Investigating the success of *Acinetobacter baumannii* in the clinical setting

QI LIU

B. Sc (Biotechnology), University of Sydney

B. Sc (Honours, first class), Macquarie University

M. Sc (Biotechnology and Commerce), Macquarie University

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

July 2017

Table of Contents

Statement of Candidature

I certify that the work presented in this thesis only consists of research generated through my own efforts with the supervision of Professor Ian T. Paulsen and Dr Karl A. Hassan in the Department of Chemistry and Biomolecular Science, a part of Faculty of Science and Engineering at Macquarie University, and has not been previously submitted to fulfil requirements for any other degree nor at any other institution. Any assistance obtained in the preparation of this work and the reported research has been acknowledged accordingly. All literature and other sources of information have been cited appropriately. Biosafety approval for this work was obtained through the Macquarie University Ethics Review Committee reference numbers 08/03/EX (19 June 2008), 5201100898 (2011-2015), and 5201401141 (2015-2020).

Qi Liu

List of figures

List of tables

List of supplementary data

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Professor Ian Paulsen, for offering me such a great research project, and his guidance, support and advice throughout my PhD period. I will always be grateful for being part of his reputed group and for the enriching experience during my studies. This will be an inspiration for the future.

I would also like to thank my co-supervisor, Dr. Karl Hassan, who helped me a great deal, providing advice in detailed laboratory techniques and data analyses, throughout the project. I will always remember his affability, patience, and scientific mind and skills over the period of my study.

I am very grateful to Dr. Anahit Penesyan, Dr. Sasha Tetu and Dr. Amanda Mackie for help me with experimental techniques and data analyses. I am also would like to thank Dr. Bridget Mabutt and her group member PhD candidate Heather Clift for their insightful suggestions on protein purification and analyses.

I would like to thank my fellow PhD candidates such as Hasinika, Liping, Varsha, Tao, Daniel, Salisa, Brodie, Alaska for their support, encouragement and most of all their friendship.

To Paul Worden, thank you for always being friendly and helpful. I am glad that I could share an office with you.

I would like to thank Macquarie University for providing a pleasant working environment and supporting me financially through the iMQRES scholarship.

Lastly, I would like to extend my thanks to my family, in particular my wife Zimeng Liu, my uncle Peishan Liu, my sister Hang Liu and my parents Pengcheng Liu/Ming ming Chen.

Publications

Qi Liu, Karl A. Hassan, Heather E. Ashwood, Hasinika K. A. H. Gamage, Liping Li, Bridget C. Mabbutt and Ian T. Paulsen. **Regulation of the *aceI* multidrug efflux pump gene in *Acinetobacter baumannii***. *J Antimicrob Chemother*. 2018.

Karl A. Hassan,[a] **Qi Liu**,[a] Peter JF Henderson [b], Ian T Paulsen [a]. **Homologs of the *Acinetobacter baumannii* AceI Transporter Represent a New Family of Bacterial Multidrug Efflux Systems**. *mBio*. *2015*, Volume 6, Issue 1, e01982-14.

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]**. Fluorescence-Based Flow Sorting in Parallel with Transposon Insertion Site Sequencing Identifies Multidrug Efflux Systems in *Acinetobacter baumannii.*** *mBio, 2016* , Volume 7, Issue 5, e01200-16.

Karl A. Hassan, **Qi Liu**, Liam D. H. Elbourne, Irshad Ahmad, David Sharples, Varsha Naidu, Chak Lam Chan, Liping Li, Steaven P. D. Harbourne, Alaska Pokhrel, Vincent L. G. Postis, Adrian Goldman, Peter J. F. Henderson, Ian T. Paulsen. Pacing across the membrane: the novel PACE family of efflux pumps is widespread in Gram-negative pathogens. *Res Microbiol*. 2018.

Karl A Hassan*, Liam DH Elbourne, Liping Li, Hasinika KA Hewawasam Gamage, **Qi Liu**, Scott M Jackson, David Sharples,Anne-Brit Kolstø, Peter JF Henderson and Ian T Paulsen*. **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*. 2015, volume 6, 00333.

Publications in Preparation

Qi Liu, Karl A. Hassan, Anahit Penesyan, Ian T. Paulsen*. **Interactions between co-cultures of clinical isolates of *Acinetobacter baumannii* and *Klebsiella pneumoniae***

For mBio.

Conference posters

Qi Liu, Karl A. Hassan, Hewawasam Gamage H.K.A, Liping Li, Ian T. Paulsen. *Acinetobacter baumannii* drug efflux pump regulation. *BacPath* 15: Molecular Analysis of Bacterial Pathogens. September 27th- 30th 2015. Phillip Island Silverwater resort, Victoria, Australia.

H. E. Clift, **Q. Liu**, K. A. Hassan, I. T. Paulsen and B. C. Mabbutt. Structure and function of drug efflux regulator proteins *in Acinetobacter baumannii*. *19th IUPAB congress and 11th EBSA congress*. July 16-20, 2017. Edinburgh International Conference Centre, Edinburgh, UK.

Karl. A. Hassan[1], Scott Jackson[2], **Qi Liu**[1], Liam DH Elbourne, Alaska Pokhrel, Emily Gibson, Peter J. F. Henderson[2], and Ian. T. Paulsen[1] Conserved intramembranous acidic residues function in transporter but not substrate binding in PACE family proteins. *BacPath* 15: Molecular Analysis of Bacterial Pathogens. September 27th- 30th 2015. Phillip Island Silverwater resort, Victoria, Australia.

Karl. A. Hassan[1], Hasinika Ariyaratne[1], Scott Jackson[2],Anahit Penesyan[1], Liping Li[1], **Qi Liu[1]**, Simon G. Patching[2], Sasha G. Tetu[1], Bart A. Eijkelkamp[3], Melissa H. Brown[3], Peter J. F. Henderson[2], Ian. T. Paulsen[1]. **An ace up their sleeve: characterization of a novel family of drug efflux systems represented by the *Acinetobacter* AceI exporter**. *BacPath* 12: Molecular Analysis of Bacterial Pathogens. September 29th-October 2nd 2013, Tangalooma Island Resort, Moreton Island, Queensland, Australia.

Karl A. Hassan[1]**, Qi Liu[1]**, Liping Li[1], Anahit Penesyan[1], Bart A. Eijkelkamp[2], Deepa R. Varkey[1], Anthony J. Brzoska[1], Liam D. H. Elbourne[1], Daniel Farrugia[1], Melissa H. Brown[2], and Ian T. Paulsen[1]. **Functional roles of efflux systems in *Acinetobacter baumannii***.

Membrane Transporters and Other Role in Human Disease meeting, Sydney, December 6th 2012, University of New South Wales, Australia.

Contributions

This thesis is presented in a thesis-by-publication format, with a blend of published and unpublished work. Each chapter constitutes a single body of work that comprises of study resulted by several co-authors. The contribution of each co-author is outlined as follows.

Chapter 2:

Homologs of the *Acinetobacter baumannii* AceI Transporter Represent a New Family of Bacterial Multidrug Efflux Systems

(Published in mBio Journal on 10 February 2015)

This work was conceived by Paulsen, Hassan, Liu, and Henderson. Experimental work including, molecular cloning and phylogenetic analysis of BTP family proteins were conducted by Hassan. Drug resistance and fluorimetric transporter assays of BTP family proteins were performed by Liu. This manuscript was written by Hassan with contributions from Liu, Paulsen and Henderson.

Chapter 3:

Regulation of the *aceI* multidrug efflux pump gene in *Acinetobacter baumannii*

This work was conceptualised by Paulsen, Hassan, and Liu. All experimental work was conducted by Liu, expect the initial cloning of aceR was done by Gamage and Li, and the protein structure comparison was conducted by Clift. Experiments results were analyzed by Liu, Paulsen, Hassan, Mabbutt, and Clift. This manuscript was written by Liu with contributions from Paulsen, Hassan, Mabbutt, and Clift, Gamage, and Li.

(Published in J Antimicrob Chemother on 21 February 2018)

Chapter 4:

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

(Published in mBio Journal on 6 September 2016)

This work was conceived by Paulsen, Hassan, Cain, Huang, Liu, Elbourne, Biinett, Li and Parkhill. The experimental design and troubleshooting was conducted by Hassan, Cain, Huang and Liu. The experimental work specifically conducted by me was preliminary data generation and concept proofing, including that culturing a number of different mutant *A. baumannii* strains, conducting ethidium accumulation assays in these *A. bamannii* mutant strains, and flow cytometric sorting of the *A. baumannii* mutant strains. The manuscript was written by Hassan with contributions from Paulsen, Cain, Huang, Liu, Elbourne, Biinett, Li and Parkhill.

Chapter 5:

Interactions between co-cultures of clinical isolates of *Acinetobacter baumannii* and *Klebsiella pneumoniae*

This work was conceived by Paulsen, Hassan, Liu, and Penesyan. All experiments were performed and analyzed by Liu, except the flow-cell imaging conducted with the assistance of Penesyan. This chapter was written by Liu, Paulsen, Hassan with contribution from Penesyan.

