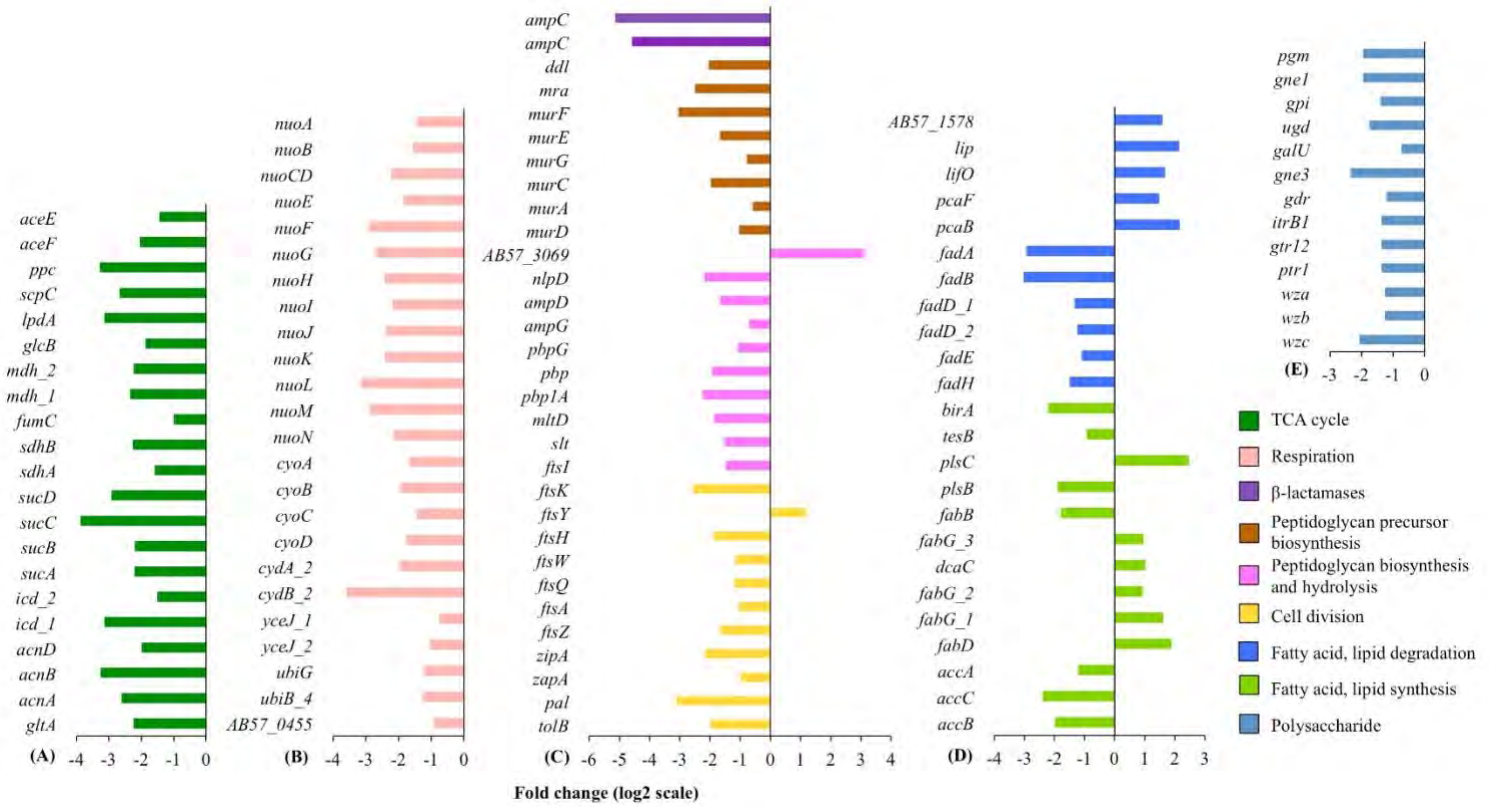

Figure 5.2. Tigecycline induced differential expression of genes in key metabolic pathways. For each cellular pathway, only the genes differentially regulated are presented, and their absolute fold changes are more than 1 (log_2 scale) with statistical significance (adjusted p value < 0.01). The bar charts are grouped by colors according to the biological functions of the genes assigned, with the color scheme under panel E. Please see Supplementary data 5.1for gene function annotation.

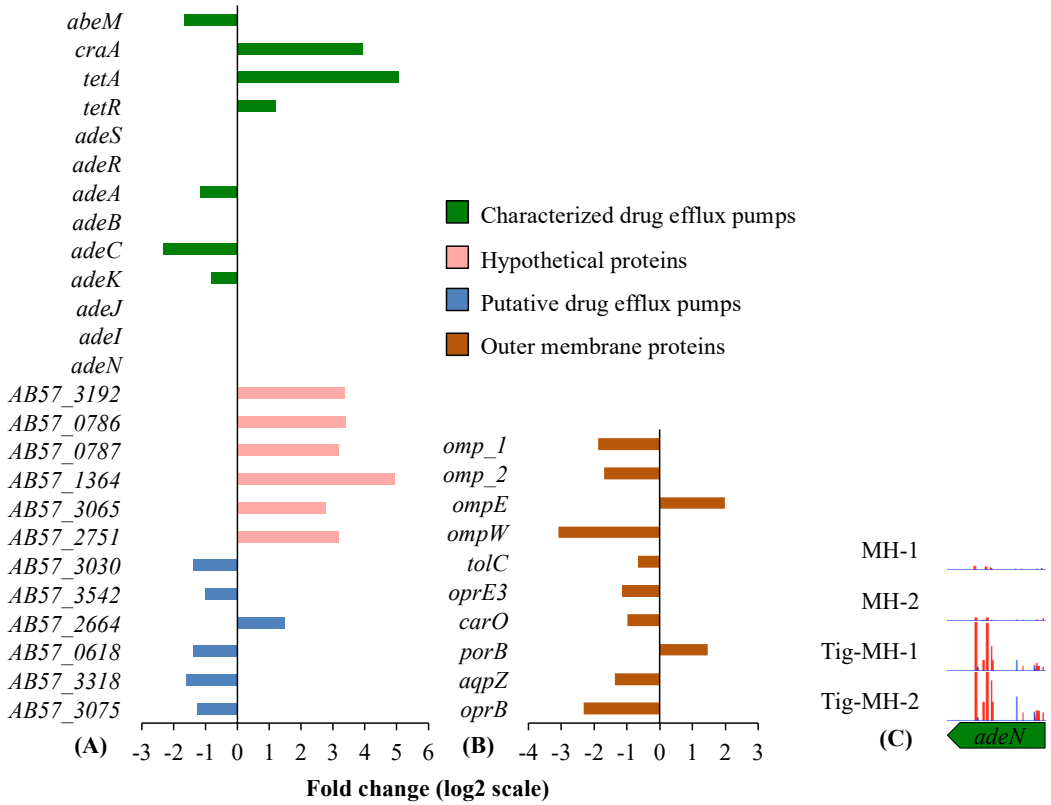

Figure 5.3. Tigecycline impact on the expression of genes involved in membrane permeability and transport. (A) The characterized drug efflux pumps presented here are either the ones differentially regulated or the ones known in conferring tigecycline resistance. The hypothetical proteins are responsive to tigecycline and predicted to have transmembrane helixes. (B) Only the differentially regulated OMPs are presented. All the genes on the panel A and B have absolute fold change more than 1 (log_2 scale) with statistical significance (adjusted p value < 0.01). Please see Supplementary data 5.1 for gene function annotation. (C) Horizontal arrow represents gene *adeN* encoding a transcriptional regulator of *adeIJK*, and shows the direction of translation. The plots above the gene cassette are the read plots of mapped Tn5 insertion sequence from two independent control cultures MH-1 and MH-2 (mutants grown in Muller Hinton [MH] broth) and two independent tigecycline cultures Tig-MH-1 and Tig-MH-2 (mutants grown in MH broth complemented with 1/10 MIC tigecycline).

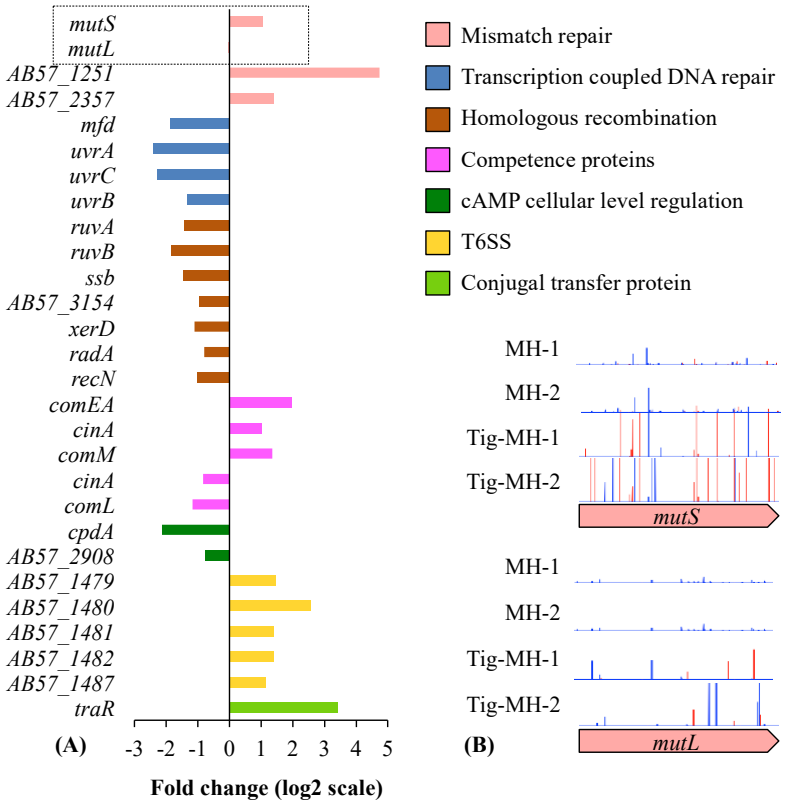

Figure 5.4. Tigecycline affects the expression of genes involved in DNA repair and HGT. (A, B) For each functional group, only the genes differentially regulated are presented, and their absolute fold changes are more than 1 (log_2 scale) with statistical significance (adjusted p value < 0.01). The bar charts are grouped by colors according to the biological functions of the genes assigned, with the color scheme above panel B. Please see Supplementary data 5.1for gene function annotation. (B) Horizontal arrow represents gens showing the direction of transcription, with assigned names within. The plots above the gene cassettes are the read plots of mapped Tn5 insertion sequence from two independent control cultures MH-1 and MH-2 (mutants grown in Muller Hinton [MH] broth) and two independent tigecycline cultures Tig-MH-1 and Tig-MH-2 (mutants grown in MH broth complemented with 1/10 MIC tigecycline).

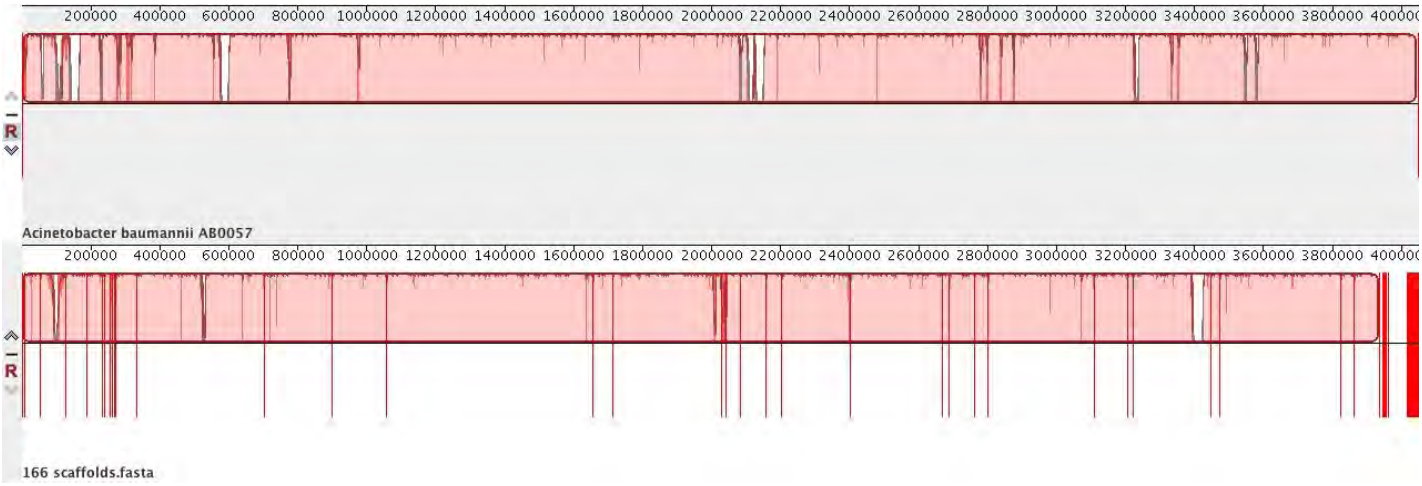

Figure S5.1. Mauve visualization of genomic identity between *A. baumannii* AB0057 and *A. baumannii* 6772166. The horizontal track on the top represents the chromosome sequence of *A. baumannii* AB0057, and the one on the bottom represents the rearranged contig sequences of *A. baumannii* 6772166 with AB0057 as the reference. The long red vertical lines on the bottom track represent the boundaries of each contig. A colored similarity plot is shown for each genome, the height of which is proportional to the level of sequence identity in that region.

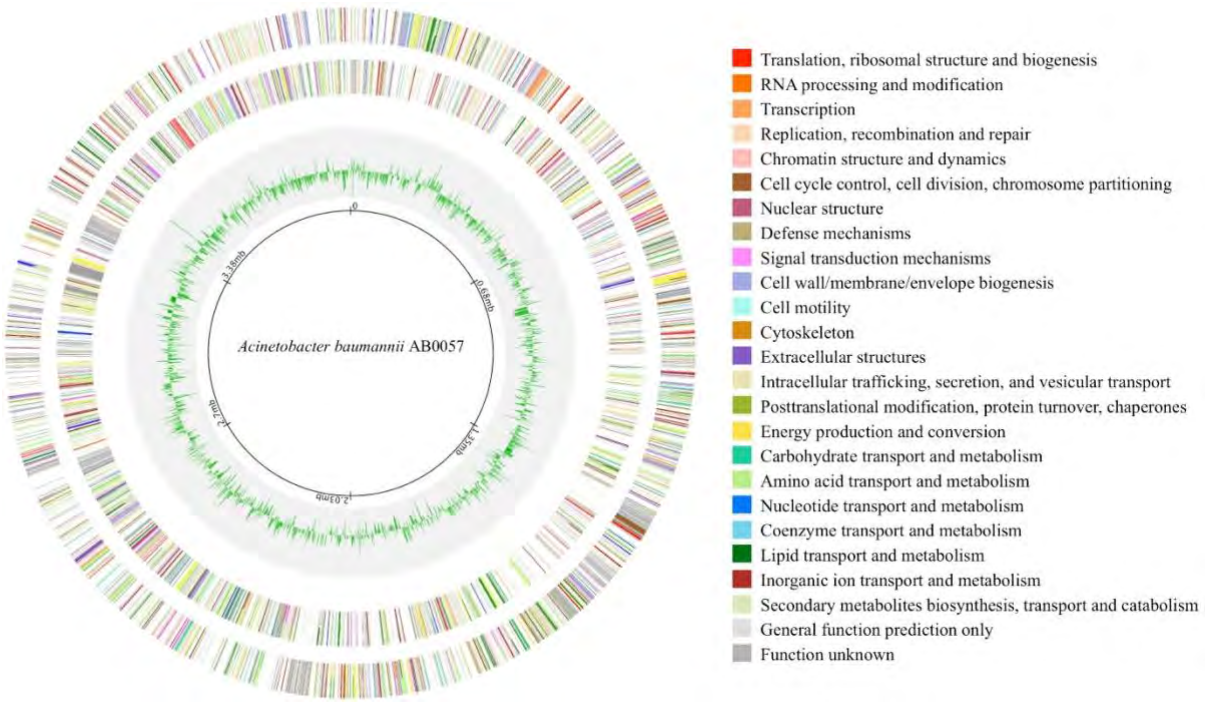

Figure S5.2. A global view of differential gene transcription induced by tigecycline. The circular map was based on the reference genome *A. baumannii* AB0057. From the outermost circle in, the circle 1 shows the ORFs on the plus strand of AB0057 chromosome, the circle 2 shows the ORFs from the minus strand, the circle 3 presents the differential transcription plots of each gene, and the circle 4 shows the coordinates of the chromosome. The ORFs are color coded according to their respective COG category, and with the color scheme on the right. This map was constructed through a web-based tool Circular Visualization for Microbial Genomes (*60*).

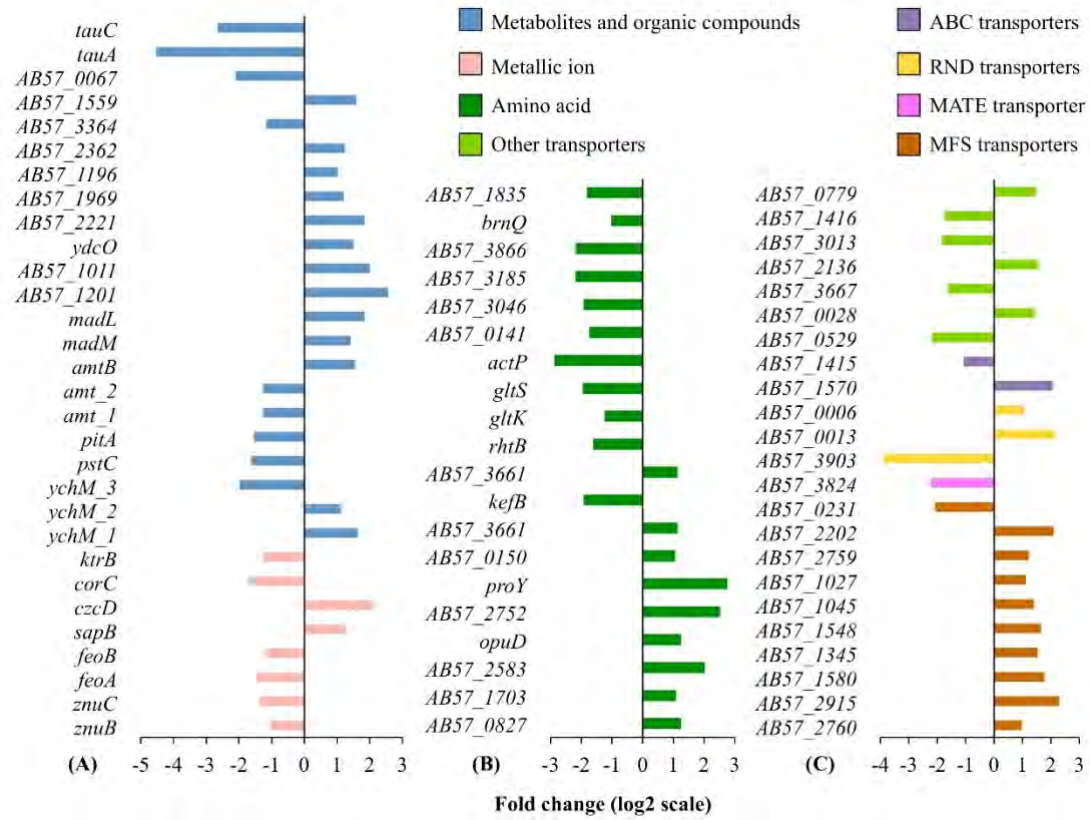

Figure S5.3. Tigecycline induced differential expression of other membrane transporter encoding genes. Only the differently regulated membrane transporter genes are presented, and their absolute fold changes are more than 1 (log_2 scale) with statistical significance (adjusted p value < 0.01). The genes on panels A and B are color grouped based on the putative substrate specificities. The genes on panel C are grouped based on transporter families, as they have no substrate predicted. Please see Supplementary data 5.1for gene function annotation.