Abstract

Acinetobacter baumannii is an increasingly problematic hospital-associated opportunistic human pathogen that causes a range of infections such as respiratory, urinary tract and blood infections. The ability of nosocomial *A. baumannii* isolates to resist a diverse range of antimicrobial compounds and persist in clinical settings makes it a growing public health problem.

Multidrug efflux pumps are significant contributors to antimicrobial resistance determinant in this microorganism. Five multidrug efflux pump superfamilies have been well described in bacteria, and characterized representatives of each of these five families are found in *A. baumannii*. In addition to drug efflux pumps and other resistance determinants, the capability of this pathogen to flourish in mixed species biofilm communities contributes to its success in the clinical setting.

Recently, AceI from *A. baumannii* has been shown to be a novel chlorhexidine efflux pump. In this study, 23 homologs of *aceI* from different bacteria were cloned and expressed, and many of the homologs were found to confer resistance to additional biocides, including benzalkonium, dequalinium, proflavine, and acriflavine. Fluorimetric transport assays indicated that an AceI homolog from *Vibrio parahaemolyticus* mediated resistance to proflavine and acriflavine via an active efflux mechanism. Thus, this group of AceI homologs represent a new multidrug efflux protein family, the proteobacterial antimicrobial compound efflux (PACE) family. This is the first new multidrug efflux family to be found in the past 15 years.

The function of AceR, a putative LysR family transcriptional regulator located adjacent to *aceI* in the *A. baumannii* genome, was investigated. AceR was demonstrated to be an activator of *aceI* gene expression, and induction is responsive to the AceI substrate

chlorhexidine. AceR was demonstrated to bind in a chlorhexidine-inducible manner with a region of DNA upstream of the putative *aceI* promoter. AceR represents the first regulator of a PACE family pump to be functionally characterized.

A novel high-throughput screening approach was developed to identify genes encoding multidrug efflux pumps and regulators in *A. baumannii*. This innovative screening method combines fluorescence-activated cell sorting (FACS) in parallel with transposon directed insertion sequencing (TraDIS). The feasibility of this method was demonstrated using a population of more than 100,000 random mutants shocked with ethidium bromide, a common substrate of multidrug efflux pumps. Ethidium bromide is differentially fluorescent inside and outside the bacterial cytoplasm due to its ability to bind nucleic acids. Cells containing the highest and the lowest concentrations of ethidium were collected, as the fluorescence intensity of the mutant cells can be distinguished by FACS. TraDIS was then applied to determine the genomic locations of transposon insertion within the collected mutants. AdeABC, AdeIJK, and AmvA efflux proteins were identified as the major ethidium efflux systems in *A. baumannii*, and a new transcriptional regulator that controls *amvA* expression was identified.

Another aspect of this work focused on clinical strains of *A. baumannii and Klebsiella pneumoniae* isolated from a respiratory tract infection from a single patient. Co-culture studies of these two pathogens showed complex, multifaceted interactions that were dependent on their growth state and media. Metabolic profiling showed that the two pathogens had very different carbon utilization capabilities, and cross-feeding studies indicated that *A. baumannii* was able to utilize a secreted metabolite from *K. pneumoniae* as a sole carbon source. Transcriptomic analysis of the two strains co-grown in a biofilm identified significant changes in the expression levels of genes encoding surface structure biogenesis, amino acid catabolism and transport, as well as biofilm formation.

Overall, this thesis provides new insight into the function and regulation of a novel class of multidrug efflux pumps, enabled the development of a new approach for identifying drug efflux pumps and their regulators, and provides a first look at the molecular and physiological interactions of two co-isolated pathogens.

Abbreviations

ABC	ATP-binding Cassette
AceI	Acinetobacter chlorhexidine efflux protein I
ATP	Adenosine-5'-triphosphate
AceR	Acinetobacter chlorhexidine efflux protein regulator
ALDH	Aldehyde dehydrogenases
BTP	Bacterial transmembrane pair
CFU	Colony forming unit
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
CL	Cardiolipin
DNA	Deoxyribonucleic acid
DBD	DNA binding domain
DIG	Digoxigenin
EMSA	Electrophoretic gel mobility shift assay
EBD	Effector binding domain
FAM	Carboxyfluorescein
GFP	Green fluorescent protein
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IC	International clone
ICU	Intensive care unit
K_d	Equilibrium dissociation constant
LB	Luria Bertani
LH	Linker helix
LPS	Lipopolysaccharide
LTTR	LysR-type transcriptional regulator
LDH	Lactate dehydrogenase
MATE	Multidrug and toxic compound extrusion
MDR	Multidrug resistance
MH	Mueller Hinton
MFS	Major facilitator superfamily
MLST	Multilocus sequence typing
MIC	Minimum inhibitory concentration
OD_{600}	Optical density at 600 nm wavelength

PM	Phenotype microarray
PCR	Polymerase chain reaction
QS	Quorum sensing
qRT-PCR	Quantitative reverse transcription PCR
RGP	Regions of genome plasticity
RNA	Ribonucleic acid
RND	Resistance nodulation-cell division
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLMM	Synthetic lung mimicking medium
SMR	Small multidrug resistance
SPR	Surface plasmon resonance
wHTH	Winged helix-turn-helix

Chapter 1: Introduction

1.1 *Acinetobacter baumannii*

Acinetobacter baumannii is a non-fermentative Gram-negative, catalase-positive, oxidase-negative, aerobic, metabolically versatile, rod-shaped bacteria commonly found in nature [1]. Acinetobacter can be isolated from many different environmental samples such as, in water, soil and sewage [1]. *A. baumannii* strains isolated in the 1970s are typically susceptible to most commercially available antibiotics [2]. However, the increasing emergence of multidrug and even pan-drug resistant strains has led it to become a major worldwide cause of Gram-negative hospital-acquired infections [3], such as meningitis, pneumonia, septicaemia, respiratory and urinary tract infections, skin and soft tissue infections, would infections, and bacteraemia. Open wounds, catheters and breathing tubes provide avenues for *A. baumannii* to enter the body. It usually infects immuno-compromised and critically ill patients, especially those who are in intensive care units (ICU) and have a history of broad antibiotic therapy [3].

The ability of *A. baumannii* to quickly adapt to changes allows them to survive for long periods of time under various harsh environmental stresses [4, 5], including dessication, and extremes of temperature, and pH. They can be recovered from many different sites in hospital environments, including bed curtains, furniture, hospital equipment, patients, and medical workers [5]. Patients colonized or infected with *A. baumanni* can carry this bacterium for days to weeks [6]. It was reported that *A. baumannii* can live in an area with 31% relatively humidity for over 27 days, and on hospital bed rails for about 9 days after the discharge of the patients [5]. Several studies have demonstrated that *A. baumannii* can form biofilms on glass, plastics and human epithelial cells, which further contributes to its resilience [7]. *A. baumannii* can also form mixed biofilms with other microbial species, and may contribute to polymicrobial infections [8]

This organism is recognized as an important and dangerous pathogen because of its propensity to cause outbreaks [9]. There are many reports about such outbreaks in different hospitals located in different geographic areas of the world, and most of these were due to multidrug or pan-drug resistant strains since the late 1990s. For example, over 400 patients from 15 different hospitals were infected by *A. baumannii* strains, which were resistant to virtually all the commercially available antibiotics in New York in 1999 [10]. Moreover, many casualties from disasters, such as the 1999 Turkey earthquake and the 2002 Bali bombing incident, or military conflicts were found to be infected with *A. baumannii* strains [9]. Approximately 240 injured US soldiers from Iraq carried bloodstream infections caused by *A. baumannii* strains in Water Reed Army Medical Centre between 2003 and 2005 [11].

1.1.1 Population structure of *A. baumannii*

It is crucial to accurately identify the strains of infectious agents that cause disease for epidemiological surveillance and public health decision-making. A number of molecular approaches can be used for investigating pathogen population structure, such as genetic fingerprinting [12], multilocus sequence typing (MLST) [13] and whole genome sequencing (WGS) [14].

The majority of clinical *A. baumannii* isolates in Europe fall into one of three lineages, International Clone groups (IC) 1-3 [15, 16], based on the discovery of three distinct ribogroups generated via ribotying [17], the hybridisation of a labelled *Escherichia coli rrnB* rRNA operon probe against digested DNA to generate a fingerprint [12]. However, ribotyping was insufficient to conclusively determine the clonality of *A. baumannii* infections due to relatively inadequate discrimination between clones. Higher resolution genetic fingerprinting methods, including pulsed-field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), and the selective amplification of restriction fragments to generate a genetic fingerprint [18], were conducted to confirm the existence of three major clonal

lineages [16]. These three clonal lineages of clinical *A. baumannii* were previously called European clones I-III (ECI-III), and a new terminology referred as international clones I-III (ICI-III) or global clones I-III (GCI-III) [4, 16, 18] was given to these *A. baumannii* clones, because the existence of the three major clonal lineages outside of Europe. For example, *A. baumannii* strains from ICI and ICII are commonly found hospitals in Australia [19].

MLST, the amplification and sequencing of short internal fragments of multiple housekeeping genes [6, 13], has largely replaced genetic fingerprinting as a typing method, due to its ability to conduct rapid and global inter-laboratory comparisons [20]. Over 400 MLST sequence types have been discovered in *A. baumannii*, suggesting a highly divergent population structure, though clinical settings are still predominated by clones I-III [21]. The Oxford [22] and Pasteur [23] schemes are two currently MLST schemes used for the typing of *A. baumannii*. Both schemes use a total of seven housekeeping genes, with *gpI, gyrB, gdhB,* and *rpoD* unique to the Oxford scheme, whereas *fusA, pyrG, rplB,* and *rpoB* are unique to the Pasteur scheme. The *cpn60, gltA* and *recA* genes common to the both schemes. Although comparison of the two methods has not been performed on *A. baumannii*, the *gpi* allele of the Oxford scheme is prone to significant variation and recombination relative to other housekeeping genes [14], thus, making it unsuitable as an epidemiological marker [24].

Recently, WGS has been used as a high resolution way to track epidemiological changes in *A. baumannii* with ability to detect single nucleotide polymorphisms, recombination events, and gene gain or loss [14, 25]. This technique has also been used in *A. baumannii* during polyclonal outbreaks for the tracking of evolutionary changes [14, 25, 26].

1.1.2 Epidemiology of *A. baumannii*

Infections caused by *A. baumannii* are more likely to occur in critically ill or otherwise debilitated individuals, due to its limited number of virulence factors [27]. However, *A. baumannii* has several factors that favour a prolonged survival rather than invasive disease including bacteriocin, encapsulation and a prolonged viability under dry conditions [5]. *A. baumannii* can survive for up to five months in hospitals, and this longevity is likely a major contributor to nosocomial spread, especially in ICU [27].

Risk factors contributing to *A. baumannii* infections include old age, presence of serious underlying diseases, immune suppression, burn injuries, mechanical ventilation, extended hospital stay, and previous treatment with antibiotics [27]. The other major characteristic of *A. baumannii* that favours its prolonged survival and spread throughout hospitals is its capacity to acquire foreign genes that confer antimicrobial resistance or to increase the expression of innate resistance genes [2].

It has been found in the United States (US) that national rates of multidrug resistant *A. baumannii* isolates grew significantly from 32.1% in 1999 to 51% in 2010 [27]. Sulbactam and group 2 carbapenems (meropene, imipenem-cilistatin and doripenem) have been the most common choice for the treatment of such MDR strains. However, a sharp rise has been observed in the rates of carbapenem resistance in *A. baumannii* from 2004 to 2014 (Figure 1.1) [28]. The National Healthcare Safety Network (NHSN) data showed a 33% rate of carbapenem resistance in *A. baumannii* across the US in 2006-2007 [29].

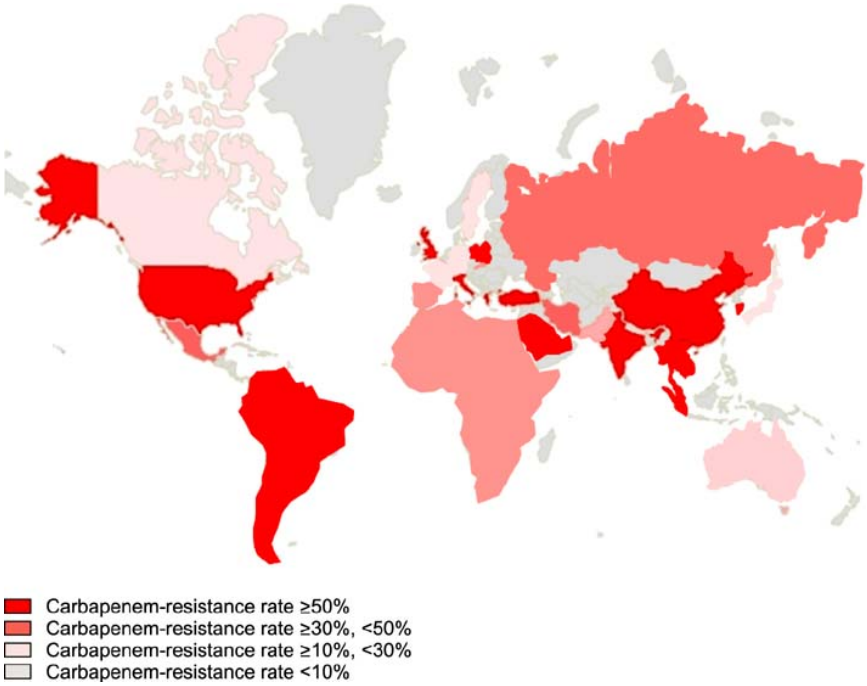


Figure 1.1. Global epidemiology of carbapenem-resistant *A. baumannii* strains from 2004 to 2014 reproduced from [28].

Apart from the high rates of carbapenem-resistant *A. baumannii* (CRAB), there has been a rising incidence of *A. baumannii* in hospital-associated infections, with the overall rate of 3% of hospital-acquired infections in 463 hospitals was reported by the NHSN in 2006-2007 in Atlanta (US) [29]. In addition, an alarming trend in Detroit (US) where CRAB rates increased from 1% in 2003, to 58% in 2008, along with an over twofold increase in total number of *A. baumannii* isolated at Detroit medical center [30].

1.1.3 Genomics of *A. baumannii*

Genome sequences are important in providing insight into both the genetic architecture and evolutionary history of *A. baumannii*, especially in isolates of diverse niches. Currently, over sixteen *A. baumannii* strains with complete genome sequences (1656-2, AB0057, AB307-0294, ACICU, ATCC17978, AYE, BJAB07104, BJAB0715, BJAB0868, D1279779, MDR-TJ, MDR-ZJ06, SDF, TCDC-AB0715, TYTH-1, ZW85-1) can be found at NCBI database (ftp://ftp.ncbi.nih.gov/genomes/archive/old_refseq/Bacteria/), and 2294 draft genomes of this species are available at NCBI webpage (https://www.ncbi.nlm.nih.gov/genome/genomes/403).

Both genome size (3.42-4.14 Mbp) and plasmid number (0-4) of *A. baumannii* strains with complete genome sequence are different, but these strains have a relatively consistent overall CG% content of 39%.

Despite the geographic variation of these *A. baumannii* strains, data from multiple comparative analyses demonstrated that they have a highly syntenic genome organizations [31, 32]. The core genome of *A. baumannii* has been predicted to be between 1455 and 2688 orthologous protein coding sequences (CDSs) based on the number, and identity of strains analyzed [21]. The majority of the core genome is involved in metabolic and general cellular processes, as well as genes encoding hypothetical proteins [33]. In contrast the accessory genome (genes that are not shared by all strains), includes genes that encode for transport, transcription regulation functions, resistance genes, and various regions of plasticity as well as laterally acquired regions [32-34].

1.1.3.1 Regions of genomic plasticity

Regions of genomic plasticity (RGP) are referred as strain-specific segments of its genome, but without any assumption about the evolutionary origin or genetic basis of these variable chromosomal segments [35]. Despite the term RGP was designated originally in the comparative genomics study of *Pseudomonas aeruginosa* [35], the use of RGP has been extended to other microorganisms [36]. This inclusive terminology includes genomic islands (GIs) and mobile genetic element (MGE) such as transposons, integrated plasmids, prophages as well as the plasticity within these elements, and variable regions resultant from genomic deletions or recombination events involving intragenic or extracellular DNA [35, 36].

1.1.4 The success of *A. baumannii* as a pathogen in the hospital setting

Three major factors that have contributed to the success of *A. baumannii* as a hospital pathogen are (I) the accumulation of a broad array of drug resistance mechanisms acquired by either lateral gene transfer or mutations, (II) the ability to form biofilms and resist desiccation on abiotic surfaces which makes this organism very difficult to eradicate from clinical settings, (III) the ability to adhere to, colonize and invade human epithelial cells [7].

1.1.4.1 Antibiotic resistance mechanisms in *A. baumannii*

There are a variety of known bacterial drug resistance mechanisms (Figure 1.2) [37]; these include antibiotic inactivation [38], enzymatic modification of antibiotics [39], antibiotic target alteration or bypass [40], and excretion via multidrug efflux pumps [41].