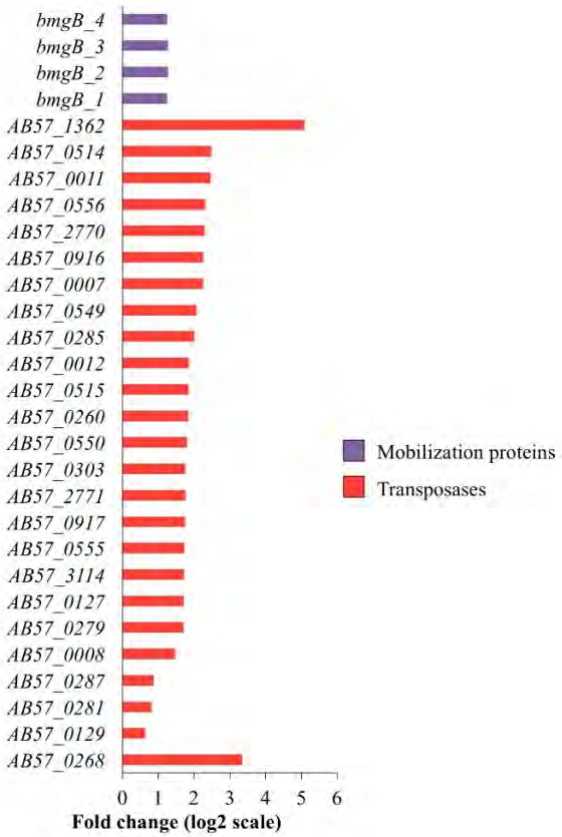

Figure S5.4. Tigecycline induced increased expression of transposase genes and DNA mobilization genes. For each functional group, only the genes differentially regulated are presented, and their absolute fold changes are more than 1 ($\log_2$ scale) with statistical significance (adjusted p value < 0.01). The bar charts are grouped by colors according to the biological functions of the genes assigned, with the color scheme indicated on the right.

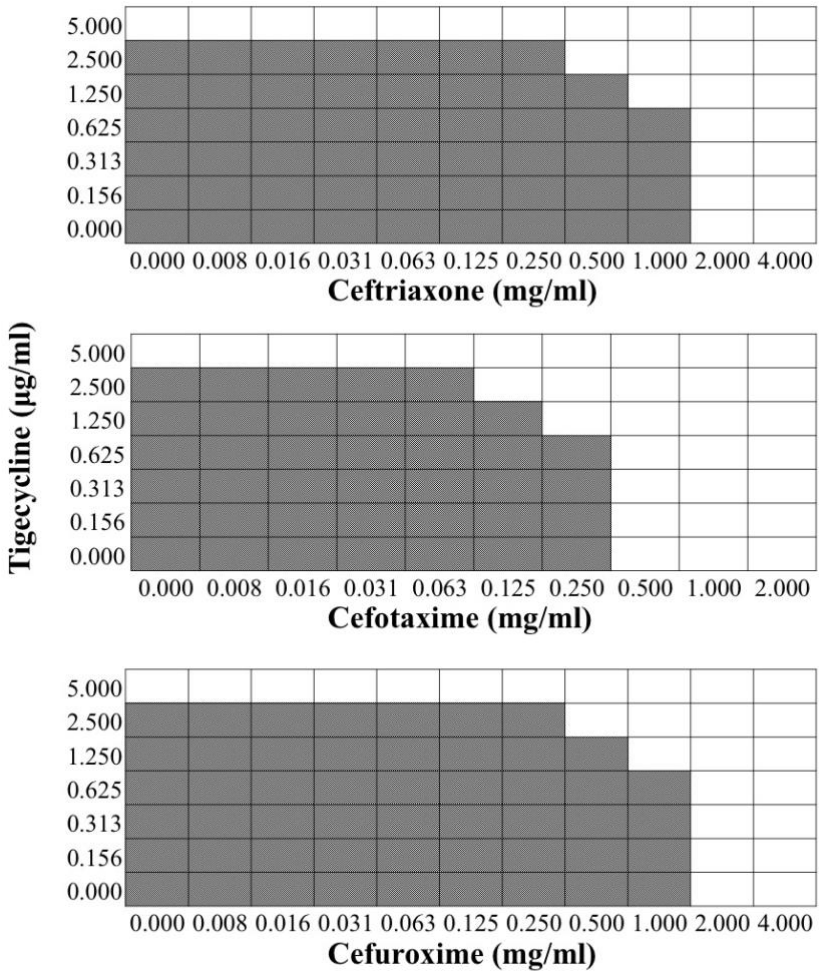

Figure S5.5. Checkerboard assays between tigecycline and β-lactam antibiotics. All the three combinations have a summation of fractional inhibitory concentration value higher than 0.5. According to "Clinical Microbiology Procedures Handbook, 3rd Edition", there is no clear synergism between tigecycline and these three β-lactam antibiotics.

Chapter 6 Transposon directed insertion-site sequencing reveals potential modes of action and resistance determinants of ten biocides in *Acinetobacter baumannii*

Title:

Transposon directed insertion-site sequencing reveals potential modes of action and resistance determinants of ten biocides in *Acinetobacter baumannii*

Liping Li[1], Karl A. Hassan[1], Amy K. Cain[2], Stephen Baker[3], Julian Parkhill[2], and Ian T. Paulsen*[1]

Authors' affiliation

1. Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia 2109

2. The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB101SA, United Kingdom

3. Centre for Tropical Medicine and Global Health, Nuffield Department of Clinical Medicine, Oxford University, Oxford, United Kingdom

***Corresponding author:** Prof. Ian T. Paulsen, ian.paulsen@mq.edu.au, Telephone: +61 2 9850 8152, Fax: +61 2 9850 8313

Running Title: Biocide fitness determinants in *A. baumannii*

6.1. Abstract

While antibiotic resistance in MDR pathogens has been extensively studied, in comparison relatively little is known about the targets and resistance mechanisms for clinically important biocides. In this study, we performed genome-wide screens, using transposon directed insertion site sequencing (TraDIS), to identify the non-essential genes of opportunistic nosocomial pathogen *Acinetobacter baumannii* that affect host fitness to ten structurally diverse biocides. A Tn*5* transposon mutant library comprising more than 100,000 unique insertion mutants was grown in the presence of sub-inhibitory concentrations of each biocide, and genomic DNA extracted for TraDIS sequencing. The locations and densities on Tn*5* insertions were mapped and compared with the library grown under permissive conditions, in order to detect dynamic changes in the mutant population caused by the biocide treatment. The data suggested that the biocides generally had pleiotropic physiological effects on the bacterial host, and genes encoding multidrug efflux pumps, lipooligosaccharide (LOS) and capsular polysaccharide biosynthesis play key roles in biocide susceptibility.

6.2. Introduction

Multiple drug resistance (MDR) in bacterial pathogens is an alarming public health issue (*1*). MDR bacteria apply various strategies to substantially reduce the intracellular concentration of active drug compounds (*2*), including expressing drug efflux pumps to export drugs out of the cell, altering outer membrane permeability to reducing drug intake, and expressing enzymes for drug inactivation. Alternatively, bacteria can avoid the effects of drugs through target site alterations or target site bypass (*3*). Many resistance genes have been spread among or between species through horizontal gene transfer (HGT) (*4*).

The role of antibiotic use in the evolution of MDR bacteria has been extensively studied. In contrast, less is known regarding the contribution of indiscriminate use of biocides, key antibacterials for infection control, to the emergence of MDR bacteria. Biocides are important antiseptics or disinfectants in hospitals, as well as in domestic households (Table 1). The recommended in-use biocide concentrations are normally tens, or hundreds of times higher, than the minimum bactericidal concentration (MBC) against the target microorganism and have multiple antibacterial targets, which in theory should make it unlikely for the bacteria to develop MDR. However, a major concern for biocide usage is whether the residual compound in a hospital or domestic environment could select for resistant strains that may display co-resistance to antibiotics.

There is evidence suggesting that exposure of bacteria to sublethal concentrations of biocides can select for antibiotic resistant mutants under laboratory conditions (*6*), and clinical antibiotic resistant isolates typically have reduced biocide susceptibilities (*7, 8*). One of the most commonly known biocide and antibiotic cross-resistance mechanisms are drug efflux pumps, such as AcrAB-TolC from *Escherichia coli* (*9*), AdeIJK and AdeABC from *Acinetobacter baumannii* (*10, 11*), and NorA from *Staphylococcus aureus* (*12, 13*). The bacterial hosts that

58 overexpress these efflux pumps show increased resistance levels to multiple structurally
59 distinct antibiotics and biocides. Increased expression of drug efflux pumps can be caused by
60 transcriptional de-repression of the respective genes, and emergence of the mutants can be
61 linked to antibacterial exposure.

62 Biocide modes of action are generally poorly characterized, but many biocides are proposed to
63 have multiple, or non-specific biological targets, including the phospholipid membrane (*14*).
64 For instance, silver ions have been found to interact with the thiol group of the exposed cysteine
65 residues of multiple enzymes, and thus to inactivate the enzymes (*15, 16*). Silver ions may
66 also disrupt cellular iron homeostasis, and it cause cell membrane proton leakage (*17*). The
67 known silver resistance determinants are majorly involved with reducing silver ion intracellular
68 concentration, including membrane transporters that could export silver ions (*18, 19*), and
69 proteins that could neutralize or reduce silver ions to the inactive metallic form (*20*). However,
70 no target-site bypass or mutations have been reported, which is not surprising given the
71 multiple targets affected by silver ions (*21*). Indeed, target bypass has rarely been reported as
72 a bacterial biocide resistance mechanism, except for triclosan, which has a specific target, an
73 essential enzyme enoyl reductase (FabI) that is involved in fatty acid synthesis (*22*). The two
74 key determinants for high-level triclosan resistance are MDR efflux pumps and either
75 mutations in the *fabI* gene or the acquisition of alternative Fab proteins that are not recognized
76 by triclosan (*23*).

77 Bacterial biofilm formation on biological or inanimate surfaces is another challenge for biocide
78 disinfection (*24*), as cells in a biofilm matrix have significantly lower susceptibility to a broad
79 range of antimicrobials than their planktonic counterparts (*25*). Rather than the resistant
80 determinants active in planktonic cells, biofilm cells adapt different mechanisms for
81 antimicrobial tolerance (*26-28*).

The Gram-negative opportunistic human pathogen *A. baumannii* has become a significant worldwide threat for immunocompromised patients who are hospitalized in an Intensive Care Unit (ICU). This is primarily due to the emergence of *A. baummannii* clonal lineages with high level resistance to antibiotics, biocides and desiccation (*29, 30*). Some of the biocide resistance mechanisms mentioned previously have also been identified in this organism, e.g. MDR efflux pumps for multiple-biocide resistance and *fabI* mutations for triclosan resistance (*23, 31*).

To advance our knowledge of both biocide modes of action and resistance mechanisms, we performed genome-wide identification of the genes that could enhance biocide tolerance or increase biocide susceptibilities in a MDR *A. baumannii* strain against ten clinically important biocides (Table 1) through transposon directed insertion-site sequencing (TraDIS) (*32*). TraDIS involves high throughput sequencing of a saturated transposon mutant library of a bacteria to identify genes that are advantageous or disadvantageous for growth under specific conditions (*32*).

6.3. Results and discussion:

6.3.1. Mutant library and TraDIS data interpretation

A random and saturated transposon mutant library of *A. baumannii* was used for global biocide resistance gene identification through TraDIS. This library originated from a global clone II multidrug resistant *A. baumannii* strain BAL062 through transposition of a Tn*5* derivative (*32*), and comprises more than 100,000 unique individual mutants, with an average of one insertion every 38 bp (Figure 6.1A). There are 347 genes with no Tn5 insertion assigned as essential for growth in Muller Hinton broth (Figure 6.2B and 6.2C). Thus, the library has sufficient insertion density to allow statistical confidence in calculations of gene importance in TraDIS assays. Ten structurally distinct biocides that are either listed as essential medicines by WHO (World Health Organization) or commonly applied in clinical settings and household products were tested in this study (Table 6.1). To impose a selective pressure each biocide was used at ¼ × minimum inhibitory concentration (MIC) of the wild-type strain. At this concentration the biocides have no observable inhibitive effect against the wild-type cells, and accordingly can impose a chemical selection that allows the identification of mutants with a fitness advantage or defect, without inhibiting the growth of the remainder of the cell population.

In comparison with control mutant pools, genes in the pools with biocide treatment that have more than a 2-fold change in transposon insertion read number and with a q-value lower than 0.01 were considered as being under significant selection (*33*). By these criteria, each of the ten biocide treatments resulted in positive selection for between 7 and 100 genes, and negative selection for between 3 and 120 genes (Table 6.1 and Figure S6.1). Genes with fewer insertion reads in the biocide treated samples are potential resistance determinants; whereas genes with an increased number of insertion reads are potential antibacterial non-essential targets or the repressors of resistance determinants. For instance, the known MDR efflux pump-encoding

operon *adeABC* and its regulatory activator genes *adeR* and *adeS* were found to have significantly decreased insertion read coverage after benzalkonium treatment, which is a known substrate of the AdeABC pump, whereas no change was seen in insertion read number after glutaraldehyde treatment, which is not a substrate of this pump (Figure 6.2A). A significant number of potential new resistance genes or new phenotypes of known resistance genes were identified, for example, the efflux pump-encoding gene *amvA* had a 19.4-fold decrease in Tn*5* insertion read number after treatment with silver nitrate, suggesting it may confer silver ion resistance; and the adjacently-encoded transcriptional regulator gene *amvR* showed a 5.2-fold increase in insertion reads, consistent with previous findings that this regulator acts as a transcriptional repressor of *amvA* (Figure 6.2A) (*33*).

For further validation of TraDIS data, the minimum inhibitory concentration change of four isogenic mutants *adeB*::T26, *lpsB*::T26, *pyrC*::T26 and *adeR*::T26, derived from *A. baumannii* AB5075 (*34*), were tested with benzalkonium, triclosan, silver nitrate and glutaraldehyde respectively (Figure 6.2B). The MIC fold changes of each of the four mutants against the respective biocide were consistent with each TraDIS results (Figure 6.2A and 6.2B).

Alternatively, some of the genes with either an increase or decrease of Tn*5* insertion read coverage might not directly contribute to resistance, rather their cellular pathways might be affected by the biocide tested. For example, one of the mechanisms by which silver nitrate exerts its antibacterial action is by collapsing cytoplasmic membrane proton motive force (*17*), correspondingly two electron transport chain related genes that encode cytochrome D ubiquinol oxidase subunits had decreased Tn*5* insertion reads in the silver nitrate treated mutant pool (Figure 6.5).

The Tn*5* insertion read coverage of 29 genes encoding hypothetical proteins or proteins with unknown functions were decreased or increased upon one, or more than one, biocide treatment,

suggesting a potential role for these genes in affecting host fitness to biocides. The biocide treatments also had a significant impact on regulatory control elements, with 21 known or putative transcriptional regulator genes impacted by one, or more than one, biocide treatment.

6.3.2. Cell envelope in *A. baumannii* biocide resistance

Several biocides tested in this study have been reported to cause damage to the bacterial cell envelope (Table 6.1). Although this effect might not be sufficient to induce lysis or cell death, it has been speculated that such damage may increase cell membrane permeability to biocides, promoting their uptake and resulting in the exposure of other cellular antibacterial targets to lethal concentrations of the biocide (*14*). In this study, TraDIS provided additional evidence for the role of the cell envelope as both an antibacterial target and a mechanism of biocide resistance.

Outer membrane lipooligosaccharide. The common architecture of lipopolysaccharide (LPS) consists of three major domains, namely the hydrophobic anchor (lipid A), the core oligosaccharide and the hydrophilic O-antigen (*35*). The homolog of O antigen ligase WaaL, responsible for transferring O antigen polysaccharide onto the outer-core of LPS has not been identified in *A. baumannii* genomes, leading to the suggestion that *A. baumannii* may produce a lipooligosaccharide (LOS) comprising only lipid A and core oligosaccharide (*36, 37*). Thus, we refer to LOS throughout this paper.

This study revealed that a lauroyl acyl-transferase encoding gene *lpxL* had 21- and 2.3-fold increases in Tn*5* insertion read number following treatment with the cationic surfactants chlorhexidine and cetrimonium bromide (CTAB) respectively, suggesting this gene plays a role in decreased fitness to these two biocides (Figure 6.3C). Additionally, although five other biocides only showed a less than two-fold increase in Tn*5* insertion read number for the *lpxL*

gene, all the changes were statistically significant with q values lower than 0.0001 (Figure 6.3C), suggesting that the product of this gene may increase the permeability of the membrane to these biocides. The predominant glycolipid molecule in the outer leaflet of *A. baumannii* OM is hepta-acylated lipid A (*38*). It was shown that LpxL can transfer one lauroyl acyl chain to lipid A, and its mutant generated hexa-acylated lipid A (*39*). Together this suggests that a different fatty acid profile (hexa-acylated lipid A) might affect the antibacterial action of these biocides against *A. baumannii* or their capacity to penetrate the *A. baumannii* outer-membrane.

Cell surface polysaccharides. In *A. baumannii*, there are two gene clusters known to be involved in capsule polysaccharide biosynthesis (K-locus) and LOS outer core oligosaccharide biosynthesis (OC-locus) respectively (*36*). We showed that nine genes on the K-locus had significant changes in the number of Tn*5* insertion reads when treated with one, or more than one, biocide (Figure 6.3C). For instance, the Tn*5* insertion read coverage of the *gna* gene encoding a UDP-glucose dehydrogenase increased 207-fold and decreased 3.6-fold upon chlorhexidine and benzalkonium treatment, respectively. This suggests that the disruption of these genes results in decreased *A. baumannii* fitness toward benzalkonium, but enhanced chlorhexidine fitness.

The K-locus has a conserved genomic location, but with substantial diversity in gene composition across *A. baumannii* isolates (*36*). The two ends of the cluster however are quite conserved in gene composition although not strictly in DNA sequences, i.e. the 5’ end of the K locus carries genes involved in capsular polysaccharide assembly and export, and the 3’ end of the K locus with UDP-sugar synthesis genes (Figure 6.3B) (*36*). It has been shown that deletion of the UDP-sugar synthesis genes in the 3’ end of the K locus not only dramatically reduced capsule production but also generated truncated LOS (*40*). In contrast, deletion of the three genes in the 5’ end of the K locus for capsular polysaccharide assembly and export

abrogated capsule production, but did not affect LOS (*40*). This indicates that disruption of the genes in the K-locus may change not only capsular polysaccharide production but also LOS sugar structure, and either of these two changes affect *A. baumannii* fitness to the biocides tested.

The number of Tn*5* insertion reads of the *pglC* gene, encoding an initiating glycosyltransferase, was significantly decreased (9.9-fold) in the silver nitrate treated mutant pool only, suggesting this gene mediates resistance to silver nitrate and not to the other nine biocides (Figure 6.3C). *pglC* is in the variable region of the K-locus, but its presence is conserved in this gene cluster across *A. baumannii* strains (*36, 41*). It is required for the construction of capsular glycan repeat-units and glycosylation of glycoproteins (*41*), and the deletion mutant affects capsule production, not LOS (*40*). Apart from *pglC*, the presence of silver nitrate also decreased the number of Tn5 insertion reads for seven genes in the K-locus (Figure 6.3C). Speculatively, this implies that the capsular polysaccharide may play a role in silver nitrate tolerance, whereas alteration of LOS glycoforms decreases the host susceptibility to all the eight biocides tested in this study, but increases the susceptibility to silver nitrate.

Seven genes in the OC-locus also had statistically significant changes of their respective Tn*5* insertion read coverage upon one, or more than one, biocide treatment (Figure 6.3C). For instance, the number of Tn*5* insertion reads for *lpsC,* encoding a glycosyltransferase decreased 5.8-fold upon treatment with benzalkonium, suggesting *lpsC* plays a role in benzalkonium resistance, and the Tn*5* insertion read coverage for the *gtrOC3* glycosyltransferase gene decreased 78.8-fold upon chlorhexidine treatment, suggesting *gtrOC3* mediates chlorhexidine resistance (Figure 6.3C). The genes in this gene cluster are mainly predicted to encode glycosyltransferases that catalyse the linking of sugars of the LOS outer oligosaccharide core, and the disruption of one of these genes in *A. baumannii* caused by insertion sequence has been

shown to produce truncated LOS (*37, 42*). Another glycosyltransferase encoding gene *lpsB*, which is outside of the two polysaccharide biosynthesis loci and involved in LOS core biosynthesis, also had significant Tn*5* insertion read changes following the treatments of seven biocides (Figure 6.3C). For instance, its transposon insertion reads increased 52-fold and decreased 5.8-fold, following chlorhexidine and triclosan treatments respectively (Figure 6.3C), suggesting that disruption of this gene results to increased chlorhexidine fitness but reduced fitness against triclosan. Previous work has shown that a *lpsB* mutant generated a truncated LOS with only two 3-deoxy-D-manno-octulosonic acid residues and lipid A (*43*). This provides further support for our speculation that truncated or altered LOS glycoforms affect *A. baumannii* susceptibilities to various biocide compounds.