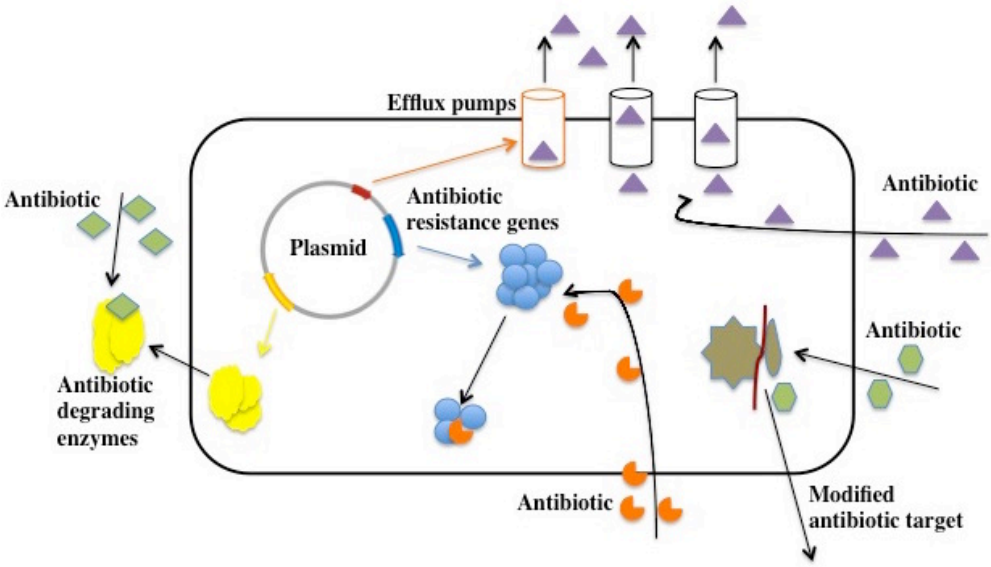


Figure 1.2. Various drug resistance strategies in bacteria.

Like many other bacteria, *A. baumannii* has developed many of the above molecular defence mechanisms to resist antimicrobial agents. This organism can enzymatically inactivate antimicrobials such as β-lactam, aminoglycosides, and rifamycin by expressing genes for corresponding enzymes [42] which destroy the antimicrobials before they can have an effect. *A. baumannii* has also acquired resistance to antimicrobials via modification of antimicrobial target sites. For example, mutations in the *gyrA* gene, which encoding DNA gyrase A (the

target of this antibiotic class) [43], can increase fluoroquinolone resistance in *A. bamannii*. Multidrug efflux pumps, which extrude the antibacterial compounds from the cell before they can reach their target sites and exert their effects, are also a major cause of resistance to antimicrobials in *A. baumannii* (see section 1.2.1.1 for examples).

The lateral acquisition of new genetic material from other resistant organisms seems to have been a key driver in the development of antimicrobial resistance in *A. baumannii*. *A. baumannii* strain AYE has the largest known multi-resistance island identified in any bacteria, which includes 45 resistance genes in an 86-kb RI. Based on sequence characteristics, the genes in this RI are thought to have been acquired from bacteria within the genera *Pseudomonas*, *Salmonella,* or *Escherichia* [2]. In this RI, 25 out of 45 resistance genes [2] are associated with resistance to various classes of antibiotics, including aminoglycosides, tetracycline, cotrimoxazole, and chloramphenicol.

1.1.4.1.1 Multidrug efflux pumps for antibiotic resistance

Efflux mediated resistance has been reported in many bacteria [44]. Five distinct efflux pump families are associated with antimicrobial resistance have been described in bacteria (Figure 1.3) [44, 45]. These five families include: the major facilitator superfamily (MFS), the resistance nodulation-cell division (RND) family, the small multidrug resistance (SMR) family, the ATP-binding cassette (ABC) family and the multidrug and toxic compound extrusion (MATE) family. All of these families have members that have been experimentally characterised in *A. baumannii* strains [46].

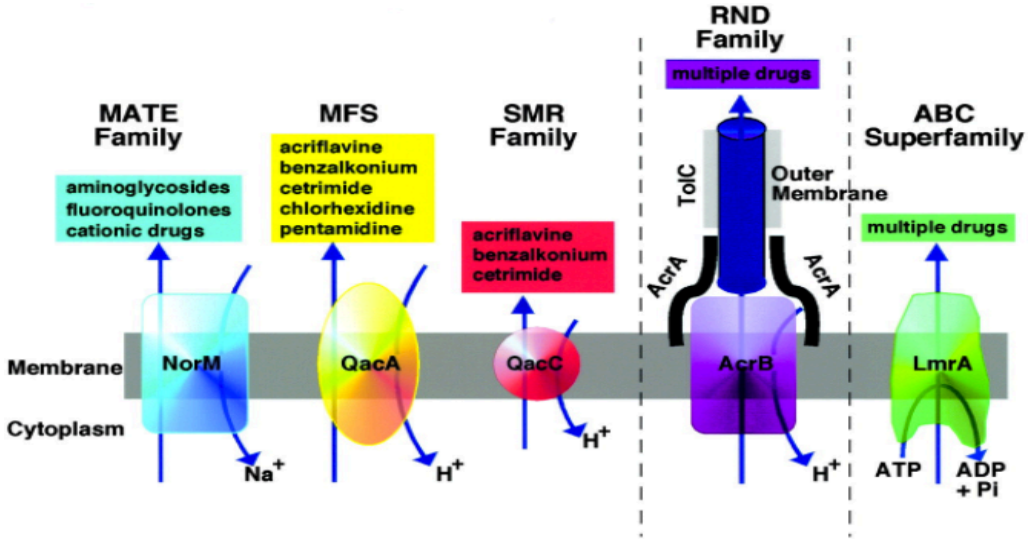


Figure 1.3. Diagrammatic comparison of the five families of efflux pumps reproduced from [44].

Single RND proteins have twelve transmembrane α-helices and a large periplasmic domain. Functional drug exporting RND transporters are trimers and are associated with outer membrane proteins, and membrane fusion proteins to form a large multi-subunit complex that spans both the inner and outer membranes in Gram-negative bacteria [47]. The first characterised RND protein in *A. baumannii* was AdeABC [48], which is a tripartite, secondary proton-motive-force dependent membrane transporter. Previous studies showed that AdeABC confers resistance to aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, tigecycline, macrolides, chloramphenicol, and trimethoprim [48]. Expression of *adeABC* is tightly regulated by the two-component regulatory system AdeR-AdeS [49]. Another two RND family transporters found in *A. baumannii* are AdeIJK and AdeFGH, which contribute to resistance to many β-lactams [50, 51]. Like *adeABC*, both *adeIJK* and *adeFGH* are also under tight regulation by AdeN [52] and AdeL [50], respectively.

The Major Facilitator Superfamily (MFS) is one of the largest transporter families and is ubiquitous to all classes of living organisms [53]. MFS proteins normally contain 12 transmembrane α-helices connected by hydrophilic loops [53]; however, there are some MFS proteins that have 14 α-helices [54]. They can transport many substrates, such as polypeptides, drugs, ions, sugars and nucleosides [55]. In *A. baumannii,* MFS transporters are

found to confer resist to many antimicrobials; for example, TetA and TetB are involved in tetracycline and minocycline resistance [56]. CraA [57] and AmvA [58] are two other MFS examples, that contribute resistance to erythromycin and chloramphenicol, respectively.

The small multidrug resistance (SMR) family proteins are small prokaryote-specific secondary proton-motive-force driven membrane transporters [59]. The SMR proteins, which are the smallest known efflux transporters, comprise 110-140 amino acid residues that form four trans-membrane α-helices [59, 60]. The majority of evidence suggests that they function as antiparallel homo-dimers or hetero-dimers. The *A. baumannii* AbeS SMR transporter has been found to be confer resistance to chloramphenicol, fluoroquinolones and erythromycin [61].

The ATP-binding cassette (ABC) superfamily proteins are the largest transporter protein superfamily, conserved across all classes of living organisms, and they depend on ATP hydrolysis to extrude drugs and other substrates out of cells [62]. ABC family transporters are composed of two cytoplasmic nucleotide-binding domains (responsible for ATP binding and hydrolysis) and two transmembrane segments that typically contain six membrane-spanning α-helices [63]. The major roles of ABC transporters are to export toxic compounds and uptake essential materials [64]. The first ABC transporter, encoded by A1S_1535, was described in *A. baumannii* ATCC 17978 in a recent publication [46]. It has been demonstrated this ABC transporter confers resistance to many antibiotics such as gentamicin, kanamycin, chloramphenicol, oxytetracycline and chloroxylenol [46].

Proteins of the multidrug and toxic compound extrusion (MATE) family have been detected in all domains of life [65, 66]. The MATE family transporters can extrude substrates coupled to H^+ or Na^+ exchange [66, 67]. Generally, each MATE protein contains 12 transmembrane α-helices with a different packing organization compared to MFS family proteins [66]. AbeM, a

member of the MATE family encoded by *A. baumannii*, extrudes aminoglycosides, fluoroquinolones, chloramphenicol, trimethoprim, and ethidium bromide [68].

1.1.4.1.2 AceI, the representative of a new family of efflux pumps

Chlorhexidine is a bisbiguanide bactericidal agent, which is widely used in hospital antiseptic products such as antibacterial soaps, mouthwashes. It is considered as an "Essential Medicine" by the World Health Organisation [69]. An increasing level of chlorhexidine resistance has been reported in hospital acquired pathogens including *A. baumannii* isolates [70].

A recent study conducted by Hassan *et al* identified AceI (Acinetobacter chlorhexidine efflux protein I) as a major chlorhexidine resistance determinant in *A. baumannii* through transcriptomic and biochemical analyses [71]. The transcriptomic data showed that the gene encoding AceI was highly expressed when cells were exposed to chlorhexidine, and the biochemical results indicated that AceI functions via an active efflux system.

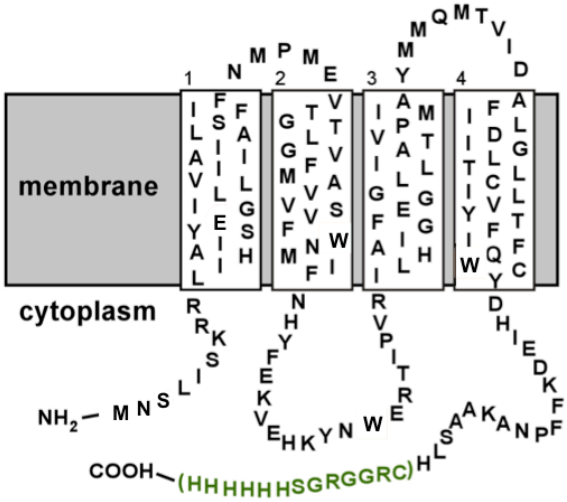


Figure 1.4. Predicted topology of AceI. This efflux protein consists of four alpha-helical transmembrane segments. It confers resistance to chlorhexidine.

The AceI chlorhexidine efflux is a small membrane protein with four alpha-helical transmembrane segments (Figure 1.4) [72]. Genes encoding proteins homologous to AceI are commonly found in the genomes of particularly proteobacterial genera, but can also be seen in other unrelated bacterial lineage such as the Firmicutes and Acinobacteria.

1.1.4.1.3 Regulation of multidrug efflux pump

Many putative and proven drug transporters exist in bacteria. Previous studies have shown that the *A. baumannii* genome typically encode representatives of each of the families of efflux systems, including seven RND, over thirty MFS, and several MATE, SMR, and ABC efflux systems [73]. It is interesting that bacteria, with their economically organized genomes, harbour such large sets of multidrug efflux genes and that many of these multidrug transporters have apparently overlapping substrate specificity.

One way to understand how bacteria utilise these multiple transporters lies in the regulation of transporter expression. It has been shown that multidrug transporters are frequently expressed under precise and elaborate transcriptional regulation [74-76]. The regulation of transporter genes is typically controlled at the RNA synthesis level [77]. In prokaryotes, such a mechanism is critical for adaptation to environmental stresses [78]. Interactions between DNA binding proteins, which act as regulatory factors and specific regulatory DNA elements determine the regulation of gene expression [77, 79]. There are two types of transcriptional regulators in prokaryotes: activators that positively regulate gene expression by acting on the promoter to stimulate RNA polymerase binding; and repressors that negatively regulate the expression of a target gene by binding to the promoter region to prevent initiation of transcription [79].

1.1.4.1.3.1 Transcriptional regulators

Various types of transcriptional regulator superfamilies have been described, such as the TetR family regulators [80], the FUR (ferric uptake regulator) superfamily [81], the Zinc cluster proteins [82], the WRKY transcription factor superfamily [83], the GntR superfamily [84], the Tubby-like proteins (TULPs) superfamily [85], the LysR-type transcriptional regulator (LTTR) superfamily proteins [86]. In Chapter 3 of this thesis, we describe an LTTR family regulator that controls the expression of *aceI*, which encodes a multidrug efflux pump from a newly discovered family of efflux proteins (described in Chapter 2).

It has been seen that many prokaryotic transcriptional regulators are homodimers, which bind to DNA sites that are palindromic or pseudopalindromic [87]. Many of these transcriptional regulatory factors contain a helix-turn-helix (HTH) DNA binding domain, and an inducer-binding domain. Basic structural features of a HTH are three helices, which form a right-handed helical bundle with an open conformation, and a sharp turn located in between the 2nd and 3rd helix of which the 3rd helix, also known as the 'recognition helix', that can insert into the DNA double helix major groove [79, 84, 87].

1.1.4.1.3.1.1 The LysR-type transcriptional regulator superfamily proteins

The LTTRs constitute the largest family of transcriptional regulators in prokaryotes [86]. They can regulate expression of operons related to basic metabolic pathways, stress-response and virulence factors [88, 89]. The LTTRs can be global or local transcriptional regulators, which have the ability to act either as activators or repressors of single genes or operons [86, 90]. Generally, co-inducers may function as signals of environmental changes, and are often necessary for transcriptional activation or repression of metabolic/synthesis pathways by LTTRs (Figure 1.5) [86, 88]. A typical LTTR consists of approximately 300 amino acids in length [86, 89, 90], of which the N-terminal HTH domain is ~70 amino acids long, and provides a DNA- binding site. The C-terminus of the protein is the inducer-binding domain,

which is ~200 amino acids in length and is separated from HTH domain by linker (~30 residues).

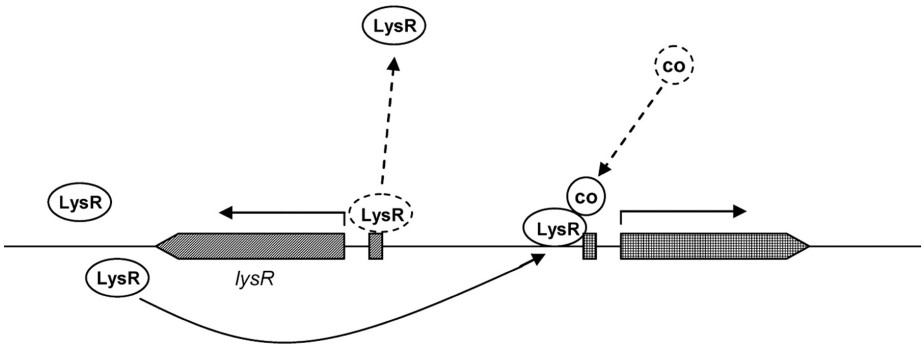
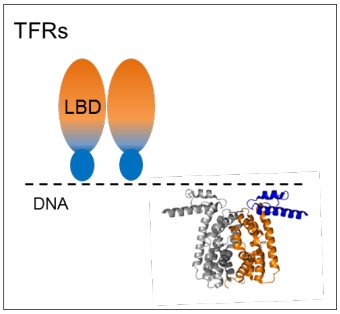


Figure 1.5. A schematic representation of the classical model for LTTR-dependent transcriptional regulation reproduced from [86]. The lysR gene is transcribed when the LysR protein is dissociated from its promoter. The LysR protein product binds upstream of the promoter of the divergently transcribed target gene. When the co-inducer interacts with the LTTR, transcription of this gene is activated.

The intergenic region between an LTTR and the target gene/operon often contains multiple LTTR protein binding sites. Broadly, LTTRs bind at -35 to +20 bp (regulatory binding site) and -40 to -20 bp (activation binding site) to the target gene [86]. Typically, LTTRs bind to imperfect palindromic DNA consisting of sequence T-N_{11}-A, but the sequence can vary in both base pair composition and length [86].

A number of LTTRs have been identified in *Acinetobacter*, which function as global and local regulators activating and/or repressing gene expression. For example, CatM acts as a global activator in catechol metabolism in *Acinetobacter calcoaceticus* in the presence of *cis-cis* muconate as co-factor [91]. BenM is another example in *Acinetobacter baylyi* ADP1 for regulating the expression of benzoate degradation [91, 92]. The research group studying BenM has come up with three oligomerisation schemes based on known structures of LTTRs (Figure 1.6).

In all schemes, effector binding domain (EBD) dimerization interfaces are the same, consisting of residues in the EBDs. The differences between the schemes are in the tetrameric interfaces. In scheme I, there is a small contribution to oligomerisation through EBD contacts, but the DNA binding domain (DBD) plays the major role in bringing together the two dimers. In scheme II, the tetrameric interface is all in the DBD. Then in scheme III, there is a larger contribution from EBD residues than in scheme I (Table in Figure 1.6). Given these forces, there could be doubt as to which dimers form first. It is suggested that one dimer binds to the regulatory binding site (RBS) in apo form, then once the effector molecule is bound to the EBD, the activation binding site (ABS) is occupied by the other dimer and they form a tetramer [92]. This would suggest that the DBD dimerises first and binds to the RBS, leaving the EBD with no binding partners. Once the second dimer binds to the ABS, the tetramer is formed through EBD contacts. This may be the case when target DNA is present, but it is assumed that in solution without the presence of DNA, LTTRs form dimers at the EBD (seen in some crystal structures).