Chaperone-usher pilus. Another potential cell surface related, biocide tolerance determinant revealed in this study is a chaperone-usher (CU) pilus assembly gene cluster *csuA/BABCDE* (Figure 6.3C) (*44*). The Tn*5* insertion reads for each of the genes in this cluster were decreased upon eight biocide treatments (Figure 6.3C), suggesting that disruption of any of the *csu* genes results in a host with increased susceptibility to these compounds. The Tn*5* insertion read changes among each of the *csu* genes to a single compound were similar, indicating that either the mature pilus system is required for a biocide tolerance phenotype or that there might be polar effect on the operon transcription caused by Tn*5* insertion. For example, Tn*5* insertion read of all the *csu* genes decreased significantly in the mutant pool treated with silver nitrate (2.2 - 3.2-fold), benzalkonium (2.8 - 3.8-fold), CTAB (7.2 - 8.4-fold), or triclosan (5.0 – 7.0-fold) (Figure 6.3C). Interestingly, a transcriptional regulator (BAL062_01328) directly upstream of the *csu* operon also had decreased Tn*5* insertion reads following treatment with the same eight biocides and the fold change was similar to *csu* gene mutants (Figure 6.3C), indicating that BAL062_01328 encodes a potential local transcriptional activator of the *csu* operon. Consistent with their known function as a transcriptional activator of the *csu* operon,

the genes of the two-component system BfmRS were also shown to have decreased Tn*5* insertion read following treatment with seven of the eight biocides (green panel on Figure 6.3C). The absolute Tn*5* insertion read fold changes of *bfmS* in all the seven biocide conditions were lower than the absolute fold change of *bfmR*, which is consistent with a previous finding that deletion of *bfmR* dramatically reduced CU pilus production, but deletion of *bfmS* had only a minor effect (*45*).

The insertion reads of Tn*5* mutants of both *bfmR* and *bfmS* were not affected by silver nitrate, even though this compound decreased Tn*5* insertion reads for the *csu* gene cluster (Figure 6.3C). In addition to its role in regulating expression of the *csu* genes, BfmS is known to negatively regulate the production of capsular polysaccharide through BfmR, and its absence leads to capsular polysaccharide hyper-production in *A. baumannii* (*40*). Speculatively, the reason that ¼ × MIC silver nitrate treatment had no observable effect on the insertion reads of the Tn*5* mutants of *bfmR* and *bfmS* might be because that deletion of either *bfmR* or *bfmS* would lead to increased production of capsular polysaccharide but reduced expression of CU pilus, both of which have been implicated in mediating silver nitrate resistance in this study.

Additionally, although the *csu* mutants were not affected by ethanol at ¼ × MIC, the Tn*5* insertion reads for *bfmRS* were decreased upon ethanol treatment, suggesting that there might be alternative ethanol tolerance determinant(s) under the regulation of BfmRS.

6.3.3. Membrane transport

Efflux pumps are inner-membrane transporter proteins that can confer drug resistance by reducing intracellular drug concentration through pumping compounds out of the cell (*46*). Seventeen drug efflux systems, including representatives from all the six known drug transporter families, have been characterized or identified in *A. baumannii* (*11, 47*). One of the

most clinically significant drug transporters in *A. baumannii* is a tripartite efflux system AdeABC, with AdeB belonging to the resistance-nodulation-division (RND) efflux pump superfamily, AdeA a membrane fusion protein (MFP) and AdeC an outer membrane factor (OMF). When overexpressed, this system confers resistance to a broad range of structurally distinct antimicrobials (*48*). In clinical *A. baumannii* MDR strains, constitutive overexpression of AdeABC is usually correlated with site mutations in the two-component transcriptional activator encoding genes *adeRS*, which are immediately upstream of *adeABC* (*49*).

Consistently, we found that the Tn*5* insertion reads of *adeB* were significantly decreased in the mutant pools that were treated with benzalkonium (115.4-fold), cetrimonium (2.2-fold), chlorhexidine (85.6-fold), triclosan (7.9-fold) and chloroxylenol (2.0-fold) respectively (Figure 6.4A), suggesting that *adeB* mediates resistance to these five compounds. Benzalkonium and chlorhexidine have previously been characterized as substrates of AdeB (*31*). Surprisingly, changes of Tn*5* insertion reads of *adeA* and *adeC* were only seen in the mutant pools treated with benzalkonium and chlorhexidine, which correlated with the high fold-change in insertions in *adeB* (Figure 6.4A). This suggests AdeABC functions as a tripartite system in pumping out these two compounds. However, these fold-changes were less significant than the changes of *adeB* caused by the same compounds. In combination with the lack of significant changes in mutants for cetrimonium, triclosan and chloroxylenol, this suggests that there might be alternative OMF and MFP proteins that could form tripartite RND efflux system with AdeB in the Tn*5* mutants lacking AdeA or AdeC. Indeed, *A. baumannii* MDR strains that do not encode AdeC, and only contain *adeAB* are common (50) and constitute more than 30 % of strains with complete genome sequences. Furthermore, *adeA* or *adeB* deletion mutants of some strains had decreased fitness to a number of antimicrobials, but *adeC* deletion mutant had no fitness change (*50, 51*), suggesting AdeC is dispensable for a functioning tripartite RND system that contains AdeA and AdeB.

When treated with benzalkonium and chlorhexidine, the two-component transcriptional activator genes of *adeABC* had significant decrease in Tn*5* insertion reads: *adeR* decreased by 7.6- and 26.7-fold, and *adeS* decreased by 7.5- and 240-fold respectively. However, no Tn*5* insertion read change in *adeRS* was observed in the other three conditions (cetrimonium, triclosan and chloroxylenol) that also negatively selected *adeB* mutants (Figure 6.4A). These observations together suggest that benzalkonium and chlorhexidine could induce the overexpression of AdeABC through AdeRS, whereas the other three compounds cannot. This suggests that transposon insertion mutations in *adeR*, *adeS*, *adeA* or *adeC* had no effect on the host fitness to cetrimonium, triclosan and chloroxylenol, probably due to AdeB coupling with alternative OMF and MFP to form tripartite RND systems (Figure 6.4B).

Another important RND system that has been characterized in clinical *A. baumannii* MDR strains is AdeIJK, which has a similar range of substrates to AdeABC (*31, 52*). The TraDIS data from this study for *adeIJK* is similar to that for *adeABC* (Figure 6.4A). For example, in comparison to the control pools, the Tn*5* insertion reads of the OMF encoding gene *adeK* were decreased 1.7-, 17-, 79.9-, 27.3-and 10.0-fold in the mutant pools treated with silver nitrate, benzalkonium, chlorhexidine, triclosan and chloroxylenol respectively (Figure 6.4A). The respective Tn*5* insertion read changes of the RND pump encoding gene *adeJ* and the MFP encoding gene *adeI* were lower than seen for *adeK*. Given the dispensability of *adeC* described above, the strong selection against insertions in *adeK* suggests that AdeK might not only form a tripartite drug efflux system with AdeI and AdeJ, but also with AdeAB (Figure 6.4B). Consistent with a previous finding that the TetR-type transcriptional regulator AdeN represses the transcription of *adeIJK* (*53*), the Tn*5* insertion reads of *adeN* had 1.5-, 99.7-, 1.7- and 1.3-fold increase in the mutant pools treated with benzalkonium, chlorhexidine, triclosan and chloroxylenol respectively (Figure 6.4A).

The Tn*5* insertion reads of another putative RND membrane transporter gene BAL062_00031 and MFP encoding gene BAL062_00030 were reduced 7.31- and 7.41-fold respectively in the mutant pools treated with triclosan, suggesting they play a role in triclosan resistance (Figure 6.4). There is no OMF gene located adjacent to BAL062_00030 and BAL062_00031. It is possible that this system uses AdeK as its OMF partner, because *adeK* is the only OMF gene that had reduced Tn*5* insertion reads (27-fold) in the mutant pool treated by triclosan. The Tn5 insertion reads of a putative transcriptional regulator gene BAL062_00029, lying immediately upstream of BAL062_00030 and BAL062_00031, were increased 1.3-fold upon triclosan treatment (Figure 6.4A), suggesting it might be a repressor of transcription for BAL062_00030 and BAL062_00031.

In addition to the RND pumps described above, three efflux pumps (AmvA, TetA, and AceI) have been implicated in mediating biocide tolerance. AmvA, from the Major Facilitator Superfamily (MFS), has been characterized as a multi-antibacterial exporter with substrates including benzalkonium, chlorhexidine, norfloxacin and erythromycin (*54, 55*). Consistent with this data, the Tn*5* insertion reads of *amvA* were decreased 1.6- and 106.2-fold in the mutant pools treated with benzalkonium and chlorhexidine respectively, and decreased 19.4-fold in the mutant pool treated with silver nitrate (Figure 6.4A). Furthermore, the Tn*5* insertion reads of *amvR*, encoding a transcriptional repressor of *amvA* (*33*), were increased 5.2- and 84.7-fold in the mutant pools treated with silver nitrate and chlorhexidine, respectively (Figure 6.4A). This suggests that in addition to the previously identified substrates such as chlorhexidine and benzalkonium, AmvA may also recognize silver ions as substrates, or may transport other compounds related to silver detoxification.

The Tn*5* insertion reads of *tetR*, located immediately upstream of a tetracycline efflux pump gene *tetA*, were increased 21.1-fold in the mutant pool treated with chlorhexidine (Figure 6.4A).

TetR is a known transcriptional repressor of *tetA* (*56*), and this result suggests that *tetA* overexpression causes reduced susceptibility to chlorhexidine. This is consistent with our previous finding that *E. coli* highly expressing *A. baumannii* TetA had a significant increase in chlorhexidine resistance level (*57*). Notably, the frequency of Tn*5* insertions in the *tetA* gene itself were not affected by chlorhexidine. We speculate that this is because *tetA* is tightly controlled by TetR, and transcriptional repression is only relieved in the presence of tetracycline antibiotics. Therefore, in the presence of a functional TetR and in the absence of a tetracycline, *tetA* is not expressed and there is no basis for selection by chlorhexidine. This result highlights a potential different in the substrate specificity of an efflux pump and the ligand recognition profile of its cognate regulator.

The *aceI* gene has been reported to confer chlorhexidine efflux (*58*), and in our TraDIS dataset, *aceI* showed an 84.4-fold decrease in Tn*5* insertion read density following chlorhexidine treatment. However, despite this large fold change, due to variability between the replicates, it was not recognised as statistically significant using our accepted threshold (Q-value = 0.22).

The other putative multidrug efflux pump highlighted from this study is an ATP-binding cassette family (ABC) transporter consisting of the genes BAL062_01229, encoding a putative membrane permease, and BAL062_01230 encoding an ATP-binding protein. In the mutant pool treated with silver nitrate, the Tn*5* insertion reads of BAL062_01229 and BAL062_01230 were decreased 2.2- and 2.6-fold respectively (Figure 6.4A), suggesting absence of this ABC efflux system decreased *A. baumannii* fitness to silver nitrate.

6.3.4. Stress responses and resistance determinants against silver nitrate

The previously proposed antibacterial action of silver nitrate is through interaction of the ion with the proteins or enzymes that have exposed cysteine residue(s) or iron-sulfur (Fe-S) cluster(s) (*15, 59*). This can lead to protein inactivation or disrupt iron homeostasis to initiate killing effects. Our silver nitrate TraDIS data identifies multiple biological pathways in *A. baumannii* affected by silver nitrate.

TCA cycle. Tn*5* insertional mutants of several TCA genes have different effects on host fitness to silver nitrate. For example, two TCA cycle related genes *sucC* and *sucD* encoding succinyl-CoA synthetase β and α subunits respectively, both had 12.3-fold increase in Tn*5* insertion reads upon silver nitrate treatment, suggesting absence of the respective proteins increased the host fitness to silver nitrate (Figure 6.5A). Previous work has demonstrated that the knockout strains of *E. coli* TCA cycle related genes (*ΔsucB*, *Δmdh*) are less sensitive to silver nitrate than the wild type (*16*). In contrast, two other TCA related genes, *acnA_1* encoding aconitate hydratase 1 and *icd_2* encoding isocitrate dehydrogenase, had 11.7- and 12.5-fold decrease in Tn*5* insertion reads respectively (Figure 6.5A), suggesting disruption of these two genes decreased host fitness to silver nitrate. Speculatively, silver nitrate targets multiple TCA related proteins such as SucC and SucD in *A. baumannii*, which could interfere with the TCA pathway that requires these two proteins. However, absence of other TCA proteins that are not affected by silver nitrate such as *acnA_1* and *icd_2* would further attenuate the growth of hosts in which the TCA pathway is already subjected to silver nitrate stress.

Cell respiration. The other known silver nitrate antibacterial effect is cytoplasmic membrane proton leakage and attenuated or ceased cell respiration (*17*). The Tn5 insertion reads of two genes *cydB* and *cydA_1*, encoding cytochrome D ubiquinol oxidase subunit I and II respectively, decreased 5.5- and 7.0-fold respectively (Figure 6.5A), suggesting disruption of these two genes decreased fitness to silver nitrate. The Tn5 insertion reads of a ubiquinone

biosynthesis gene BAL062_03562 decreased 4-fold, indicating this gene also affects *A. baumannii* susceptibility to silver nitrate. Ubiquinone transfers electron to the cytochrome *bd*-I oxidase, the terminal acceptor of the aerobic respiration chain, and this process generates energy in the form of proton-motive force (PMF) across the cytoplasmic membrane (*60*). We hypothesize that these genes might be involved in maintaining the PMF to resist silver nitrate induced cytoplasmic membrane proton leakage and the compromised electron suppliers from TCA cycle.

Silver nitrate's effect on reducing membrane proton potential can affect the efficacy of transporter proteins in the cytoplasmic membrane. Although the mutant library was grown in rich media, due to the presence of silver nitrate which may compromise the efficiency of amino acid uptake through collapsing the PMF, the bacterial host may need to rely on amino acid biosynthesis for replenishing its amino acid pool. The mutants of 9 genes involved in amino acid biosynthesis identified in this study with statistically significant Tn5 insertion read changes all had decreased insertion reads when treated by silver nitrate. For example, 13.3-fold decrease for homoserine dehydrogenase gene *hom_2*, 14.0-fold decrease for threonine synthase gene *thrC*, and 9.5-fold decrease for argininosuccinate lyase gene *argH* (Figure 6.5A). Two genes of cysteine biosynthesis showed around 2-fold increase (Figure 6.5A). The other amino acid biosynthesis genes are either essential for growth or show no significant Tn5 insertion read changes.

Biotin biosynthesis. Biotin is a crucial co-enzyme for a class of important metabolic enzymes that need to be biotinylated before becoming biologically active, for instance acetyl-CoA carboxylase (ACC) involved in the first step of fatty acid synthesis (*48*). We showed that silver nitrate treatment led to a 2.2- to 2.4-fold increase of Tn*5* insertion reads in genes in a biotin biosynthesis operon (Figure 6.6), suggesting that a defect in biotin biosynthesis renders the

406 host less silver nitrate susceptible. Four genes in a gene cluster related to CoA processing also
407 had increased Tn5 insertion reads upon silver nitrate treatment, i.e. BAL062_02318,
408 BAL062_02319, BAL062_02320 and BAL062_02321 were increased 7.0-, 8.5-, 6.8- and 3.1-
409 fold respectively. In contrast, disruption of two genes (BAL062_03606 and BAL062_03607)
410 encoding putative fatty acid oxidases, likely involved in fatty acid degradation (*61*), had
411 decreased Tn*5* insertion reads (3.1- and 2.4-fold respectively), suggesting absence of these two
412 proteins resulted in a host with decreased fitness to silver nitrate. Silver nitrate treatment has
413 been reported to cause detachment of the bacterial cytoplasmic membrane from the
414 peptidoglycan layer (*62*), which may be connected with these effects on fatty acid biosynthesis.

415 **Other cellular pathways**. Twelve genes responsible for purine and pyrimidine biosynthesis
416 had a significant increase in Tn*5* insertion reads upon silver nitrate treatment, suggesting
417 absence of these proteins resulted in decreased silver nitrate susceptibility of the host. For
418 instance, two genes for pyrimidine biosynthesis *pyrC_2* encoding dihydroorotase and *pyrD*
419 encoding dihydroorotate dehydrogenase had 35.9- and 9.9-fold increase in Tn5 insertion reads
420 respectively (Supplementary data 6.1, section "nucleotide").

421 Seven genes encoding hypothetical proteins had significantly increased Tn*5* insertion read
422 coverage after silver nitrate selection, suggesting that insertional disruption of these genes may
423 increase *A. baumannii* fitness in the presence of silver nitrate. A further seven genes encoding
424 transcriptional regulators had significant changes in Tn*5* insertion read coverage upon silver
425 nitrate treatment, indicating that silver nitrate has broad pleiotropic effects against *A.*
426 *baumannii*.

427 ### 6.3.5. Proton motive force and cell division

428 Various genes that are involved in peptidoglycan (PG) synthesis, cell shape determination and
429 cell division were shown to have decreased Tn*5* insertion reads following treatment with silver

nitrate or other biocides (Figure 6.5B). The bacterial rod shape determining genes *mreB*, *mreC*, *mreD*, *rodA* and *pbp2* had decreased Tn*5* insertion reads (4.8-, 6.3-, 4.7-, 4.8- and 4.0-fold respectively) when treated by silver nitrate, suggesting disruption of these genes resulted in decreased host fitness to silver nitrate. These five genes are required for maintenance of the cylindrical shape of the cell in diverse bacterial species, such as *E. coli*, *Bacillus subtilis*, and *Caulobacter crescentus* (*63-66*). Silver ion solution has been shown to cause distortion in bacterial cell membranes and morphology (*67*), which suggests a link between the role of these genes in cell shape maintenance and murein biosynthesis, and the targeting of the cell envelope by silver nitrate. Hypochlorite and ethanol also showed similar effects on these genes, though with a lower fold change (Figure 6.5B), suggesting they may also affect cell membranes and morphology.

The FtsZ-ring associated genes (*zipA*, *zapA* and *rlpA*) and six genes involved in PG synthesis and hydrolysis were also found to have a role in protecting cells from the challenges of multiple structurally distinct biocides (Figure 6.5B). The Tn*5* insertion reads of *zipA* were decreased in mutant pools treated with eight different biocides, for example a 6.9-fold decrease in the silver nitrate pool, a 3.0-fold decrease in the chloroxylenol pool, and a 2.5-fold decrease in the glutaraldehyde pool. ZipA is an essential component of the FtsZ ring and an essential protein in mediating septum formation in *E. coli* (*68*). The numbers of Tn*5* insertion reads of the genes in the *pal-tolQ* operon, which is required for the OM invagination during cell division, were significantly reduced (59.3 – 61.7-fold) in the mutant pool treated with chlorhexidine. The *pal-tolQ* operon in *Delftia acidovorans* has previously been reported to play a role in chlorhexidine resistance in biofilms (*69*).