Scheme	Domain	Interface (Å²)	ΔG (kcal/mol)
I (1IXC)	DBD	1094.4	-15.3
	EBD (dimer)	1355.3	-12.6
	EBD (tetramer)	842.3	1.1
II (3ISP)	DBD	1491.2	-21.1
	EBD (dimer)	1175.1	-13.4
III (3FZV)	DBD	1224.5	-12.3
	EBD (dimer)	1411.8	-25.4
	EBD (tetramer)	589.6	-6.7

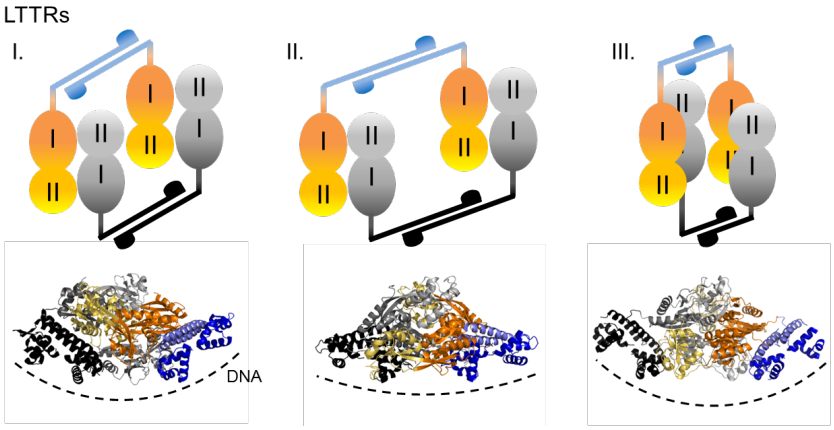


Figure 1.6. LysR oligomerisation and subsequent DNA binding reproduced from [92]. Top left is the arrangement of a TFR dimer in relation to bound DNA (crystal structure CmeR, PDB: 2QCO). The table to the right contains interface statistics from each of the tetrameric oligomerisation schemes seen blew. These are represented figuratively and with crystal structures with the approximate curve of the bound DNA. The coloured chain and black and white chain form an EBD dimer, whereas chains of the same colours form DBD dimers.

1.2 Bacteria interactions

The vast majority of studies into pathogenic bacteria examine individual strains in isolation. However, real infections can include multiple bacterial species that exist within complex polymicrobial communities, and as such several pathogenic organisms can be co-isolated from the same patient [93-95]. One example pursued in this thesis stems from a respiratory infection caused by both *A. baumannii* and *Klebsiella pneumoniae*. Dual-colonization with different antimicrobial resistant bacteria has been well described with prevalence percentages range from 1-11% [96, 97]. Microorganisms have also been shown to have a remarkable interactivity with their neighbouring species, ranging from fierce competition for nutrients and chemical warfare to collaborative cross-feeding, and protective shielding in order to survive and proliferate in complex consortia [98].

1.2.1 Synergistic interactions

In recent years, synergistic interactions between different types of microorganisms have been found increasingly investigated. There are many different ways for microorganisms to cooperate, such as marginal support and absolute mutual dependence [99]. Studies frequently reported where one microorganism excretes metabolites, such as certain amino acids or precursors of vitamins, which can be used by a partner organism that lacks specific synthetic pathways and can profit from this interaction [99].

In nutrient limited conditions, inter-species interactions could be facilitated among microorganisms. Such interactions can be best illustrated by the degradation of polymeric organic compounds. Many studies have demonstrated that extracellular hydrolytic enzyme producing bacteria can cross-feed other bacteria by degrading polymers which cannot be metabolized directly by other microorganism [100]. As a consequence, the enzyme-producing bacteria may only partially consume the nutrients from their efforts in polymer degradation, because other bacteria also benefit from that without producing these enzymes [101].

Cooperation and mutual interdependence are frequently found among anaerobic bacteria. One such example in certain anaerobic niches is the interactions between two hyperthermopilic bacteria, *Thermotoga maritima* and *Methanococcus jannaschii*. Hydrogen, formed as the by-product (inhibitor) of the degradation of sugar or peptide by *T. maritima*, can be utilized to generate methane by *M. jannaschii* [102, 103]. Such syntrophic interactions improve cell growth through the interspecies transfer of hydrogen [104]. This syntrophy can be easily found in the mammalian digestive tract, and in anaerobic digesters used for domestic waste treatment [105].

1.2.2 Antagonistic interactions

A population of microorganisms inhabiting a common environment may compete for nutrients and other resources in the environment. In some circumstances, the populations even generate chemicals that have a toxic or inhibitory effect on their competitors in the environment [106]. Two populations within a single niche incline to compete with each other for energy and space, especially when competition is focused on a single resource [107]. Previous studies have shown that microbial interactions resulted in either bacteriostatic or bacteriocidal responses, which were attributed to antagonistic effects of metabolic products with antimicrobial properties or changes in the physico-chemical environment [108].

These metabolic products include production of organic acids, hydrogen peroxide, inhibitory enzymes, low molecular weight metabolites and bacteriocins, which can have adverse effects on other bacteria [109-112]. Many bacteria species have been shown to produce a variety of peptide bacteriocins, for example, *Streptococcus mutans* strain is able to produce at least five different bacteriocins, mutacins I to V, which can inhibit the growth of *Streptococcus sanguinis* strains [113]. Other bacteria, such as *Prevotella intermedia, Capnocytophaga ochracea, Acinobacillus actinomycetemcomitans* and *Eikenella corrodens*, have also been shown to use bacteriocin or bacteriocin-like compounds to compete with other species [114, 115].

Studies have revealed that both environmental conditions, such as cell density, nutritional availability, and pH, as well as genetic factors can affect production of antimicrobials in a well-regulated process [116-118]. These regulatory systems ensure that inhibitory metabolites are produced at the right time and place [118]. The production of those metabolites among bacteria could shape and establish a specific composition of bacterial species within a polymicrobial community [118].

1.2.3 *A. baumannii* coexistence with other bacteria

In chapter 4 of this thesis, we describe interactions between two co-isolated *A. baumannii* and *K. pneumoniae* from a single patient with a respiratory infection. Both *A. baumannii* and *K. pneumoniae* have been classified into the six top priority dangerous multidrug resistant microorganisms, named the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniea, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) by the Infectious Diseases Society of America [119]. The co-isolation of these strains provided a unique opportunity with *A. baumannii* and *K. pneumonia* to investigate bacteria interactions, thus, some background information of *K. pneumoniae* was provided in this chapter.

1.2.3.1 General features of *K. pneumoniae*

Similar to *A. baumannii*, *K. pneumoniae* is also a Gram-negative, γ-*Proteobacteria*; however, it is non-motile, encapsulated and ubiquitous in natural environments [120]. The conspicuous distinction between *K. pneumoniae* and other enterobacteria is the presence of a thick polysaccharide capsule. This is considered to be a significant virulence factor which helps the bacterium to avoid phagocytosis [121]. *K. pneumoniae* has been emerging as a major worldwide cause of drug-resistant infections and has increasingly been identified globally [122]. Since the 1960s, this microorganism has become an important cause of community-acquired pneumonia in the US and elsewhere [123]. The global pattern of community-acquired *K. pneumoniae* bacteremia differs according to geographic area [122]. The community-acquired *K. pneumoniae* in urinary tract infections, vascular cathethers and cholangitis were found in the United States, Europe, Australia and Argentina, whereas elsewhere classic *K. pneumoniae* strains still exist and have remained important over the last two decades [122]. In addition, an invasive form of *K. pneumoniae* infection, associated with primary bacteremic liver abscesses, endophthalmitis, and meningitis has been reported almost exclusively in Asia [124].

From a public health point of view, the spread of *K. pneumoniae* is worrying, because it is a significant cause of many hospital-acquired infections in severely ill patients, and is well-known for its ability to accumulate and transfer resistance determinants such as extended-spectrum β-lactamases and carbapenemases [125]. Several studies have shown that the spread of multidrug-resistant isolates in the clinic is a result of commonly shared plasmids across *K. pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, *Enterobacter* sp, and S*almonella* sp [126].

1.2.3.1.1 Genomics of *K. pneumoniae*

At the time of writing, genomes of nine *K. pneumoniae* were fully sequenced, including KP-HS11286, KP-JM45, KP342, KP-CG436, KP-KCTC2242, KP1084, KP-NTUH-K2044, KP-MGH78578 and KP-SB3432 (ftp://ftp.ncbi.nih.gov/genomes/archive/old_refseq/Bacteria/). There are 2487 draft genomes of *K. pneumoniae* updated in the NCBI database (https://www.ncbi.nlm.nih.gov/genome/genomes/815). Comparative analysis showed that genome size of these *K. pneumoniae* lies within 5.16-5.64 Mbp with a CG% content of approximately 57%, and different numbers of plasmids (0-6). Previous studies have revealed that the majority of proteins encoded within the core genome of *K. pneumoniae* are involved in metabolic process, such as energy metabolism and transporters, which are essential functions required for survival of microorganisms [127].