The cellular localization of MreB and FtsA (FtsZ-ring associated) proteins are modulated by the PMF, suggesting the PMF affects cell division (*70*). This may explain why TraDIS could

simultaneously reveal that both the PMF related genes and cell division related genes affect *A. baumannii* fitness to silver nitrate. An electron transport related operon encoding cytochrome -o (*cyo*) ubiquinol oxidase subunits had significant reduced Tn*5* insertion reads upon chloroxylenol, benzalkonium or ethanol treatment (Figure 6.5B), suggesting the electron transport chain is also affected by these compounds. Correspondingly, multiple genes responsible for amino acid biosynthesis had reduced Tn*5* insertion reads when treated by 9 out of the 10 biocides tested, including the three compounds that affect *cyo* operon (Figure 6.5A). These compounds are all structurally distinct. Speculatively, the collapse of the PMF might be the direct or downstream antibacterial effect of multiple antimicrobials through affecting electron transport chain, and the subsequent result is the malfunction of cytoplasmic transport systems and hence reduced nutrient uptake and increased accumulation of toxins. Like silver nitrate, collapse of the PMF by the other biocides might also be linked to the cell division related genes, which were shown to affect *A. baumannii* fitness to these compounds.

6.3.6. *Stress responses of A. baumannii to the other biocides*

Phenol biocides, triclosan and chloroxylenol. The major antibacterial effect of triclosan is blocking fatty acid biosynthesis by interfering with an essential protein enoyl-acyl reductase FabI (*71*). However, this effect is not detectable through TraDIS, because *fabI* is essential and TraDIS is based on fitness changes resulting from transposon mutants of non-essential genes. However, the Tn5 insertion reads of the five biotin biosynthesis genes were 1.6-fold increased upon triclosan treatment, suggesting absence of these genes also slightly increased the host fitness to triclosan, and possibly connected with triclosan targeting fatty acid biosynthesis. The Tn5 insertion reads for six ribonuclease genes were decreased 2.2 to 2.9-fold, a transcriptional elongation factor gene *greA* was decreased 2.4-fold and an RNA polymerase factor gene *sigX* was decreased 3.1-fold in response to triclosan (Figure 6.6), suggesting that triclosan may also

affect RNA metabolism, or that these genes are involved in a stress response to triclosan. The *dsbA* and *dsbB* genes, that are critical for disulfide bond formation in periplasmic proteins, showed 6.0- and 4.9-fold decrease of Tn5 insertion reads in the triclosan-treated mutant pool. This suggests that disulfide bonds in periplasmic proteins may have a protective effect against triclosan.

The antibacterial mechanism of chloroxylenol is poorly characterized. The *ddc* and *dat* genes involved in 1,3-diaminopropane biosynthesis, which is critical for amine and polyamine biosynthesis, had 2.2- and 2.7-fold increase in Tn5 insertion read coverage in the mutant pool treated with chloroxylenol.

Cationic surfactants. The only known antibacterial target of cationic surfactants is the phospholipid membrane, through binding to the anionic sites on the acidic phospholipid membrane. This causes displacement of cell surface associated divalent cations (Mg^{2+} and Ca^{2+}), thus affecting membrane fluidity and at bactericidal concentrations, leading to cell lysis (*72*).

The disruption of biotin synthesis genes made the host more sensitive to both benzalkonium and CTAB (Figure 6.6, with all Tn5 insertion read change Q-value < 10^{-15}). A similar trend was observed with the chlorhexidine-treated library, although it was not statistically significant (Supplementary data 6.1, section "Others"). This likely reflects the importance of the biotin-dependent fatty acid biosynthesis pathway for the robustness of the phospholipid membrane.

Halogens, glutaraldehyde and ethanol. Hypochlorite is a strong oxidative agent that has been reported to disrupt nucleotide bases and oxidize thiol groups in proteins (*73, 74*). Consistent with this mode of action, DNA repair and recombination genes (*ruvA*, *ruvB*, *ruvC*, *nudJ* and *nudH*) and sulfur metabolism genes (*cysI*, *cysN*, *cysD*, *cysH* and *cysG*) had decreased Tn*5*

insertion reads in the hypochlorite selected mutant pool (Figure 6.6), indicating the presence of these genes is important in protecting the host from chlorine challenge. Hypochlorite slightly reduced Tn*5* insertion read coverage of several nucleoside biosynthesis genes (*purK*, *purE*, *pyrC_1* and *pyrB*, Supplementary data 6.1, section "nucleotide").

Glutaraldehyde is proposed to exert a fixative action on the cell surface, reacting with unprotonated amines and making the membrane impermeable; it also has intracellular targets causing partial inhibition of protein and DNA synthesis, although such effects alone are not lethal (*14, 75*). The antibacterial action of ethanol is not clear. Glutaraldehyde and ethanol had similar effects to hypochlorite on Tn*5* insertion read coverage for the *ruv* genes and *nudH*, suggesting these agents also target DNA (Figures 6). Biotin biosynthesis genes had decreased Tn*5* insertion reads in the mutant pool with glutaraldehyde treatment (Figure 6.6), probably connected to their role in affecting fatty acid biosynthesis, which is consistent with the findings of the cationic surfactants described previously.

6.4. Conclusions

TraDIS enabled us to investigate the potential resistance determinants, stress responses and modes of action of 10 diverse biocides that are commonly used for disinfection in hospital and in personal hygiene products. The TraDIS data suggested that these compounds are likely to have intracellular targets, and need to penetrate through the cell envelope to exert antibacterial effects. Transposon insertion of genes related to LOS and lipid A biosynthesis affects *A. baumannii* fitness to all these 10 compounds. In contrast to LOS, capsular polysaccharide may affect host susceptibility to only silver nitrate out of the ten biocides tested. Various *A. baumannii* drug efflux pumps, such as AdeABC, AdeIJK and AmvA were shown as resistance determinants to multiple biocides, further suggesting that these compounds have intracellular target(s).

525 Our data also suggested that the biocides tested in this study cause a range of pleiotropic
526 physiological effects on *A. baumannii*. Genes from multiple cellular pathways had statistically
527 significant changes in Tn*5* insertion read coverage as a result of the biocide treatments. These
528 pathways included the TCA cycle, electron transport chain, amino acid biosynthesis,
529 nucleoside biosynthesis and biotin biosynthesis. Based on these observations, we propose that
530 one of the downstream or direct antibacterial effects of silver nitrate and several other biocides
531 is the collapse of the PMF. Collectively, these ten biocide TraDIS data sets suggest that the
532 mode of action of these biocides is more complicated than previously appreciated.

533

6.5. Methods and materials

Bacterial strain and mutant library. A multiple drug resistant strain *A. baumannii* BAL062, belonging to global clonal II, was used as parental strain for mutant library construction. The transposome used was a derivative of EZ-Tn5 γori/KAN-2> (Epicentre Biotechnologies) made in Sanger Centre (*32*). A saturated Tn5 mutant library, with more than 100k unique mutants through Tn*5* derivative electroporation.

Biocides and biocide susceptibility test. The minimum inhibitory concentrations of the parental strain *A. baumannii* BAL062 against 10 biocides were tested using a two-fold serial broth dilution method. Due to the different solubility of each compound, the media used were varied. Triclosan and chloroxylenol were tested in DMSO complemented Muller Hinton broth, silver nitrate in Luria Bertani broth without sodium chloride, and the rest of 7 compounds in MH broth. Three biological repeats were performed to confirm MIC values. Eight of the ten biocides tested here were purchased from Sigma; ethanol was purchased from Chemsupply and 12.5% hypochlorite (in H_2O) from POCD Scientific.

TraDIS assay. Two biological replicate experiments were performed for each compound. Aliquots containing more than 1×10^9 Tn5 *A. baumannii* mutant library cells were taken from glycerol stocks and inoculated into 10 mL of growth media. In total this equated to 18 sets of MH broth, 6 sets of DMSO complemented MH broth, and 4 sets of Luria Bertani broth without sodium chloride, and grown overnight at 37ºC with shaking. The next day, each overnight culture was diluted 1:100 in respective fresh media, containing the respective biocide compound at ¼ × MIC, except for the six negative controls (without biocide), i.e. two DMSO complemented MH, two standard MH, and two LB without NaCl. The cultures were then grown at 37ºC with shaking for 16 hours. Genomic DNA was extracted from 2 mL of biocide treated culture using the DNeasy Blood & Tissue Kit (Qiagen), and the genomic DNA

558 concentration measured using the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher
559 Scientific).

560 **Sequencing and data analysis.** Genomic DNA was fragmented and ligated with adaptors. Tn5
561 adjacent sequences were enriched through PCR, and the PCR products contained 10 bp
562 matching the 3' end of the Tn5 transposon (*32*). The 28 PCR libraries were multiplexed and
563 sequenced on Hiseq2500 Illumina platform, generated 500k 50 bp single-end reads per sample.
564 The resulting FASTQ files were filtered for Tn5 3' end 10 bp sequence, and the Tn5 tagged
565 reads were mapped to *A. baumannii* BAL062 genome sequence using SMALT-0.2.3. The
566 precise transposon insertion sites was determined using the Bio::Tradis tools
567 (https://github.com/sanger-pathogens/Bio-Tradis). Insertion sites and read counts were
568 tabulated per gene, and further analysis was conducted using R scripts.

569 To identify genes important for survival under ¼ × MIC biocide selection, edgeR was used to
570 identify significant differences in read counts over genes before and after selection. The TMM
571 (trimmed mean of M values) normalization was applied, and tagwise dispersion was estimated.
572 Only genes exhibiting greater than 10 reads in both replicates of at least one of the conditions
573 being assayed were tested for differences in the prevalence of mutants. P values were corrected
574 for multiple testing by the Benjamini-Hochberg method, and genes with a corrected P value (Q
575 value) of <0.01 (a hypothetical 1% false discovery rate [FDR]) and an absolute log fold change
576 >0.5 were considered significant.

577

578

6.6. References

1. J. O'Neil, "Tackling a crisis for the health and wealth of naions," *The review on antimicrobial resistance* (World Health Organization, 2014).
2. J. M. A. Blair, M. A. Webber, A. J. Baylay, D. O. Ogbolu, L. J. V. Piddock, Molecular mechanisms of antibiotic resistance. *Nat. Rev. Microbiol.* **13**, 42-51 (2015).
3. J. Poehlsgaard, S. Douthwaite, The bacterial ribosome as a target for antibiotics. *Nat Rev Microbiol* **3**, 870-881 (2005).
4. L. S. Frost, R. Leplae, A. O. Summers, A. Tourssaint, Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol.* **3**, 722-732 (2005).
5. D. E. Karageorgopoulos, M. F. Falagas, Current control and treatment of multidrug-resistant *Acinetobacter baumannii* infections. *Lancet Infect. Dis.* **8**, 751-761 (2008).
6. M. A. Webber *et al.*, Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. *J. Antimicrob. Chemother.* **70**, 2241-2248 (2015).
7. F. Fernández-Cuenca *et al.*, Reduced susceptibility to biocides in *Acinetobacter baumannii*: association with resistance to antimicrobials, epidemiological behaviour, biological cost and effect on the expression of genes encoding porins and efflux. *J. Antimicrob. Chemother.* **70**, 3222-3229 (2015).
8. S. E. Brooks, M. A. Walczak, R. Hameed, P. Coonan, Chlorhexidine resistance in antibiotic-resistant bacteria isolated from the surfaces of dispensers of soap containing chlorhexidine. *Infect Control Hosp Epidemiol* **23**, 692-695 (2002).
9. M. N. Alekshun, S. B. Levy, The mar regulon: multiple resistance to antibiotics and other toxic chemicals. *Trends Microbiol.* **7**, 410-413 (1999).
10. Rajamohan G, Srinivasan VB, W. A. Gebreyes, Novel role of *Acinetobacter baumannii* RND efflux transporters in mediating decreased susceptibility to biocides. *J. Antimicrob. Chemother.* **65**, 228-232 (2010).
11. K. A. Hassan, Q. Liu, P. J. F. Henderson, I. T. Paulsen, Homologs of the *Acinetobacter baumannii* AceI transporter represent a new family of bacterial multidrug efflux systems. *mBio* **6**, e01982-01914 (2015).
12. A. A. Neyfakh, C. M. Borsch, G. W. Kaatz, Fluoroquinolone resistance protein NorA of *Staphylococcus aureus* is a multidrug efflux transporter. *Antimicrob. Agents Chemother.* **37**, 128-129 (1993).
13. T. Conceicão, C. Coelho, H. Lencastre, M. Aires-de-Sousa, High prevalence of biocide resistance determinants in *Staphylococcus aureus* isolates from the three African countries. *Antimicrob. Agents Chemother.* **60**, 678-681 (2015).
14. G. McDonnell, A. D. Russell, Antiseptics and disinfectants: activity, action, and resistance. *Clini Microbial Rev.* **12**, 147-179 (1999).
15. S. Y. Liau, D. C. Read, W. J. Pugh, J. R. Furr, A. D. Russell, Interaction of silver nitrate with readily identifiable groups: relationship to the antibacterial action of silver ions. *Lett. Appl. Microbiol* **25**, 279-283 (1997).
16. J. R. Morones-Ramirez, J. A. Winkler, C. S. Spina, J. J. Collins, Silver enhances antibiotic activity against Gram-negative bacteria. *Sci Transl Med.* **5**, 190ra181 (2013).
17. P. Dibrov, J. Dzioba, K. K. Gosink, C. C. Häse, Chemiosmotic mechanism of antimicrobial activity of Ag(+) in *Vibrio cholerae*. *Antimicrob Agents Chemother* **46**, 2668-2670 (2002).
18. A. Gupta, K. Matsui, J. F. Lo, S. Silver, Molecular basis for resistance to silver cations in *Salmonella*. *Nat. Med.* **5**, 183-188 (1999).
19. A. Odermatt, R. Krapf, M. Solioz, Induction of the putative copper ATPase, CopA and CopB, of *Enterococcus hirae* by Ag+ and Cu2+ extrusion by CopB. *Biochem. Biophys. Res. Commun.* **202**, 44-48 (1994).

20. S. Franke, G. Grass, D. H. Nies, The product of the *ybdE* gene of the *Escherichia* chromosome is involved in detoxification of silver ions. *Microbiology* **147**, 965-972 (2001).
21. J. Y. Maillard, P. Hartemann, Silver as an antimicrobial: facts and gaps in knowledge. *Crit Rev Microbiol* **39**, 473-483 (2013).
22. L. M. McMurry, M. Oethinger, S. B. Levy, Triclosan targets lipid synthesis. *Nature* **394**, 531-532 (1998).
23. Y. Chen, B. Pi, H. Zhou, Y. Yu, L. Li, Triclosan resistance in clinical isolates of *Acinetobacter baumannii*. *J. Med. Microbiol.* **58**, 1086-1091 (2009).
24. J. A. Otter *et al.*, Surface-attached cells, biofilms and biocides susceptibility: implications for hospital cleaning and disinfection. *J. Hosp. Infect.* **89**, 16-27 (2015).
25. H. Ceri *et al.*, The calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. *J. Clin. Microbiol.* **37**, 1771-1776 (1999).
26. T. F. C. Mah, G. A. O'Toole, Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol.* **9**, 34-39 (2001).
27. S. P. Bernier *et al.*, Starvation, together with the SOS repsonse, mediates high biofilm-specific tolerance to the fluoroquinolone ofloxacin. *PLoS Genet* **9**, e1003144 (2013).
28. A. Penesyan, M. Gillings, I. T. Paulsen, Antibiotic discovery: combatting bacterial resistance in cells and in biofilm communities. *Molecules* **20**, 5286-5298 (2015).
29. A. Y. Peleg, H. Seifert, D. L. Paterson, *Acinetobacter baumannii*: emergence of a successful pathogen. *Clini Microbial Rev.* **21**, 538-582 (2008).
30. Y. Doi, G. L. Murray, A. Y. Peleg, *Acinetobacter baumannii*: evolution of antimicrobial resistance-treatment options. *Semin Respir Crit Care Med* **36**, 85-98 (2015).
31. G. Rajamohan, V. B. Srinivasan, W. A. Gebreyes, Novel role of *Acinetobacter baumannii* RND efflux transporters in mediating decreased susceptibility to biocides. *J Antimicrob Chemother* **65**, 228-232 (2010).
32. G. C. Langridge *et al.*, Simultaneous assay of every *Salmonella* Typhi gene using one million transposon mutants. *Genome Res.* **19**, 2308-2316 (2009).
33. Hassan KA *et al.*, Fluorescence-Based Flow Sorting in Parallel with Transposon Insertion Site Sequencing Identifies Multidrug Efflux Systems in *Acinetobacter baumannii*. *mBio* **7**, e01200-01216 (2016).
34. L. A. Gallagher *et al.*, Resources for Genetic and Genomic Analysis of Emerging Pathogen *Acinetobacter baumannii*. *J Bacteriol* **197**, 2027-2035 (2015).
35. C. R. Raetz, C. Whitfield, Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**, 635-700 (2002).
36. J. J. Kenyon, R. M. Hall, Variation in the complex carbohydrate biosynthesis loci of *Acinetobacter baumannii* genomes. *PLoS ONE* **8**, e62160 (2013).
37. J. J. Kenyon, K. E. Holt, D. Pickard, G. Dougan, R. M. Hall, Insertions in the OCL1 locus of *Acinetobacter baumannii* lead to shortened lipooligosaccharides. *Res Microbiol* **165**, 472-475 (2014).
38. L. A. Arroyo *et al.*, The *pmrCAB* operon mediates polymyxin resistance in *Acinetobacter baumannii* ATCC 17978 and clinical isolates through phosphoethanolamine modification of lipid A. *Antimicrob Agents Chemother* **55**, 3743-3751 (2011).
39. J. M. Boll *et al.*, Reinforcing Lipid A Acylation on the Cell Surface of *Acinetobacter baumannii* Promotes Cationic Antimicrobial Peptide Resistance and Desiccation Survival. *mBio* **6**, e00478-00415 (2015).
40. E. Geisinger, R. R. Isberg, Antibiotic modulation of capsular exopolysaccharide and virulence in *Acinetobacter baumannii*. *PLoS. Pathogens* **11**, e1004691 (2015).

41. R. G. Lees-Miller *et al.*, A common pathway for O-linked protein-glycosylation and synthesis of capsule in Acinetobacter baumannii. *Mol. Microbiol.* **89**, 816-830 (2013).
42. J. J. Kenyon, S. J. Nigro, R. M. Hall, Variation in the OC locus *Acinetobacter baumannii* genomes predicts extensive structural diversity in the lipoologosaccharide. *PLoS ONE* **9**, e107833 (2014).
43. N. R. Luke *et al.*, Identification and characterization of a glycosyltransferase involved in Acinetobacter baumannii lipopolysaccharide core biosynthesis. *Infect. Immun.* **78**, 2017-2023 (2010).
44. N. Pakharukova *et al.*, Structural insight into archaic and alternative chaperone-usher pathways reveals a novel mechanism of pilus biogenesis. *PLoS Pathog* **11**, e1005269 (2015).
45. A. P. Tomaras, M. J. Flagler, C. W. Dorsey, J. A. Gaddy, L. A. Actis, Characterization of a two-component regulatory system from *Acinetobacter bauamnnii* that controls biofilm formation and cellular morphology. *Microbiology* **154**, 3398-3409 (2008).
46. X. Z. Li, P. Plésiat, H. Nikaido, The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin Microbiol Rev* **28**, 337-418 (2015).
47. S. Coyne, P. Courvalin, B. Périchon, Efflux-mediated antibiotc resistance in *Acinetobacter* spp. *Antimicrob. Agents Chemother.* **55**, 947-953 (2011).
48. A. Chapman-Smith, J. E. Cronan, The enzymatic biotinylation of proteins: a post-translational modification of exceptional specificity. *Trends Biochem. Sci.* **24**, 359-363 (1999).
49. E. J. Yoon, P. Courvalin, C. Grillot-Courvalin, RND-type efflux pumps in multidrug-resistant clinical isolates of *Acinetobacter baumannii*: major role for AdeABC overexpression and AdeRS mutations. *Antimicrob Agents Chemother* **57**, 2989-2995 (2013).
50. A. Nemec, M. Maixnerová, T. J. van der Reijden, P. J. van den Broek, L. Dijkshoorn, Relationship between the AdeABC efflux system gene content, netilmicin susceptibility and multidrug resistance in a genotypically diverse collection of *Acinetobacter baumannii* strains. *J Antimicrob Chemother* **60**, 483-489 (2007).
51. I. Marchand, L. Damier-Piolle, P. Courvalin, T. Lambert, Expression of the RND-type efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two-component system. *Antimicrob Agents Chemother* **48**, 3298-3304 (2004).
52. E. J. Yoon *et al.*, Contribution of resistance-nodulation-cell division efflux systems to antibiotic resistance and biofilm formation in *Acinetobacter baumannii*. *mBio* **6**, e00309-00315 (2015).
53. N. Rosenfeld, C. Bouchier, P. Courvalin, B. Périchon, Expression of the resistance-nodulation-cell division pump AdeIJK in *Acinetobacter baumannii* is regulated by AdeN, a TetR-type regulator. *Antimicrob Agents Chemother* **56**, 2504-2510 (2012).
54. G. Rajamohan, V. B. Srinivasan, W. A. Gebreyes, Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant resistance in *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* **65**, 1919-1925 (2010).
55. K. A. Hassan *et al.*, Roles of DHA2 family transporters in drug resistance and iron homeostasis in *Acinetobacter* spp. *J Mol Microbiol Biotechnol* **20**, 116-124 (2011).
56. P. Orth, D. Schnappinger, W. Hillen, W. Saenger, W. Hinrichs, Structural basis of gene regulation by the tetracycline inducible Tet repressor-operator system. *Nat Struct Biol* **7**, 215-219 (2000).
57. L. Li, K. A. Hassan, M. H. Brown, I. T. Paulsen, Rapid multiplexed phenotypic screening identifies drug resistance functions for three novel efflux pumps in *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* **71**, 1223-1232 (2016).

58. K. A. Hassan *et al.*, Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad Sci U S A* **110**, 20254-20259 (2013).
59. F. F. Xu, J. A. Imlay, Silver (I), mercury(II), cadmium(II), and zinc(II) target exposed ezymic iron-sulfur clusters when they toxify *Escherichia coli*. *Appl. Environ. Microbiol.* **78**, 3614-3621 (2012).
60. L. Auseel *et al.*, Biosynthesis of physiology of coenzyme Q in bacteria. *Biochim. Biophys. Acta* **1837**, (2014).
61. Y. Fujita, H. Matsuoka, K. Hirooka, Regulation of fatty acid metabolism in bacteria. *Mol. Microbiol.* **66**, 829-839 (2007).
62. Q. L. Feng *et al.*, A mechanistic study of the antibacterial effect of silver ions on *Eshcerichia coli* and *Staphylococcus aureus*. *J. Biomed. Mater. Res.* **52**, 662-668 (2000).
63. L. J. Jones, R. Carballido-López, J. Errington, Control of cell shape in bacteria: helical, actin-like filaments in *Bacillus subtilis*. *Cell* **104**, 913-922 (2001).
64. M. Leaver, J. Errington, Roles for MreC and MreD proteins in helical growth of the cylindrical cell wall in *Bacillus subtilis*. *Mol Microbiol* **57**, 1196-1209 (2005).
65. A. V. Divakaruni, R. R. Loo, Y. Xie, J. A. Loo, J. W. Gober, The cell-shape protein MreC interacts with extracytoplasmic proteins including cell wall assembly complexes in *Caulobacter crescentus*. *Proc Natl Acad Sci U S A* **102**, 18602-18607 (2005).
66. R. M. Figge, A. V. Divakaruni, J. W. Gober, MreB, the cell shape-determining bacterial actin homologue, co-ordinates cell wall morphogenesis in *Caulobacter crescentus*. *Mol Microbiol* **51**, 1321-1332 (2004).
67. W. K. Jung *et al.*, Antibacterial activity and mechanism of action of the silver ion in *Staphylococcus aureus* and *Escherichia coli*. *Appl Environ Microbiol* **74**, 2171-2178 (2008).
68. C. A. Hale, P. A. J. de Boer, Direct binding of FtsZ to ZipA, an essential component of the septal ring structure that mediates cell division in *E. coli*. *Cell* **88**, 175-185 (1997).
69. T. Rema, J. R. Lawrence, J. J. Dynes, A. P. Hitchcock, D. R. Korber, Microscopic and spectroscopic analyses of chlorhexidine tolerance in Delftia acidovorans biofilms. *Antimicrob Agents Chemother* **58**, 5673-5686 (2014).
70. H. Strahl, L. W. Hamoen, Membrane potential is important for bacterial cell division. *Proc Natl Acad Sci U S A* **107**, 12281-12286 (2010).
71. R. J. Heath, C. O. Rock, A triclosan-resistant bacterial enzyme. *Nature* **406**, 145-146 (2000).
72. P. Gilbert, L. E. Moore, Cationic antiseptics: diversity of action under a common epithet. *J. Appl. Microbiol.* **99**, 703-715 (2005).
73. C. L. Hawkins, M. J. Davies, Hypochlorite-induced damage to nucleosides: formation of chloramines and nitrogen-centered radicals. *Chem Res Toxicol* **14**, 1071-1081 (2001).
74. C. L. Hawkins, M. J. Davies, Hypochlorite-induced damage to proteins: formation of nitrogen-centred radicals from lysine residues and their role in protein fragmentation. *Biochem J* **332 (Pt 3)**, 617-625 (1998).
75. P. V. McGucken, W. Woodside, Studies on the mode of action of glutaraldehyde on *Escherichia coli*. *J. Appl. Microbiol.* **36**, 419-426 (1973).
76. M. Solioz, A. Odermatt, Copper and silver transport by CopB-ATPase in membrane vesicles of *Enterococcus hirae*. *J. Biol. Chem* **270**, 9217-9221 (1995).
77. V. B. Srinivasan, G. Rajamohan, W. A. Gebreyes, Role of AbeS, a novel efflux pump of the SMR family of transporters, in resistance to antimicrobial agents in *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* **53**, 5312-5316 (2009).

78. H. Ogase, I. Nagal, K. Kameda, S. Kume, S. Ono, Identification and quantitative analysis of degradation products of chlorhexidine with chlorhexidine-resistant bacteria with three-dimensional high performance liquid chromatography. *J. Appl. Bacteriol* **73**, 71-78 (1992).
79. X. Z. Su, J. Chen, T. Mizushima, T. Kuroda, T. Tsuchiya, AbeM, an H+-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. *Antimicrob. Agents Chemother.* **49**, 4362-4364 (2005).
80. L. Zhu, J. Lin, J. Ma, J. E. Cronan, H. Wang, Triclosan resistance of *Pseudomonas aeruginosa* PAO1 is due to FabV, a triclosan-resistant enoyl-acyl carrier protein reductase. *Antimicrob. Agents Chemother.* **54**, 689-698 (2010).
81. J. A. Chesney, J. W. Eaton, J. R. Mahoney, Bacterial glutathione: a sacrificial defense against chlorine compounds. *J. Bacteriol* **178**, 2131-2135 (1996).
82. V. A. Fried, A. Novick, Organic solvents as probes for the structure and function of the bacterial membrane: effects of ethanol on the wild type and an ethanol-resistant mutant of *Escherichia coli* K-12. *J. Bacteriol* **114**, 239-248 (1973).

Table 6.1. Current knowledge of biocide antibacterial actions and resistance mechanisms and summary of TraDIS data

Biocide (abbreviation)	Examples of clinical application	Proposed antibacterial action	Resistance mechanisms (resistance genes previously identified in *A. baumannii*)	Tn*5* insertion read change [a]		References
				Genes > 2-fold increase	Genes > 2-fold decrease	
Silver nitrate ($AgNO_3$)	Silver impregnated nylon cloth as wound dressing, Ag coated catheters	Inactivation of proteins containing exposed thiol groups and Fe-S clusters, inhibition of DNA synthesis	Efflux, silver efflux system periplasmic chaperone, cation/copper transporter,	100	120	(*18-20, 76*)
Benzalkonium (BZK)	Preoperative skin disinfection, hard-surface disinfection	Cell envelope disruption	Efflux (*abeS*, *adeABC*, *adeIJK*, *amvA*)	7	45	(*10, 54, 77*)
Cetrimonium bromide (CTAB)	Preoperative skin disinfection, cosmetics additive	Cell envelope disruption	Efflux (*abeS*)	35	42	(*77*)
Chlorhexidine (CHL)	Pharmaceutical preservative, skin, hand and surgical disinfections	Phospholipid bilayer damage and unknown intracellular targets	Efflux (*aceI*, *abeS*, *adeABC*, *adeIJK*, *amvA*), unidentified chlorhexidine-degrading enzyme	68	34	(*10, 54, 77, 78*)
Triclosan (TRC)	Antiseptic soap, hand rinses, dental hygiene	Inhibits the FabI enoyl-acyl carrier protein reductase and blocks fatty acid biosynthesis	Efflux (*abeM*, *adeABC*), mutations on enoyl-(acyl-carrier-protein) reductase FabI (*fabI*), expression of expression of alternative enoyl reductase FabK, FabL or FabV	24	57	(*10, 22, 23, 79, 80*)
Chloroxylenol (CRL)	Surgical hand scrubs and other hand-washing products	Probable cell membrane destabilization	Efflux (A1S_2795 and ABAYE_0913 MFS family, A1S_1535 ABC family)	13	44	(*57*)
Polyvidone iodine (PVPi)	First aid and treatments for skin infection and wounds	Probable binding to key proteins with exposed cysteine and methionine residues, nucleotides and fatty acids	None identified	9	3	
Sodium hypochlorite (HClO)	Hard-surface disinfection, disinfection of blood spillages	DNA damage and inhibition of DNA biosynthesis	Glutathione, antioxidant against HClO	14	34	(*81*)
Glutaraldehyde (GLU)	Endoscopes and surgical equipment disinfection	Probable cell envelope disruption, inhibition of DNA, RNA and protein synthesis	None identified	11	17	
Ethanol (EtOH)	Hand sanitising	Probable disruption of phospholipid bilayer and cell lysis	Cell membrane alteration	13	18	(*82*)

[a] Tn*5* read coverage in the mutant pool treated with biocide compare to the control pool with no treatment. The fold-change was based on two biological replicates and with q-value lower than 0.01.

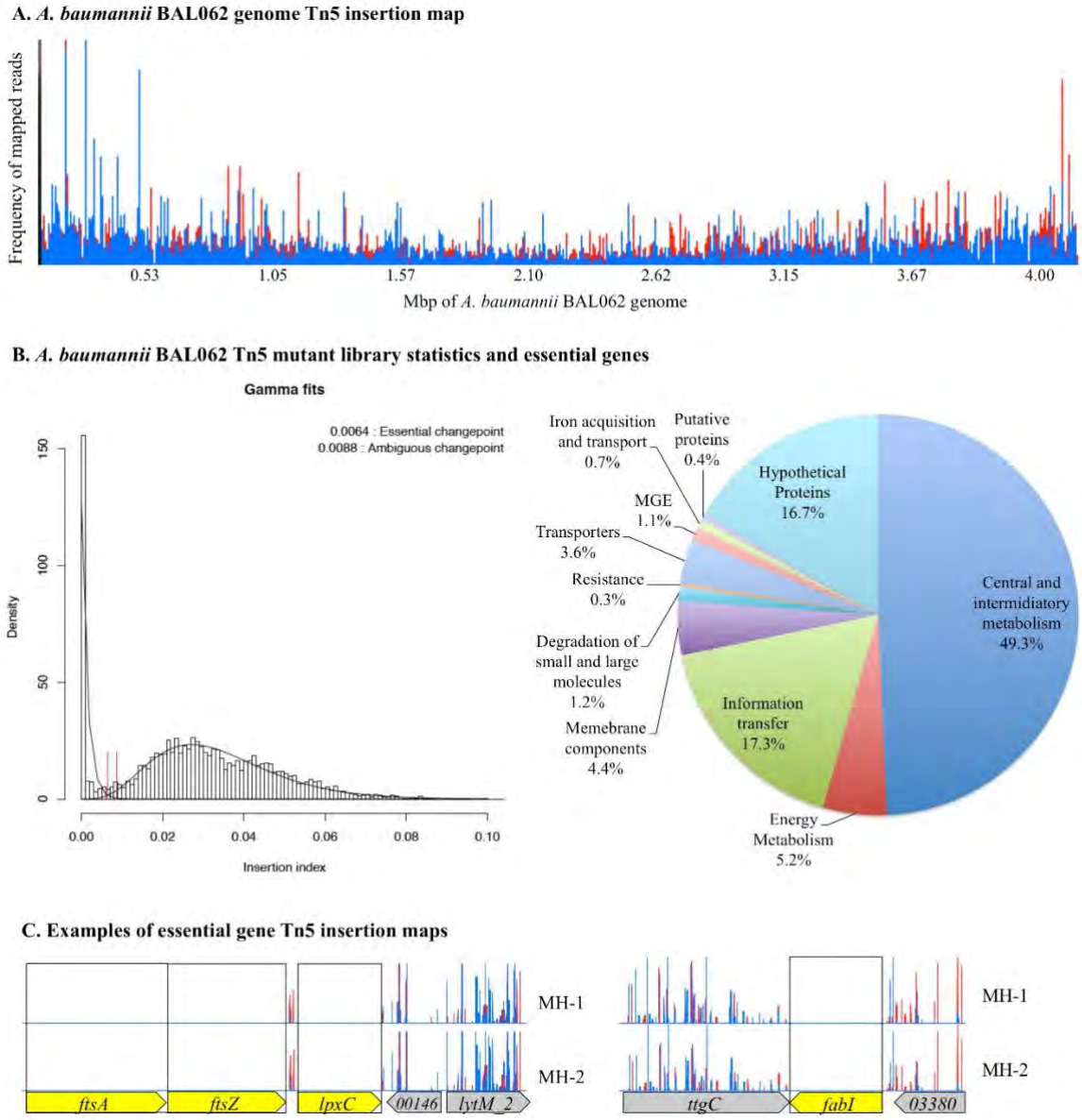

Figure 6.1. Chromosomal Tn5 insertion map and essential genes in *A. baumannii*. (A) Transposon directed insertion-site sequence reads frequency and distribution across the entire *A. baumannii* BAL062 genome for a pool of 103,116 unique transposon insertion mutants. The y-axis shows the number of mapped sequence reads with maximum 2500 reads. The blue line refers to the transposon (the ORF) inserts in the sense DNA strand, and red line the anti-sense strand. (B) Left: bimodal frequency distribution of insertion index (number of inserts per gene length). This allows differentiation of genes that do not tolerate transposon insertions in the sharp left-hand peak and genes containing insertions in the broad right-hand peak. Right: KEGG classification of essential genes required for *A. baumannii* growth in Muller Hinton broth. (C) Essential genes have no insertion reads, but each of the non-essential genes have multiple Tn5 insertion plots.

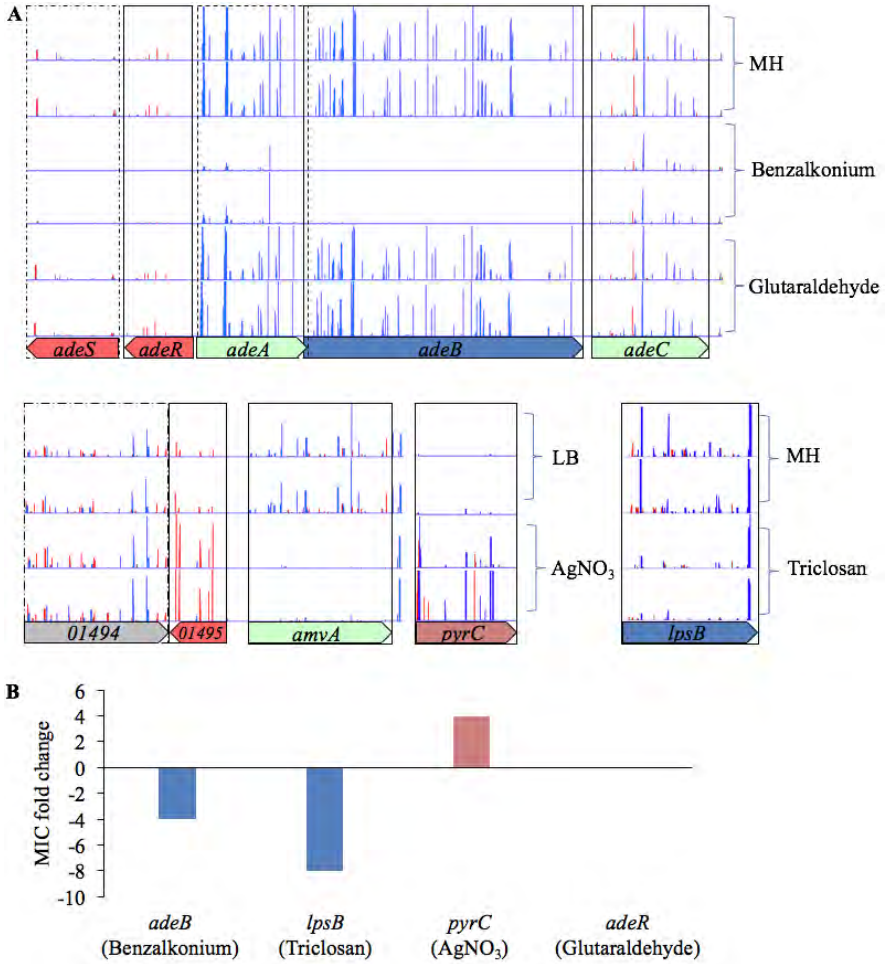

Figure 6.2. Biocide TraDIS data interpretation. (A) Example genes with Tn*5* insertion plots. Horizontal arrows represent genes showing the direction of translation, with assigned gene names within. Above the ORF cassettes are the Tn*5* read plots of mapped insertion sequence (the box height represents a maximum of 100 reads in all conditions, apart from *lpsB* with 10 reads). LB and MH are control media. Two biological replicates were shown for each biocide treatment. (B) MIC fold change of four different mutants against four different biocide compounds compared with the background strain AB5075. The MIC results are consistent with the change of Tn*5* insertion read coverage of each gene presented in Figure 6.2A.

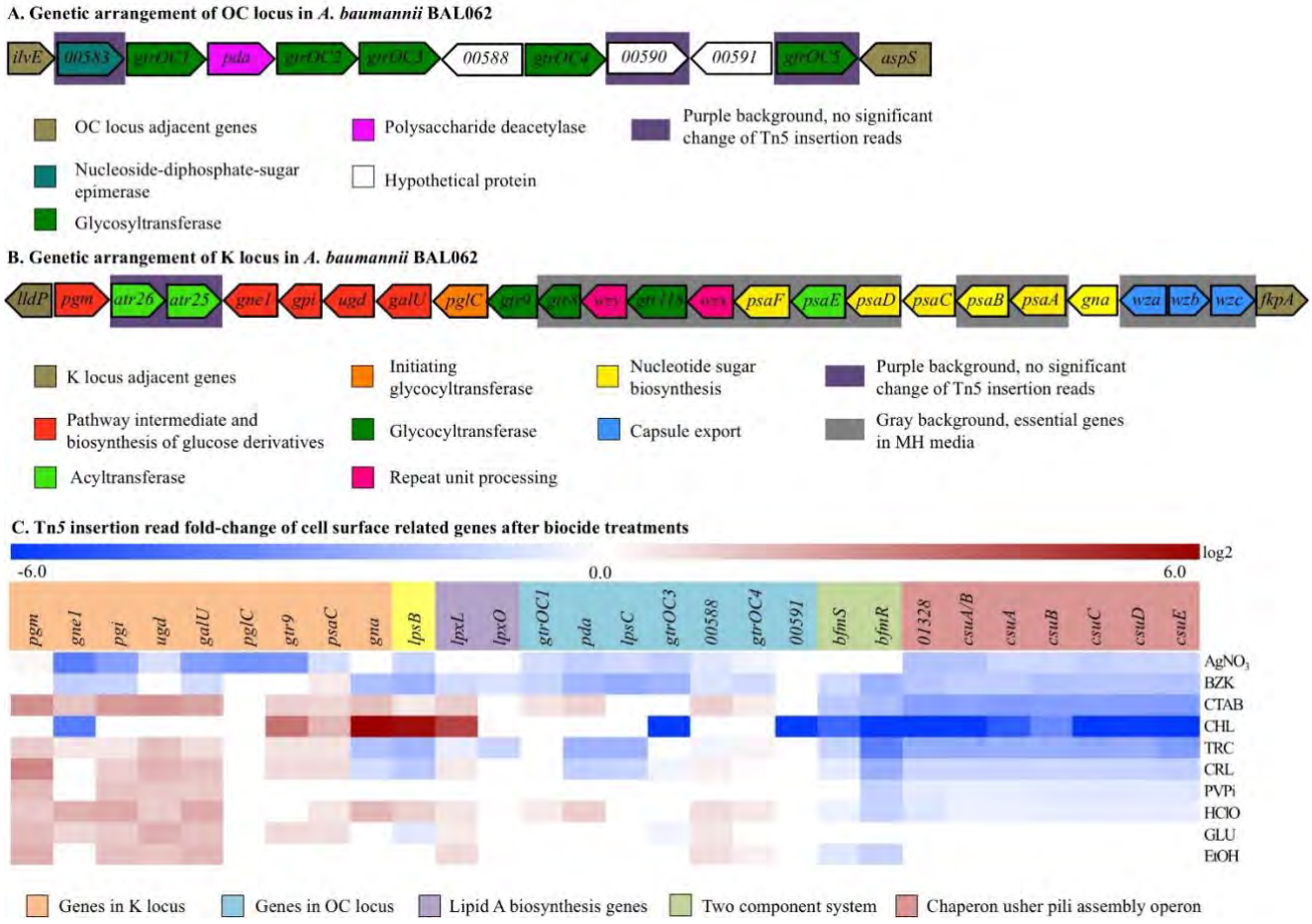

Figure 6.3. Role of the cell envelope in *A. baumannii* biocide resistance. (A and B) horizontal arrows represent genes showing the direction of translation, with assigned gene names within. Genes are coloured by the predicted functional group, essentiality for growth and change of Tn*5* insertion read coverage, with the colour scheme shown underneath. The genes are not drawn to scale. For gene annotations please see (Supplementary data 6.1, section "K-CSU-LOS"). (C) As shown in the gradient bar at the top, the wells are colour coded by the change of Tn*5* insertion read coverage. Darker blue represents higher Tn*5* insertion read decrease and darker red higher Tn*5* insertion read increase of a gene in a biocide library, in comparison with the respective mutant in the control library. The fold change is in log2 scale. Gene names are coloured according to their predicted biological functional group, with colour scheme underneath. Biocide abbreviations are shown on the right. For biocides' full names please refer to Table 1.