1.2.3.1.2 Epidemiology of *K. pneumoniae*

K. pneumoniae is implicated in hospital outbreaks, increasingly associated with high drug-resistant profiles through β-lactamase production [128, 129]. The first outbreak of extended-spectrum β-lactamase (ESBL) producing bacteria, especially *K. pneumonia*, was recorded in French hospitals in the 1980s, and has existed as a threat since then [130]. In the United States of America, historically the predominant β-lactamase encoded in *K. pneumoniae* were of the Temoneria (TEM)- or sulphydryl variable (SHV) SHV-type, but a shift has occurred to the Cefotaximase-Munich (CTX-M)-type being the most commonly detected ESBL [130, 131]. Worldwide distribution of *K. pneumoniae* encoding CTX-M has been recorded with an increase in prevalence over recent years [132].

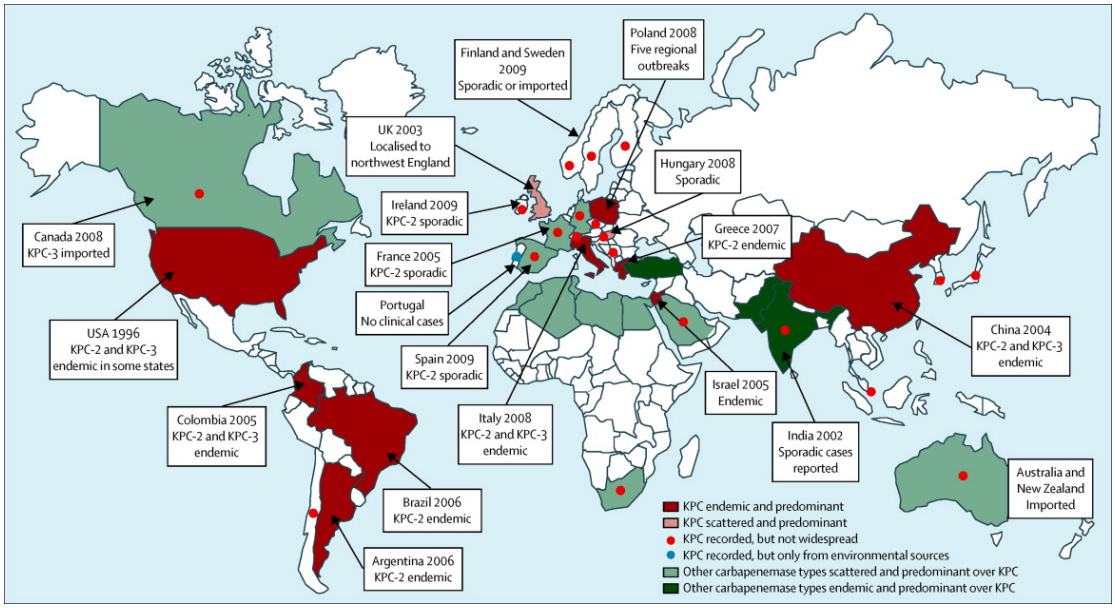


Figure 1.7. Epidemiological features of producers of *Klebsiella pneumoniae* carbapenemases by country of origin reproduced from [133].

Infections by ESBL-producing *K. pneumoniae* ranging from urinary tract infections to complicated sepsis are preferentially treated with the carbapenem antibiotic [134-136]. Carbapenem resistance in *K. pneumoniae* has been detected at alarming frequencies globally (Figure 1.7), including in Africa [137], Asia [138], Europe [139], North America and South America [140]. Various *K. pneumoniae* stains have different prevalence and geographical

distribution, but a particularly important strain involved in national epidemics is the carbapenem-resistant sequence type 258 [141].

1.2.3.2 Epidemiology of *A. baumannii* and *K. pneumoniae* co-infections

Co-infections with multidrug resistant bacteria are associated with remarkable morbidity and mortality. It has been shown that such co-infections are continuing to increase in frequency [142-144]. Rates of co-infection with multidrug resistant *Acinetobacter* spp. and extended spectrum β-lactamase producing microorganisms can go up to 38% of patients in long-term care facilities [145].

While there is only very limited data in the literature on dual-infections, one study indicated that dual-infections by *A. baumannii* and *K. pneumoniae* appears to be a frequent event, 40% of 86 patients, who had carbapenem-resistant Enterobacteriaceae infections, proved to be co-colonized with *A. baumannii* or *P. aeruginosa* in ICUs in Detroit, Michigan [146]. A second study similarly found that 40% of 30 patients who had a carbapenem-resistant *Klebsiella pneumoniae* infection were co-colonized with *A. baumannii* in an ICU in Sicily. Dual-colonized patients in these studies were reported to be very ill, of advanced age, and more likely than the non co-colonized patients to have had a stay in an ICU, long-term care facility, and long-term acute care facility and to have undergone surgery [146].

1.2.4 Bacterial interactions in biofilms

Microorganisms frequently live in spatially structured communities, called biofilms, which can be surfaced bound or free floating and are generally encased in a secreted polymer matrix [147]. Biofilms, which are the dominant lifestyle of bacteria, can cause antimicrobial resistant infections and the destruction of surfaces and flow systems [98, 147]. Thus, they are great concerns in medical and industrial settings [148, 149]. Bacterial biofilms are frequently found with multiple populations of different bacterial species [98]. Figure 1.8 illustrates a model of

interspecific interactions in multispecies biofilms [150]. Each population is confronted with dynamic changes in nutrient profile due to either environmental changes or metabolism, and migration of other population [98, 151]. Therefore, the success rate of any given population in a multispecies biofilm is strongly based on the behavior of other species [151, 152].

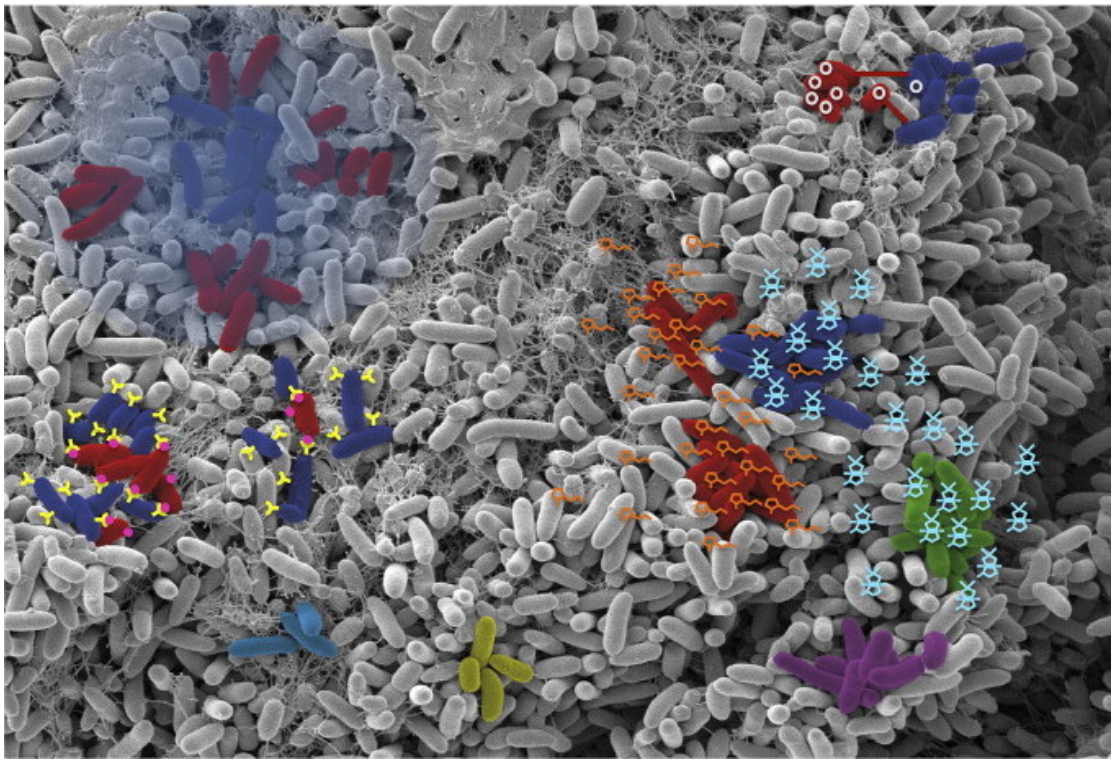


Figure 1.8. Illustration of interspecific interactions in polymicrobial biofilms reproduced from [150]. Top left: con-metabolism where one bacterial species (blue cells) produces substrates (blue zone) allowing growth of another species (red cells). Bottom left: co-aggregation where specific cell-cell attachment occurs between cells of different species (blue and red cells) via specific surface-associated components (yellow and pink). Top right: horizontal gene transfer where plasmid DNA (white circles) is transferred from one species (red cells) to another (blue cells) through the conjugative pili (red lines connecting blue and red cells). Bottom right: quorum sensing through intraspecific (red cells, orange N-acyl homoserine lactone compounds) and interspecific (blue and green cells, cyan furanosyl borate diester compounds) communication via diffusible compounds. Intraspecific communication signals (red cells, orange compounds) may also reach and affect cells of other species (blue). Yellow, cyan, and purple cells are not affected by the interactions.