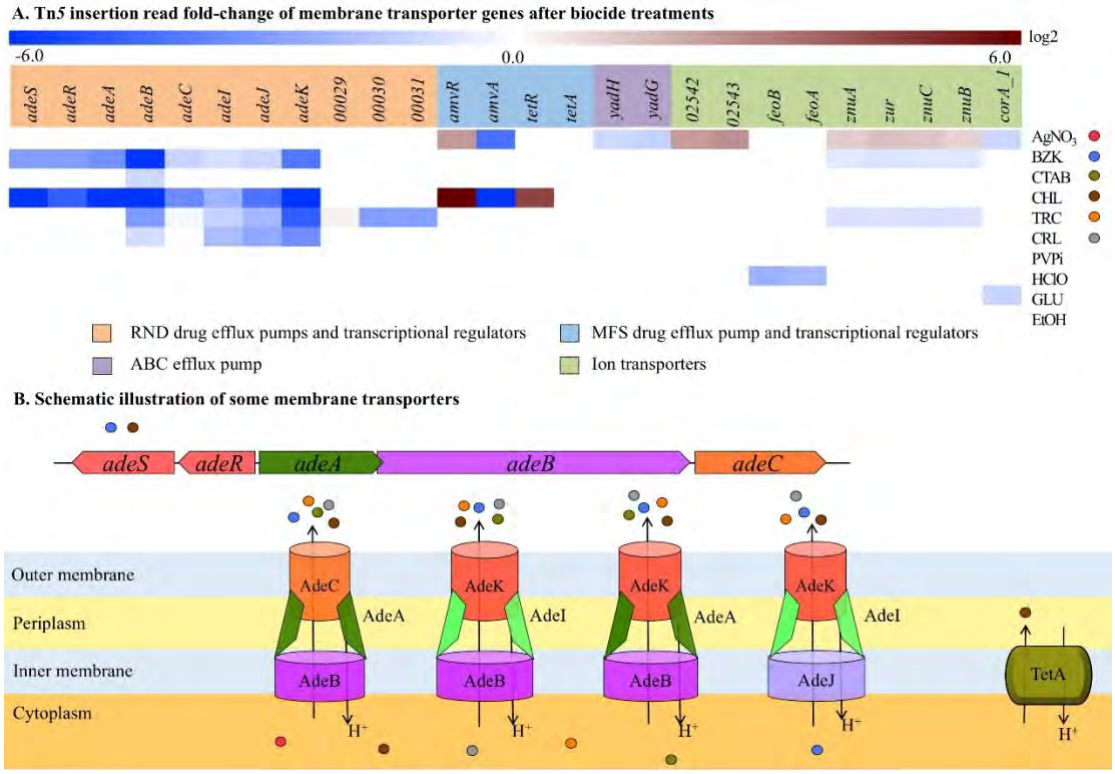

Figure 6.4. Membrane transport. (A) As shown in the gradient bar at the top, the wells are colour coded by the change of Tn*5* insertion read coverage. Darker blue represents higher Tn*5* insertion read decrease and darker red higher Tn*5* insertion read increase of a gene in a biocide library, in comparison with the respective mutant in the control library. The fold change is in log2 scale. Gene names are coloured according to their predicted biological functional group, with colour scheme underneath. Biocide abbreviations are shown on the right. For biocides' full names please refer to Table 1. Transporter genes are grouped by their predicted protein families, with colour scheme underneath. Coloured circles next to the biocide abbreviations represent the same substrates of efflux pumps in Figure 6.4B. (B) The ORFs of *adeRS* and *adeABC* are shown as horizontal arrows. The blue and brown circles represent benzalkonium and chlorhexidine, which could induce the increase of *adeABC* expression through AdeRS. The RND efflux protein AdeB is proposed to form tripartite system with alternative MFP such as AdeI and OMF such as AdeK, and correspondingly AdeJ with AdeA and AdeC. We proposed that although five biocide compounds are the potential substrates of AdeB, only chlorhexidine and benzalkonium could induce the increased expression level of *adeABC*. The transport driving forces (proton motive force) and substrates (coloured circles) are also noted.

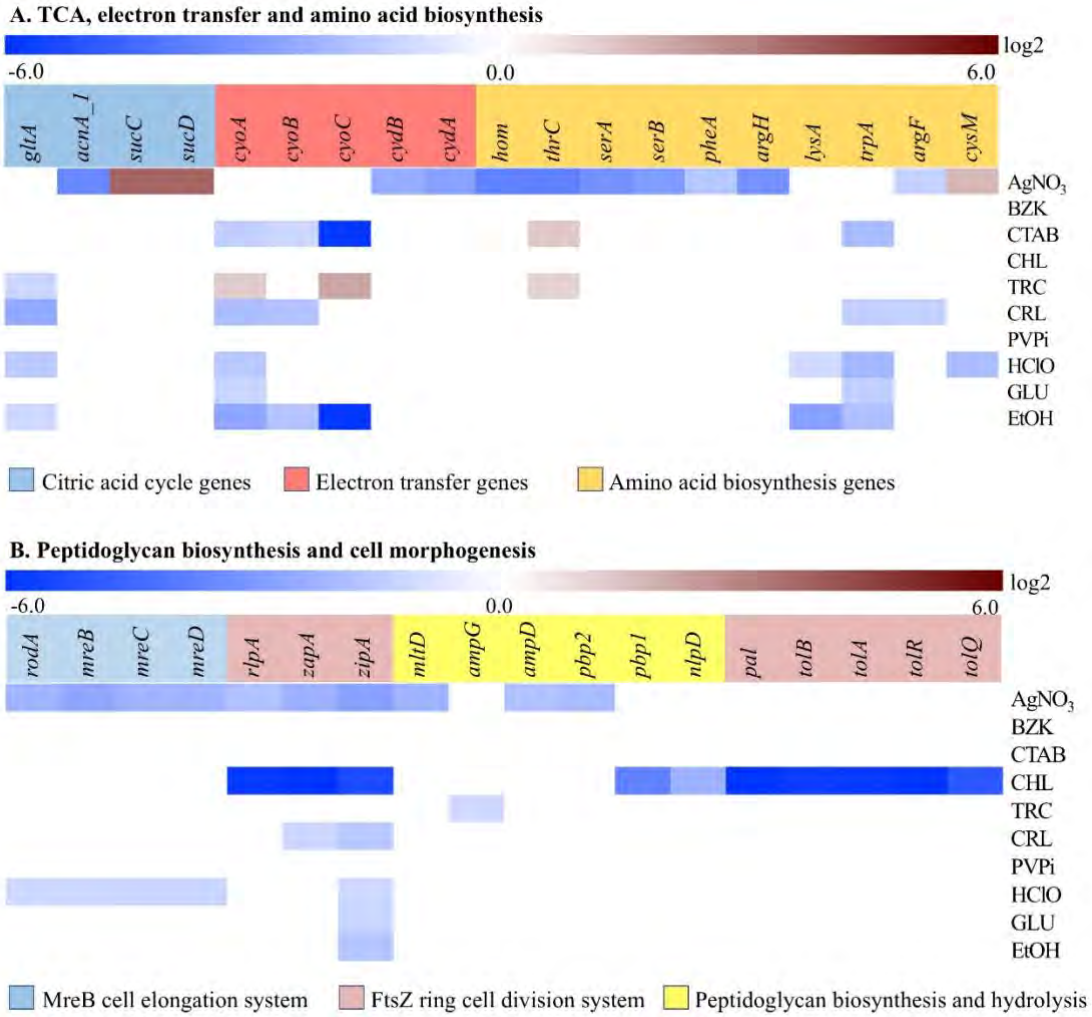

Figure 6.5. Proton motive force and cell division. As shown in the gradient bar at the top, the wells are colour coded by the change of Tn*5* insertion read coverage. Darker blue represents higher Tn*5* insertion read decrease and darker red higher Tn*5* insertion read increase of a gene in a biocide library, in comparison with the respective mutant in the control library. The fold change is in log2 scale. Gene names are coloured according to their predicted biological functional group, with colour scheme underneath. Biocide abbreviations are shown on the right. For biocides' full names please refer to Table 1.

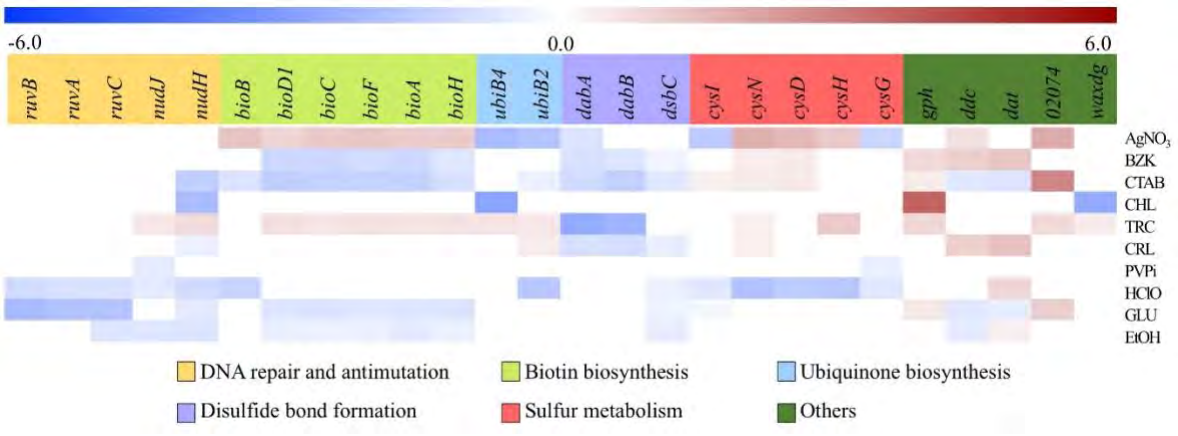

Figure 6.6. Other genes that affect *A. baumannii* fitness to biocides. As shown in the gradient bar at the top, the wells are colour coded by the change of Tn*5* insertion read coverage. Darker blue represents higher Tn*5* insertion read decrease and darker red higher Tn*5* insertion read increase of a gene in a biocide library, in comparison with the respective mutants in the control library. The fold change is in log2 scale. Gene names are coloured according to their predicted biological functional group, with colour scheme underneath. Biocide abbreviations are shown on the right. For biocides' full names please refer to Table 1.

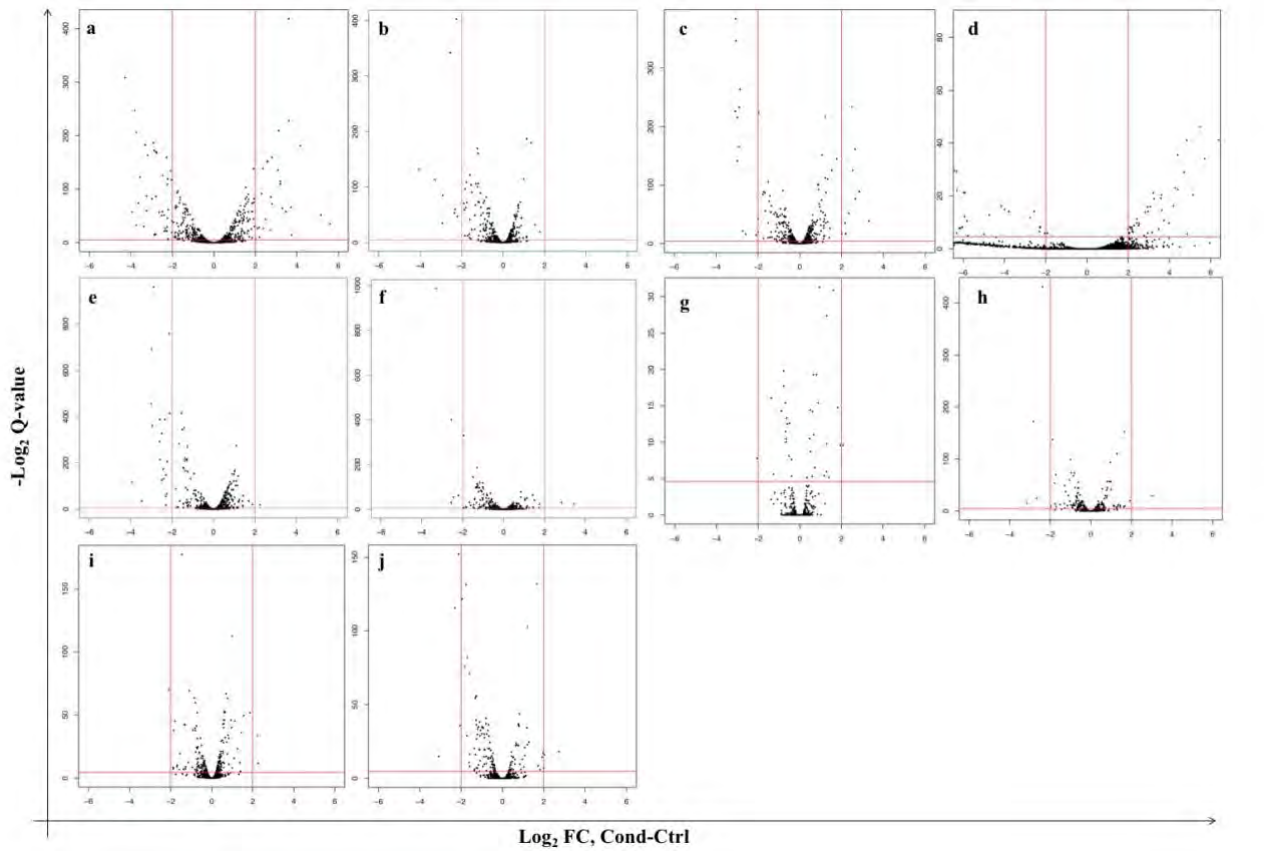

Figure S6.1. Biocide TraDIS data overview. Changes in prevalence of mutants between the control mutant library in rich media and mutant library in biocide supplemented media. Red lines show the cut-off criteria of 1% false discovery rate (horizontal) and a $\log_2$ fold change (Log_2FC, vertical). Biocides: a- $AgNO_3$, b- benzalkonium, c- cetyltrimethylammonium bromide, d- chlorhexidine, e- triclosan, f- chloroxylenol, g- polyvidone iodine, h- hypochlorite, i- glutaraldehyde and j- ethanol.

Chapter 7 General discussion and future perspectives

A. baumannii has become a significant clinical issue, due to its resistance to multiple antibiotics, antiseptics and desiccation. It has been responsible for endemic or epidemic outbreaks of nosocomial infections. With the emergence of hyper-virulent and MDR strains, it is urgent to develop alternative therapies for *A. baumannii* infections, as well as slow down or avoid the emergence of new MDR hyper-virulent strains. This requires a thorough understanding of MDR mechanisms and the biomolecular cues in *A. baumannii* that induce the development of MDR. In this thesis three novel high throughput technologies (Biolog-qPCR, TraDIS and RNA-Seq) were utilised to investigate the resistance mechanisms of *A. baumannii* to a broad range of antibacterials at a global scale. The data support the notion that antibacterial resistance phenotypes are frequently linked not to a single gene but to a combination of multiple genes. For example, multiple genes encoding efflux systems, plus genes involved in the TCA cycle and electron transport contribute to the fitness of *A. baumannii* when treated with silver nitrate (Chapter 6).

7.1 Efflux pumps and their transcriptional regulation

A major focus of this thesis is on efflux pumps, as they are major MDR systems that reduce drug intracellular concentrations. Chapter 2 describes the development of a novel high throughput efflux pump phenotypic screening method combining multiplexed Biolog phenotype microarray assays and qPCR, which can be applied to screen for the functions of heterologously expressed putative efflux genes. This method enabled the identification of novel drug resistance phenotypes for three new drug transporters in *A. baumannii* and surprisingly, identified chlorhexidine as a potential substrate of the TetA transporter. TetA is a well characterised efflux system previously thought to be a tetracycline-specific transporter. Quantitative RT-PCR on *A. baumannii* showed that both *tetA* and its transcriptional repressor encoding gene *tetR* are not inducible by chlorhexidine. This suggests that chlorhexidine cannot induce *tetA* overexpression through TetR. Consistent with this, the TraDIS chlorhexidine data shows no Tn*5* insertion read change in *tetA*, presumably because expression of *tetA* is not induced by chlorhexidine. However, *tetR* Tn5 mutants had significantly increased fitness

when exposed to chlorhexidine (chapter 6), as deletion of the repressor gene would lead to increased expression of *tetA*, which could then confer chlorhexidine resistance.

The RNA-Seq and TraDIS data in Chapter 5 demonstrated that tigecycline can induce expression of both *tetA* and *tetR*, but is not actually a substrate of the TetB efflux pump, which is consistent with previous findings (*1*). Drug efflux pump genes are typically under stringent transcriptional regulation, and the data in this thesis and in other publications suggests that not all efflux pump substrates can necessarily induce expression of the efflux pump gene, for instance chlorhexidine to TetA (Chapter 2) and tigecycline to AdeB and AdeJ (*2-4*). However, resistance to these non-inducing substrates can easily evolve through mutations in the regulator gene resulting in constitutive expression of the efflux pump gene. Such efflux pump substrates will not be discovered through standard gene knockout experiments of the efflux gene in its native host. Instead, our high throughput heterologous expression based Biolog-qPCR method could be an efficient way for characterizing the full spectrum of antibacterial substrates of many more novel efflux pumps.

The biocide TraDIS work described in Chapter 6 also provided insight into the interactions between different components of RND tripartite efflux systems. The RND transporter AdeB is known to interact with the outer membrane factor (OMF) AdeC and the membrane fusion protein (MFP) AdeA to form a functional efflux complex. The biocide TraDIS work suggested that AdeB may also form a functional complex with alternate OMF and MFP proteins (Chapter 6). Similar TraDIS results were found for another RND system AdeIJK, indicating this pattern might be common for the other RND efflux proteins. Double or multiple knockout mutants will need to be generated of the different OMF and MFP genes to confirm this hypothesis. Transcriptional studies using qRT-PCR may also provide further support.

It's important to verify whether the activation of AdeR is due to the compound's direct binding with the sensor protein AdeS, or whether the expression of *adeRS* and *adeABC* are also under the regulation of the other transcriptional regulators or regulatory RNAs that are responsive to the compound directly or indirectly. This is because biocide TraDIS revealed that the impacts on biocide

resistance of the transcriptional regulator mutants were less significant than the respective efflux gene mutants, indicating that there might be additional transcriptional or post-transcriptional regulation mechanisms yet determined.

There are several other drug efflux genes responsive to antibacterial challenges. For instance, expression of *craA* and *aceI* is induced by chloramphenicol and chlorhexidine, respectively. Unpublished work from the Paulsen group has demonstrated that *aceR,* which is divergently transcribed upstream of *aceI,* encodes a transcriptional activator of *aceI* gene expression and is responsive to chlorhexidine. Through ChIP-Seq on the wild type *A. baumannii* strain, and RNA-Seq on the *aceI* and *aceR* deletion mutant, the genes directly co-regulated and the cellular pathways directly or indirectly affected by AceR can be identified. Mass spectrometry-based metabolomics can be applied to assess metabolic response of *A. baumannii* with/without human serum, chlorhexidine or polyamines treatment, to determine the metabolites that may be affected by these conditions. Some of these metabolites might be directly transported by AceI or interact with AceR. ^{14}C labelled target metabolites could be used for AceI transport assay. Electrophoretic mobility shift assay (EMSA), DNase I Footprinting and protein thermal stability assay could be used to assess the interaction between AceR and the target metabolites. The regulation circuit of *craA* under chloramphenicol stress remains to be characterized. The *amvA* efflux gene confers resistance to silver and chlorhexidine (Chapter 6), as well as ethidium bromide (*5*). Both the biocide TraDIS data as well as published ethidium bromide TraDISort data (*5*) support that the divergently transcribed *amvR* gene is a transcriptional repressor of *amvA*.

Additionally, there are various putative ion transporters that were implicated in conferring increased fitness on exposure to biocides such as silver nitrate (Chapter 6). It is unclear how they are transcriptionally regulated, and whether their biocide fitness phenotypes are directly due to transport of these compounds or indirectly through the physiological changes induced by the compounds. Twenty uncharacterized transcriptional regulators were shown to play a role in biocide fitness in *A. baumannii* in the TraDIS experiments. Future work could focus on making targeted deletion mutants

of these genes, and either directly examining efflux gene expression through qRT-PCR, or looking at gene expression globally using RNA-Seq. A range of techniques including electrophoretic mobility shift assay EMSA (*6*), DNase I Footprinting, (*7*), protein thermal stability (*8*), ChIP-seq and isothermal titration calorimetry (ITC) (*9*) could then be used to verify direct compound and promoter region binding with the potential regulator proteins *in vitro*.

In TraDISort experiment, the major mutant population that were sequenced by TraDIS were cells with aberrant accumulation of ethidium bromide. The factors that can affect fluorescence intensity are cell size and intracellular quantity of fluorescent compound. Efflux pumps directly export ethidium, and thus their respective regulators can also affect ethidium accumulation. Alternatively, some of the other genes that were also selected by TraDISort affect cell morphology. To answer why mutants related to biosynthesis of biotin and polysaccharides were counter-selected by TraDISort, it's important to find out the composition of these mutants in the population within normal fluorescence intensity range. This information will help to clarify whether these mutants were excluded by gating or they just fell into the normal fluorescence intensity range. A normal or near normal distribution of fluorescence intensity are often observed for any population of bacterial strain, suggesting in any cell population there are always cells with aberrant intracellular fluorescent compound accumulation. By considering this phenomenon, it's not surprising to see some mutants could be counter-selected by TraDISort. But why these mutants not the others? The answer may be related to how a single cell population forms normal distribution, and which genetic or epigenetic factor(s) can affect the population distribution

7.2 Potential biocide fitness determinants and modes of antibacterial action

The majority of the biocides tested in this thesis, except for silver nitrate and triclosan, have been reported to target the cell envelope, and cell lysis has previously been proposed as the primary bactericidal effect (*10*). In chapter 6, TraDIS revealed that disruption of genes in the K-locus gene cluster affected *A. baumannii* fitness to all the biocides tested. The K-locus includes genes that are involved in capsular polysaccharide biosynthesis only, as well as other genes that play a role in the

biosynthesis of both capsular polysaccharide and the outer core component of lipooligosaccharide (LOS) (*11, 12*). One of the genes specific for capsular biosynthesis, *pglC*, only affected cellular fitness under silver nitrate selection. These observations led to a hypothesis that capsular polysaccharide only provides protection against silver nitrate and has no effect against the other nine biocides. Whereas variation of LOS sugar morphology might affect the antibacterial potencies of all ten biocides. This leads to the speculation that these biocides might have intracellular targets and a change of LOS morphology might affect OM permeability and thus compound uptake. Supporting the possibility that these biocides have intracellular targets, several membrane efflux transporters were shown as potential biocide resistance determinants. Although the position of the K-locus is conserved in *A. baumannii* strains, the gene content of the locus is highly variable, and can be used to differentiate lineages (*12*). An interesting question is whether biocides and antibiotics play a selective role on the K-locus variation patterns of *A. baumannii* strains from different lineages.

A type I pili operon *csuA/BABCD* was proposed as a potential biocide resistance determinant (Chapter 6). Knockouts of this gene cluster are known to affect biofilm formation on abiotic surfaces (*13, 14*). The TraDIS studies were undertaken in planktonic culture, one possibility is that the mechanism of biocide resistance is increased *A. baumannii* self-aggregation. This could be validated by comparing the background strain and isogenic *csu* operon mutants through a combination of biocide MIC analyses, cell morphology and aggregation analysis by scanning electron microscopy, and analysis of intracellular biocide concentrations using fluorescent or radiolabelled compounds.

A two-component regulatory system BfmRS is responsible for transcriptional regulation of the K-locus genes and *csuA/BABCD* operon (*11, 15*). Translation-inhibitor antibiotics, such as chloramphenicol, can induce the expression of K-locus genes and increase the virulence of *A. baumannii* in a mouse infection model, indicating such antibiotics may have influenced the emergence of *A. baumannii* as an opportunistic nosocomial pathogen (*11*). It will be interesting to investigate whether antibacterial compounds also induce expression of other virulence determinants, such as *csuA/BABCD*; and whether the induction is caused by antibacterial compounds binding to

BfmRS or through physiological change(s) induced by the compounds. Future work could focus on assessing how antibacterials affect the production of type I csu pili, the production of exopolysaccharide of capsule and LOS (through gel electrophoresis, mass spectrometry or chromatography), lipid A structure, cell surface morphology, cell lysis, biocide intracellular concentration, biocide susceptibility, and virulence levels using isogenic *bfmRS*, *csu* and K-locus mutants.

Several structurally distinct biocides caused statistically significant Tn*5* insertion read change for multiple electron transport genes (Chapter 6). This led to the speculation that decreased PMF might be the direct or downstream antibacterial effect of these biocides through affecting electron transport chain, resulting in subsequent cytoplasmic transport deficiencies. It has been reported that silver ions can collapse the PMF of the bacterial cytoplasmic membrane (*16*). Multiple genes responsible for amino acid biosynthesis had reduced Tn*5* insertion reads when treated by 9 out of the 10 biocides. This suggests the possibility that these biocides may compromise the efficiency of amino acid uptake through collapsing the PMF, and instead the cell relies on amino acid biosynthesis rather than transport for replenishing its amino acid pool. These hypothesises could potentially be validated and further investigated through measuring PMF change and the uptake of fluorescence- or radio-labelled compounds (antimicrobials and nutrients) of bacterial host upon biocide treatment. There may well be a broad range of pleiotropic effects on the cell due to an altered PMF, for example, the PMF has been shown to modulate the distribution of several conserved cell division proteins, and could affect bacterial cell division (*17*).

This leads to several provocative questions: 1) Whether collapsing the PMF is one of the downstream antibacterial effects of the biocides tested in this thesis and other clinically important antibiotics, 2) Are other fitness determinants detected by TraDIS such as TCA cycle and cell division genes linked to changes in the PMF or are they directly affected by the biocides? Collectively, the answers to these questions might affect our big picture view of how antimicrobials kill bacteria or inhibit their growth.

7.3 Cellular adaption to tigecycline

RNA-Seq analysis showed that a sub-inhibitory concentration of tigecycline induced significant differential expression of more than 1600 genes (Chapter 5). The gene expression changes included decreased expression of genes for protein translation, and increased expression of ribosome synthesis and assembly genes, which is consistent with tigecycline's antibacterial effect of ribosome stalling and translation interference. TCA cycle, cell respiration and cell division genes showed decreased expression which is consistent with the nature of tigecycline as a bacteriostatic antibiotic. Genes associated with mobile genetic elements showed increased gene expression, whereas two important DNA repair systems showed decreased gene expression, suggesting that treatment with tigecycline may increase rates of mutation and lateral gene transfer. A toxin-antitoxin system, which has been previously linked to persister cell formation (ref), showed increased expression. Lastly, differential expression of approximately 660 hypothetical genes of unknown function was observed. Two hypotheses and one question were raised from these observations: 1) whether tigecycline and other translation inhibitor antibiotics can induce persister cell formation, which in turn might be the outcome of the bacteriostatic effects; 2) whether the increased HGT and mutagenesis rates correlate with slow growth rate caused by antibacterial stress; 3) what are the regulatory circuits controlling the widespread transcriptional changes in response to tigecycline?

Since single cell RNA-Seq is becoming feasible in bacteria (*18, 19*), the first hypothesis, which proposed that persisters are the outcome of tigecycline-induced bacteriostasis, could be validated through comparing the transcriptomes of tigecycline-induced persister cells and tigecycline stressed cells. The second hypothesis could be investigated by examining mutation rates in cells stressed by antibiotics targeting different cellular components, while simultaneously examining transcriptomic responses. Changes in rates of lateral gene transfer are more difficult to assess as these may not occur within laboratory timescales. The question about regulatory circuits controlling cellular responses to tigecycline could be unravelled through a combination of regulatory gene knockout mutants, RNA-Seq and ChIP-Seq transcriptomics.

7.4 New technologies for deciphering deep biology of MDR pathogenic bacteria

The major advantages of RNA-Seq and TraDIS are that they both assess genes responsive to antimicrobial stimuli at a global scale, and together they have the potential to decipher biological reactions from the bacterial cellular target(s) to eventual physiological outcomes (bacteriostatic or biocidal). Transcriptomics has been successfully applied for the discovery of a novel drug efflux transporter (*20*), studies of the physiological characteristics of bacterial biofilm cells (*21*), and identification of important factors in bacterial pathogenesis and antimicrobial resistance (*22, 23*). In addition, as shown by our tigecycline RNA-Seq study in *A. baumannii* (Chapter 5) transcriptomics can also provide insight into the downstream physiological effects of a drug. In this example, tigecycline binds to the 16S rRNA, resulting in downstream effects such as stalling translation and boosting ribosome production.

Alternatively, TraDIS and other saturated transposon mutant library technologies are powerful approaches for the identification of genes that are essential for growth (*24*), while simultaneously assessing the role of non-essential genes in response to the environmental stimuli tested. Transposon insertion sequencing (TIS) technologies (*25*) have been widely applied for the discovery of genes involved in bacterial virulence, sporulation and antibiotic resistance (*26-30*). The biocide TraDIS study (Chapter 6) provided insight into the potential antimicrobial modes of action of the 10 biocides through non-essential gene mutants from known cellular pathways. However, due to current technical and knowledge bottlenecks, both RNA-Seq and TIS related technologies have not yet reached their full potential (*31-33*).

7.4.1 Limitations of RNA-Seq and TraDIS

For reaching the deeper potential of RNA-Seq, it's critical to functionally characterize the genes that are annotated as hypothetical proteins, and to clearly define the genetic regions of protein coding sequences (CDS), and regulatory RNA species including transcriptional start sites (TSS), 5' and 3' untranslated regions (UTR) of ORFs, transcriptional attenuators, small RNAs (sRNA) and other non-

coding RNAs (ncRNA). The 5' UTR regions often contain riboswitches, that form secondary structures to facilitate or interfere with transcription (*34*). Transcriptional attenuators are within the transcript of mRNAs, and also form secondary structures to interrupt transcription (*35*). Small RNAs are non-coding RNAs, which are present in either intergenic or intragenic regions and can modulate protein expression and functionality through base pairing with target mRNAs or binding to proteins (*36, 37*). These RNA species have been increasingly recognized as a significant regulatory layer in tuning gene expression and affecting protein function by sensing environmental stimuli such as metabolites and antibiotics, and further impact bacteria metabolism, virulence, and antimicrobial resistance (*38, 39*). Acquisition of this information is mostly beyond current bioinformatic annotation and prediction capacity and thus requires further experimentation through alternative or new technologies, which will be discussed in the following sections.

TraDIS and other TIS technologies are efficient ways of speeding up the discovery of the phenotypes of genes encoding hypothetical proteins (*24*), which could release further potential of transcriptomics in deciphering the biology of MDR in pathogenic bacteria. However, due to the absence of essential genes in the mutant library, TIS cannot determine how these genes affect bacterial survival upon antimicrobial challenges, and importantly antimicrobials frequently exert their bacteriostatic or biocidal effects through interrupting essential biological molecules or cellular pathways (*40*). Similar to RNA-Seq, the potential of TIS is also restricted by current genome annotation capacity, particularly the limited information of regulatory RNA species. The phenotype variations mediated through single nucleotide polymorphisms (SNP) also cannot by assessed by TIS alone. The other challenge is the bottlenecks of TIS related experiments, especially the conditions that could cause stochastic alterations in mutant population composition, which could mask the transposon insertion read changes in response to selection conditions (*31*). New technologies that might be feasible for bypassing TIS bottlenecks and characterizing essential genes in response to antimicrobial challenges will be addressed in the following sections.

7.4.2 Potential approaches for the discovery of regulatory RNAs and their roles in MDR

Recently, a new cost effective RNA-Seq protocol that directly sequences exposed RNA 3' termini (Term-seq) was developed for genome-wide discovery of riboswitches on the 5' untranslated region of mRNA encoding sequences in bacterial hosts (*41*). For instance, it revealed that the transcriptional upregulation of a lincomycin specific ABC efflux pump in *Listeria monocytogenes* is mediated by ribosome stalling caused by lincomycin, which releases the antiterminator to facilitate the mRNA read-through and hence overexpression of the efflux pump (*41*). An ABC efflux pump gene *msrC* mediating erythromycin resistance in *Enterococcus faecalis* is under coordinated regulation by both a riboswitch and a transcriptional regulator protein (*41*). Such information is very limited for *A. baumannii,* but would provide important insights into many important questions. For example, it could illuminate the genetic regulatory circuit(s) that governs the constitutive overexpression of AdeABC in MDR *A. baumannii* isolates and its tight regulation in drug susceptible isolates. More broadly, it would be feasible to apply this technology and targeted mutagenesis for identification of the potential antibacterial responsive riboswitches of *adeRS*, *adeABC*, *adeN*, *adeIJK*, other drug efflux genes and their transcriptional regulators in *A. baumannii*.

The identification of potential non-coding regulatory RNA species also responsive to antibacterials could potentially be achieved through comparative strand-specific RNA-Seq, comparative ribosome profiling and TraDIS. Strand-specific RNA-Seq preserves information about which DNA strand were the transcripts transcribed from, precisely defines the 5' ends (promoters) and the 3' ends (terminators) of the transcripts, and thus could enable the discovery of functional non-coding RNAs that are missed in the conventionally annotated genome sequences (*42, 43*). Beyond bioinformatic predictions, it will be interesting to redefine *A. baumannii* BAL062 genome sequence through strand-specific RNA-Seq and ribosome profiling. Ribosome profiling is also a relatively new deep sequencing technology based on ribosome protected mRNAs and excluding other transcripts that are not translated (*44*), which provides a more complete expression profile than proteomics, particularly with respect to detecting proteins with low solubility or low expression levels. It can experimentally define ORFs in an unbiased manner, evaluate each individual protein synthesis rate at different

environmental stimuli, and measure the protein translational pause landscape at specific positions along the transcript (*45, 46*). In combination with comparative strand-specific RNA-Seq and comparative ribosome profiling, TraDIS based on the redefined genome and using highly saturated libraries can serve for even more precise determination of the impacts of the regulatory non-coding RNAs and non-essential fitness determinants in response to stresses induced by a broader range of antibacterials, on a genome-wide scale.

The identification of novel regulatory ncRNAs involved in adaption to antimicrobial challenges, leads to the question: what are the genes or other ncRNAs that are regulated by the ncRNA. The answers could potentially be revealed through sRNA interactome studies (*32*), which aim to discover sRNA targets and illuminate their roles in post-transcriptional control in bacteria. Lately, two novel sRNA interactome sequencing technologies, RNase E UV-crosslinking ligation and sequencing hybrids (CLASH) and RNA interaction by ligation and sequencing (RIL-Seq), were successfully developed for investigating RNase E-related and RNA chaperone Hfq-related global sRNA interactome in bacteria, respectively (*47, 48*). Both studies identified novel sRNAs and their potentially targeted mRNAs. For instance, RNase E-CLASH indicated that sRNA Esr41 regulates multiple mRNAs involved in colicin resistance as well as iron transport. RIL-Seq revealed a novel sRNA GadF co-transcribed from part of the complementary sequence of the ORF of a transcriptional regulator protein GadE, both of which respond to acidic stress through regulating the expression of multiple acidic stress response proteins. RIL-Seq also revealed that various environmental stimuli that could trigger substantial changes in the Hfq-mediated sRNA interactome.

The limitation of both CLASH and RIL-Seq is that they can only reveal the sRNAs that were bonded to a single known ribonucleoprotein (RNP), such as Hfq, RNase E, and CsrA (*49*), and thus would not detect sRNAs that are not recognized by any of the currently identified RNPs. Gradient profiling by sequencing (Grad-Seq) has the potential to unveil the global RNP landscape of a cell at single RNP resolution, through RNA-RNP complex partitioning and cellular transcript sequencing (*50*).

This approach enabled the discovery of ProQ as an additional global RNA chaperone in *E. coli* and *Salmonella* (*50*).

7.4.3 CRISPR systems, beyond essential genes

Individual targeted gene knockout mutants are required for validation of hypotheses and results derived from TraDIS and transcriptomics studies, however, *A. baumannii* and many other pathogenic bacteria are highly recalcitrant for generating gene knockouts. In the last five years, targeted gene deletion or disruption approaches, such as homologous recombination mediated by *A. baumannii* specific λ recombineering system (*51*) and transposon mutagenesis (*52*), have successfully generated deletion or transposon insertion mutants of various functionally different genes in certain *A. baumannii* strains. In addition, CRISPR-Cas9 could potentially be used for targeted mutagenesis for the genes that are hard to be knocked out by the traditional approaches (*53, 54*).

Bacterial essential genes cannot be deleted or studied by conventional methods. TIS-related technologies have identified the minimal gene sets that are required for bacterial growth in rich media (*24, 55*). Based on the essential genes identified through TraDIS, CRISPR interference (CRISPRi) (*56*) could be applied to investigate the essential genes' role in mediating antimicrobial modes of killing. This novel technology uses a nuclease deactivated variant of *Streptococcus pyogenes* Cas9 (dCas9) paired with a single guide RNA (sgRNA) to sterically hinder transcription at the sgRNA base-pairing genomic locus, thus lowering the expression of the essential gene (*57*). For validation of the hypothesis regarding biocide mode of action (Chapter 6), which were based on the observations from non-essential genes, CRISPRi could be used to directly assess the impact of down-regulation of the essential gene(s) from the same cellular pathway(s). For example, the expression of essential cell division genes (*ftsZ* involved in septum formation, and *ftsI* involved in cross-linking of peptidoglycan at the division septum) or cellular respiratory genes (*ubiH* encoding ubiquinone biosynthesis hydroxylase, and *nuoCD* encoding bifunctional NADH:ubiquinone oxidoreductase subunit C/D) could be decreased using CRISPRi to investigate whether these genes may play a role in susceptibility to silver nitrate. In addition to essential genes, CRISPRi can also be used to

knockdown the non-essential genes or ncRNAs that affect bacterial host fitness to antimicrobials, as an alternative to traditional gene knockouts.

7.5 Conclusions

High throughput sequencing technologies, such as TraDIS and RNA-Seq, have the potential to reveal a complete picture of the targets and resistance factors for all available antibiotics and biocides. This could conceivably provide insights that allow for synergistic use of existing agents or suggest strategies that might allow the revitalization of older drugs. With a better understanding of antimicrobial resistance, it may be possible to develop small molecule inhibitors that block the function of resistance proteins, interfere with the regulatory circuits controlling resistance gene expression, or even affect rates of lateral gene transfer. Given the urgent global threat represented by antimicrobial resistant pathogens, the hope is that such compounds may have the potential to preserve our current antibacterial arsenal, and broaden combination therapy options for the infections caused by *A. baumannii* and other bacterial pathogens.

7.6 References

1. T. Hirata, A. Saito, K. Nishino, N. Tamura, A. Yamaguchi, Effects of efflux transporter genes on susceptibility of Escherichia coli to tigecycline (GAR-936). *Antimicrob Agents Chemother* **48**, 2179-2184 (2004).
2. M. Hornsey *et al.*, AdeABC-mediated efflux and tigecycline MICs for epidemic clones of Acinetobacter baumannii. *J Antimicrob Chemother* **65**, 1589-1593 (2010).
3. M. Deng *et al.*, Molecular epidemiology and mechanisms of tigecycline resistance in clinical isolates of Acinetobacter baumannii from a Chinese university hospital. *Antimicrob Agents Chemother* **58**, 297-303 (2014).
4. L. Damier-Piolle, S. Magnet, S. Bremont, T. Lambert, P. Courvalin, AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in Acinetobacter baumannii. *Antimicrob Agents Chemother* **52**, 557-562 (2008).
5. K. A. Hassan *et al.*, Fluorescence-Based Flow Sorting in Parallel with Transposon Insertion Site Sequencing Identifies Multidrug Efflux Systems in Acinetobacter baumannii. *MBio* **7**, (2016).
6. L. M. Hellman, M. G. Fried, Electrophoretic mobility shift assay (EMSA) for detecting protein-nucleic acid interactions. *Nat Protoc* **2**, 1849-1861 (2007).
7. P. Pattnaik, Surface plasmon resonance: applications in understanding receptor-ligand interaction. *Appl Biochem Biotechnol* **126**, 79-92 (2005).
8. J. J. Lavinder, S. B. Hari, B. J. Sullivan, T. J. Magliery, High-throughput thermal scanning: a general, rapid dye-binding thermal shift screen for protein engineering. *J Am Chem Soc* **131**, 3794-3795 (2009).
9. G. Klebe, Applying thermodynamic profiling in lead finding and optimization. *Nat. Rev. Drug Discov.* **14**, 95-110 (2015).
10. G. McDonnell, A. D. Russell, Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev* **12**, 147-179 (1999).
11. E. Geisinger, R. R. Isberg, Antibiotic modulation of capsular exopolysaccharide and virulence in Acinetobacter baumannii. *PLoS Pathog* **11**, e1004691 (2015).
12. J. J. Kenyon, R. M. Hall, Variation in the complex carbohydrate biosynthesis loci of Acinetobacter baumannii genomes. *PLoS One* **8**, e62160 (2013).
13. A. P. Tomaras, C. W. Dorsey, R. E. Edelmann, L. A. Actis, Attachment to and biofilm formation on abiotic surfaces by Acinetobacter baumannii: involvement of a novel chaperone-usher pili assembly system. *Microbiology* **149**, 3473-3484 (2003).
14. A. de Breij *et al.*, CsuA/BABCDE-dependent pili are not involved in the adherence of Acinetobacter baumannii ATCC19606(T) to human airway epithelial cells and their inflammatory response. *Res Microbiol* **160**, 213-218 (2009).
15. A. P. Tomaras, M. J. Flagler, C. W. Dorsey, J. A. Gaddy, L. A. Actis, Characterization of a two-component regulatory system from Acinetobacter baumannii that controls biofilm formation and cellular morphology. *Microbiology* **154**, 3398-3409 (2008).
16. P. Dibrov, J. Dzioba, K. K. Gosink, C. C. Häse, Chemiosmotic mechanism of antimicrobial activity of Ag(+) in Vibrio cholerae. *Antimicrob Agents Chemother* **46**, 2668-2670 (2002).
17. H. Strahl, L. W. Hamoen, Membrane potential is important for bacterial cell division. *Proc Natl Acad Sci U S A* **107**, 12281-12286 (2010).
18. J. Wang, L. Chen, Z. Chen, W. Zhang, RNA-seq based transcriptomic analysis of single bacterial cells. *Integr Biol (Camb)* **7**, 1466-1476 (2015).
19. A. E. Saliba, A. J. Westermann, S. A. Gorski, J. Vogel, Single-cell RNA-seq: advances and future challenges. *Nucleic Acids Res* **42**, 8845-8860 (2014).
20. K. A. Hassan *et al.*, Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc. Natl. Acad. Sci. U.S.A* **110**, 20254-20259 (2013).
21. A. Dotsch *et al.*, The Pseudomonas aeruginosa transcriptome in planktonic cultures and static biofilms using RNA sequencing. *PLoS One* **7**, e31092 (2012).

22. A. J. Westermann *et al.*, Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions. *Nature* **529**, 496-501 (2016).
23. G. Koch *et al.*, Evolution of resistance to a last-resort antibiotic in Staphylococcus aureus via bacterial competition. *Cell* **158**, 1060-1071 (2014).
24. G. C. Langridge *et al.*, Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants. *Genome Res.* **19**, 2308-2316 (2009).
25. T. van Opijnen, A. Camilli, Transposon insertion sequencing: a new tool for systems-level analysis of microorganisms. *Nat Rev Microbiol* **11**, 435-442 (2013).
26. R. R. Chaudhuri *et al.*, Comprehensive assignment of roles for Salmonella typhimurium genes in intestinal colonization of food-producing animals. *PLoS Genet* **9**, e1003456 (2013).
27. W. Z. Stephens *et al.*, Identification of Population Bottlenecks and Colonization Factors during Assembly of Bacterial Communities within the Zebrafish Intestine. *MBio* **6**, e01163-01115 (2015).
28. J. P. Santa Maria, Jr. *et al.*, Compound-gene interaction mapping reveals distinct roles for Staphylococcus aureus teichoic acids. *Proc Natl Acad Sci U S A* **111**, 12510-12515 (2014).
29. D. Skurnik *et al.*, Enhanced in vivo fitness of carbapenem-resistant oprD mutants of Pseudomonas aeruginosa revealed through high-throughput sequencing. *Proc Natl Acad Sci U S A* **110**, 20747-20752 (2013).
30. M. Dembek *et al.*, High-throughput analysis of gene essentiality and sporulation in Clostridium difficile. *mBio* **6**, e02383 (2015).
31. M. C. Chao, S. Abel, B. M. Davis, M. K. Waldor, The design and analysis of transposon insertion sequencing experiments. *Nat Rev Microbiol* **14**, 119-128 (2016).
32. J. Hör, J. Vogel, Global snapshots of bacterial RNA networks. *EMBO J* **36**, 245-247 (2017).
33. A. E. Saliba, S. C. Santos, J. Vogel, New RNA-seq approaches for the study of bacterial pathogens. *Curr Opin Microbiol* **35**, 78-87 (2017).
34. T. J. Santangelo, I. Artsimovitch, Termination and antitermination: RNA polymerase runs a stop sign. *Nat Rev Microbiol* **9**, 319-329 (2011).
35. C. Yanofsky, Attenuation in the control of expression of bacterial operons. *Nature* **289**, 751-758 (1981).
36. L. S. Waters, G. Storz, Regulatory RNAs in bacteria. *Cell* **136**, 615-628 (2009).
37. M. Nitzan, R. Rehani, H. Margalit, Integration of Bacterial Small RNAs in Regulatory Networks. *Annu Rev Biophys* **46**, 131-148 (2017).
38. G. Oliva, T. Sahr, C. Buchrieser, Small RNAs, 5' UTR elements and RNA-binding proteins in intracellular bacteria: impact on metabolism and virulence. *Fems Microbiol Rev* **39**, 331-349 (2015).
39. D. Dar, R. Sorek, Regulation of antibiotic-resistance by non-coding RNAs in bacteria. *Curr Opin Microbiol* **36**, 111-117 (2017).
40. M. A. Kohanski, D. J. Dwyer, J. J. Collins, How antibiotics kill bacteria: from targets to networks. *Nat. Rev. Microbiol.* **8**, 423-435 (2010).
41. D. Dar *et al.*, Term-seq reveals abundant ribo-regulation of antibiotics resistance in bacteria. *Science* **352**, 9822 (2016).
42. J. Z. Levin *et al.*, Comprehensive comparative analysis of strand-specific RNA sequencing methods. *Nat Methods* **7**, 709-715 (2010).
43. T. Conway *et al.*, Unprecedented high-resolution view of bacterial operon architecture revealed by RNA sequencing. *mBio* **8**, e01442-01414 (2014).
44. N. T. Ingolia, S. Ghaemmaghami, J. R. Newman, J. S. Weissman, Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling. *Science* **324**, 218-223 (2009).
45. Mohammad F, Woolstenhulme CJ, Green R, A. R. Buskirk, Clarifying the Translational Pausing Landscape in Bacteria by Ribosome Profiling. *Cell Rep* **14**, (2016).
46. G. W. Li, D. Burkhardt, C. Gross, J. S. Weissman, Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. *Cell* **157**, 624-635 (2014).

47. S. A. Waters *et al.*, Small RNA interactome of pathogenic E. coli revealed through crosslinking of RNase E. *EMBO J* **36**, 374-387 (2017).
48. S. Melamed *et al.*, Global Mapping of Small RNA-Target Interactions in Bacteria. *Mol Cell* **63**, 884-897 (2016).
49. E. Holmqvist *et al.*, Global RNA recognition patterns of post-transcriptional regulators Hfq and CsrA revealed by UV crosslinking in vivo. *EMBO J* **35**, 991-1011 (2016).
50. A. Smirnov *et al.*, Grad-seq guides the discovery of ProQ as a major small RNA-binding protein. *Proc Natl Acad Sci U S A* **113**, 11591-11596 (2016).
51. A. T. Tucker *et al.*, Defining gene-phenotype relationships in Acinetobacter baumannii through one-step chromosomal gene inactivation. *mBio* **5**, e01313-01314 (2014).
52. L. A. Gallagher *et al.*, Resources for Genetic and Genomic Analysis of Emerging Pathogen Acinetobacter baumannii. *J Bacteriol* **197**, 2027-2035 (2015).
53. G. Gasiunas, R. Barrangou, P. Horvath, V. Siksnys, Cas9-crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria. *Proc Natl Acad Sci U S A* **109**, E2579-2586 (2012).
54. M. Jinek *et al.*, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821 (2012).
55. N. Wang, E. A. Ozer, M. J. Mandel, A. R. Hauser, Genome-wide identification of Acinetobacter baumannii genes necessary for persistence in the lung. *mBio* **5**, e01163-01114 (2014).
56. L. S. Qi *et al.*, Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**, 1173-1183 (2013).
57. J. M. Peters *et al.*, A Comprehensive, CRISPR-based Functional Analysis of Essential Genes in Bacteria. *Cell* **165**, 1493-1506 (2016).

Other outputs

Oral presentation

- Liping Li, Karl A. Hassan, Amy K. Cain, Stephen Baker, Julian Parkhill, Ian T. Paulsen (2017) Transposon Directed Insertion-site Sequencing revealed potential modes of action and resistance determinants of ten biocides in *Acinetobacter baumannii*. Bugs by the Beach, Wollongong.

Posters

- Karl A. Hassan, Liping Li, Scott M. Jackson, Hasinika K.A.H. Gamage, Qi Liu, Peter JF. Henderson, Ian T. Paulsen (2015) Transcriptomics exposes a family of multidrug efflux pumps and reveals the drug efflux potential hidden in many microbial pathogens. Gordon Research Seminars, Renaissance Tuscany II Ciocco Lucca (Barga), Italy.
- Liping Li, Karl A. Hassan, Anahit Penesyan, Deepa R. Varkey, Hasinika K.A.H. Gamage, Qi Liu, Bart A. Eijkelkamp, Melissa H. Brown, Ian T. Paulsen (2013) Characterization of putative drug efflux pumps in *Acinetobacter baumannii*. 9th International Symposium on the Biology of *Acinetobacter*, Cologne, Germany.
- Karl A. Hassan, Liping Li, Anahit Penesyan, Bart A. Eijkelkamp, Deepa R. Varkey, Anthony J. Brzoska, Liam DH. Elbourne, Daniel Farrugia, Melissa H. Brown, and Ian T. Paulsen (2011) Functional roles of efflux systems in *Acinetobacter baumannii*. BacPath 11: Molecular Analysis of Bacterial Pathogens, Mercure Kooindah Waters Resort in Wyong, NSW, Australia.

Book chapter:

- Liping Li, Sasha G. Tetu, Ian T. Paulsen, and Karl A. Hassan (2017) A transcriptomic approach to identify novel drug efflux pumps in bacteria. In Akihito Yamaguchi and Kunihiko Nishino, eds. Bacterial multidrug exporters: Methods and Protocols, Methods in Molecular Biology, vol. 1700. Springer Science+Business Media LLC (in press).

Awards

- Postgraduate Research Fund ($5000 AUD) for conference travel from Macquarie University, Australia
- Macquarie University International Postgraduate Research Scholarship to undertake Doctor of Philosophy degree

Abbreviations

AAC	Acetyltransferase
ABC	ATP-binding cassette transporter family
ACR	Acriflavine
$AgNO_3$	Silver nitrate
AMK	Amikacin
ANT	Nucleotidyltransferase
APH	Phophotransferase
BZK	Benzalkonium
CAT	Acetyltransferase
CAZ	Ceftazidime
CDS	Coding sequence
CHL	Chlorhexidine
CHO	Chloramphenicol
CIP	Ciprofloxacin
CLASH	UV-crosslinking ligation and sequencing hybrids
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRi	CRISPR interference
CRL	Chloroxylenol
CTAB	Cetyltrimethylammonium bromide
CU	chaperone-usher
DOX	Doxycycline
ECI	European clonal lineage I
ERY	Erythromycin
ETB	Ethidium bromide
EtOH	Ethanol
FUS	Fusidic acid
GCI	Global clonal lineage I
GEN	Gentamicin
GLU	Glutaraldehyde
GNAT	GCN5-related N-acetyltransferase
Grad-Seq	Gradient profiling by sequencing
HClO	Hypochlorite
HGT	Horizontal gene transfer
ICU	Intensive care unit
IM	Inner membrane

IS	Insertion sequence
ITC	Isothermal titration calorimetry
KAN	Kanamycin
LB	Luria Bertani
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
MATE	Multidrug and toxic-compound extrusion transporter family
MBC	Minimum bactericidal concentration
MDR	Multiple-drug resistant
MFP	Membrane fusion protein
MFS	Major facilitator super family
MGE	Mobile genetic elements
MH	Mueller Hinton
MIC	Minimum inhibitory concentration
MIN	Minocycline
MIT	Mitomycin C
ncRNA	non-coding RNA
NOR	Norfloxacin
OM	Outer membrane
OMF	Outer membrane factor
OMP	Outer membrane protein
ORF	Opening reading frame
PACE	Proteobacterial antimicrobial compound efflux transporter family
PBP	Penicillin binding protein
PDR	Pan-drug resistant
PG	Peptidoglycan
PHE	1,10-phenanthroline
PMF	Proton motive force
PVPi	Polyvidone iodine
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RI	Resistance island
RIL-Seq	RNA interaction by ligation and sequencing
RNA-Seq	RNA sequencing
RND	Resistance nodulation division transporter family
RRF	Ribosome recycling factor
SFZ	Sulfathiazole

sgRNA	Single guide RNA
SMR	Small multidrug resistance transporter family
SNP	Single nucleotide polymorphisms
sRNA	small RNA
STR	Streptomycin
SUL	Sulfamethoxazole
TA	Toxin-antitoxin
TCA	Tricarboxylic acid cycle
TCR	Transcription-coupled nucleotide excision repair
Term-seq	RNA 3' termini sequencing
TET	Tetracycline
TIS	Transposon insertion sequencing
TMP	Trimethoprim
TOB	Tobramycin
TraDIS	Transposon Directed Insertion-site Sequencing
TransAAP	Transporter Automated Annotation Pipeline
TRC	Triclosan
TSS	Transcriptional start site
UTR	Untranslated region
WHO	World Health Organization
XDR	Extensively drug resistant

Office of the Deputy Vice-Chancellor (Research)

Research Office
C5C Research HUB East, Level 3, Room 324
MACQUARIE UNIVERSITY NSW 2109 AUSTRALIA

Phone **+61 (0)2 9850 4194**
Fax +61 (0)2 9850 4465
Email biosafety@mq.edu.au

6 February 2015

Professor Ian Paulsen
Department of Chemistry and Biomolecular Sciences
Faculty of Science and Engineering
Macquarie University

Dear Professor Paulsen,

Re: "Characterizing bacterial transporter, regulator and metabolic genes" (Ref: 5201401141)

NOTIFICATION OF A NOTIFIABLE LOW RISK DEALING (NLRD)

The above application has been reviewed by the Institutional Biosafety Committee (IBC) and has been approved as an NLRD, effective 6 February 2015.

This approval is subject to the following standard conditions:

- The NLRD is conducted by persons with appropriate training and experience, within a facility certified to either Physical Containment level 1 (PC1) or PC2.
- Working requiring Quarantine Containment level 2 (QC2) does not commence until the facility has been certified
- Only persons who have been *assessed by the IBC* as having appropriate training and experience may conduct the dealing. This includes persons involved in all parts of the dealing e.g. researchers, couriers and waste contractors. A copy of the IBC's record of assessment must be retained by the project supervisor.
- NLRDs classified under Part 1 of Schedule 3 of the Regulations must be conducted in a facility (or class of facilities) that is certified to at least a PC1 level **and** that is mentioned in the IBC record of assessment as being appropriate for the dealing;
- NLRDs classified under Part 2.1 of Schedule 3 of the Regulations must be conducted in a facility (or class of facilities) that is certified to at least a PC2 level **and** that is mentioned in the IBC record of assessment as being appropriate for the dealing;
- Any transport of the GMO must be conducted in accordance with the Regulator's *Guidelines for the Transport of GMOs* available at: http://www.ogtr.gov.au/internet/ogtr/publishing.nsf/Content/transport-guide-1
- A copy of the IBC's record of assessment has been attached to this approval.
- The record of assessment must be kept by the person or organisation for 8 years after the date of assessment by the IBC (regulation 13 C of the *Gene Technology Regulations 2001*).
- All NLRDs undertaken by Macquarie University will be reported to the OGTR at the end of every financial year.
- If the dealing involves organisms that may produce disease in humans, the NLRDs must be conducted in accordance with the vaccination requirements set out in the Australian Standard AS/NZS 2243.3:2010

- The Chief Investigator must inform the Biosafety Committee if the work with GMOs is completed or abandoned.

Please note the following standard requirements of approval:

1. Approval will be for a period of 5 years subject to the provision of annual reports. If, at the end of this period the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to submit a Final Report. If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. Please contact the Committee Secretary at biosafety@mq.edu.au for a copy of the annual report.

A Progress/Final Report for this study will be due on: 1 February 2016

2. If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email.

If you need to provide a hard copy letter of approval to an external organisation as evidence that you have approval, please do not hesitate to contact the Committee Secretary at the address below.

Please retain a copy of this email as this is your formal notification of final Biosafety approval.

Yours Sincerely

Associate Professor Subramanyam Vemulpad

Chair, Macquarie University Institutional Biosafety Committee

Encl. Copy of record submitted by Macquarie University to the OGTR.

Biosafety Secretariat
Research Office
Level 3, Research Hub, Building C5C East
Macquarie University
NSW 2109 Australia

T: +61 2 9850 6848
F: +61 2 9850 4465

http://www.mq.edu.au/research

MACQUARIE UNIVERSITY
SYDNEY ~ AUSTRALIA
CRICOS Provider Number 00002J