Bacteria within biofilms interact intimately and influence each other's evolutionary fitness via social phenotypes [153, 154]. Many such interactions are involved in cooperation that benefit neighboring cells, such as the secretion of signaling molecules [155], surface adhesins [156], nutrient chelators [157], structure polymers [158] and digestive enzymes [159]. For example,

Vibrio cholera forms biofilms on environmental particles of the structural polymer chitin, which it digests through communally beneficial chitinases [160]. Cooperative and collective behavior of microorganisms within biofilms has substantial advantages compared with solitary cells, including an increased resilience against external threats and higher efficiency in digesting complex nutrients [160]. Bacteria are therefore fundamentally social organisms, and their cooperative phenotypes are vital to how they influence the world around them [161].

However, biofilm-dwelling cells should not be assumed to work together harmoniously, their social interactions can also be competitive for limited space and resources, and many social phenotypes harm other strains and species [162]. The direct injection of toxins into adjunct cells, antibiotic secretion, and mechanisms for displacing or suffocating neighbors all target competitors for elimination, and can substantially change the composition of biofilms [163, 164]. One example is type VI secretion system mediated attack by *P. aeruginosa* in response to antagonism from other bacteria [165]. *Pseudomonas fluorescens* and *V. cholera* can produce extracellular matrix materials that provide secreting cells a positional advantage over competitors, which are physically displaced from nutrient access [166] or cut off from nutrient access [167].

The relative fitness benefits of cooperative and competitive phenotypes can be strongly influenced by the spatial arrangement of different biofilm-dwelling cells. Alteration of social phenotypes by changing the reproductive rates of neighbouring cells can cause compositional and structural changes in microbial biofilms formation, which in turn shape their overall function, such as the virulence of some pathogens [168, 169]. The arrangement of microbial communities in space is thus pivotal to whether competitive or cooperative interactions are advantageous in a given environmental context [170].

1.2.4.1 *A. baumannii* biofilm formation

Biofilms provide another effective way for bacteria to survive in the presence of antibiotics [171]. This may be especially true for *A. baumannii* which is associated with biofilm contamination of medical devices [93]. *A. baumannii* forms biofilms on abiotic surfaces, such as polystyrene and glass, as well as biotic surfaces such as epithelial cells and fungal filaments [172]. Biofilm formation in *A. baumannii* has been shown to resist antimicrobial treatment, dehydration and nutrient starvation [172]. These traits are important factors in *A. baumannii* infections such as ventilator-acquired pneumonia, urinary tract infections, septicemia, wound infections, and more recently in severe cases of necrotizing fasciitis [173].

Biofilm-specific resistance has been demonstrated to be significantly higher than antibiotic resistance of planktonic bacteria [174]. Biofilm-related infections are thus more difficult to treat and prone to relapse [175]. Intriguingly, several studies have revealed that low doses of certain antibiotics can induce biofilm formation in *A. baumannii* [176]. A recent study of 272 clinical *A. baumannii* isolates indicated a correlation between antibiotic resistance and biofilm formation [177].

Biofilm formation in *A. baumannii* is multifactorial and dependent on multiple molecular determinants, including genes encoding a biofilm-associated protein (*bap*) [178], outer membrane protein A (*ompA*) [179], synthesis of poly-β-1-6-N-acetylglucosamine (*pgaABCD*) [180], and a chaperone-usher pili assembly system (*csuA/BABCDE*) [7]. The multifactorial nature of biofilm formation has been investigated by comparative transcriptomics of *A. baumannii* cells in biofilm and planktonic stages, revealing several other genes associated with biofilm formation, such as genes encoding homoserine lactone synthesis (A1S_0109, A1S_0112 and A1S_0113), TetR family regulator encoding gene (A1S_2042) and genes encoding fimbrial proteins (A1S_1507 and A1S_3168) [181].

1.2.4.2 *K. pneumoniae* biofilm formation

K. pneumoniae strains were reported to form biofilms *in vitro* as early as the end of the 1980s [182], and subsequently solid evidence of an *in vivo* biofilm formed by this organism [183]. It has been demonstrated that 40% of *K. pneumoniae* isolated from urine, sputum, blood and wound swabs, were able to form biofilms [184].

This bacteria pathogen has been frequently found to be able to form mixed biofilms together with other species, such as *P. aeruginosa* [185, 186]. *K. pneumoniae* was reported to form a thicker biofilm with an increased resistance to disinfection in a dual species mixture than in isolation [187].

Key factors in *K. pneumoniae* biofilm formation include type 1 or type 3 fimbriae, lipopolysaccharide (LPS) and the capsule [94]. Type 3 fimbriae have been demonstrated as the major appendages that mediate the formation of biofilms on biotic and abiotic surfaces, and the attachment to endothelial and bladder epithelial cell lines [188-190]. In particular, growth of *K. pneumoniae* on both abiotic and biotic surfaces is enhanced by type 3 fimbrial proteins, MrkA and MrkD, respectively [188, 191].

Capsule and LPS have been proven to contribute to the structure of biofilm communities of *K. pneumoniae*. Gene disruption and microscopic analyses suggested that LPS is involved in the initial adhesion on abiotic surfaces, and that the capsule is essential for a proper initial coverage of substratum and construction of mature biofilm architecture [192].

1.2.4.3 Quorum sensing of bacteria in biofilm

Many cooperative bacterial traits are regulated by quorum sensing, a microbial regulatory mechanism that involves the secretion of and response to diffusible molecules called autoinducers [193]. Quorum sensing (QS) is typically used to assess population density and species complexity monitoring in the immediate environment [194]. Previous studies have indicated that quorum sensing could be used to fine-tune the timing of extracellular matrix secretion, as the matrix confers an advantage in competition for limited space by reducing dispersal ability [195]. Biofilm simulations showed that quorum sensing might act to predict when clonal patches occur along cell group fronts, enabling cells to time the secretion of public goods to avoid exploitation [196].

QS has also been found to regulate competitive secretion phenotypes such as the production of bacteriocin by *Streptococcus* spp. [197] and *Lactobacillus* spp. [198]. It is consistent with the hypothesis that toxin-secreting strains can initiate effective attacks only at a sufficiently high cell density [199]. Toxin secretors can detect nearby target cells by sensing many other diffusible cues that are not necessarily autoinducers but still correlated with population density [200]. For example, *P. aeruginosa* releases the toxin pyocyanin in response to N-acetylglucosamine that is shed from the cell walls of Gram-negative bacteria [201].

1.2.4.3.1 Quorum sensing in *A. baumannii* and *K. pneumoniae*

There has only been limited work on QS in *A. baumannii* and *K. pneumoniae*. In *A. baumannii*, N-(3-hydroxydodecanoyl)-L-homoserine lactone (3-hydroxy-C12-HSL) is the major quorum molecule identified, with at least five other N-acyl homoserine lactone (AHL) molecules produced in significantly lower amounts [202]. Recently, interspecies interaction between *A. baumannii* and *P. aeruginosa* showed that biofilm formation, bacterial growth and virulence of *A. baumannii* were not affected by metabolites produced by *P. aeruginosa*. Instead, biofilm formation was stimulated when a heterologous pool of AHLs from both

species was used, suggesting that complementary quorum interactions were responsible for symbiotic co-existence [203].

High-resolution mass spectrometry analyses of *K. pneumoniae* have shown that it produces two AHL molecules, N-octanoylhomoserine lactone and N-3-dodecanoyl-L-homoserine lactone [204]. In addition, hypervirulent strains of *K. pneumoniae* have been shown to enhance inherent virulence characteristics by releasing a siderophore-related quorum molecule for iron acquisition from the surrounding environment [205].

1.3 Objectives of thesis

Acinetobactor baumannii is an increasingly problematic human pathogen that represents a significant public health threat in recent decades. The acquisition of a broad array of antibiotic resistance genes has led to the spread of multidrug resistant strains globally. Its virulence and ability to persist in hospital settings remains incompletely understood, especially in the context of mixed species biofilms with other pathogens, such as *K. pneumoniae,* within a single infection.

The objectives of this project were to investigate two aspects of the success of *A. baumannii* in the clinical settings. The first aim was to directly study mechanisms of drug resistance of *A. baumannii*; specifically, the regulation and activity of a recently identified AceI drug efflux system. The second aim was to examine interactions at a phenotypic and molecular level between two of the ESKAPE pathogens, *A. baumannii* and *K. pneumoniae*, which were co-isolated from a single respiratory infection within one patient.

1.4 References

1. Bergogne-Berezin, E. and K.J. Towner, *Acinetobacter spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features*. Clin Microbiol Rev, 1996. **9**(2): p. 148-65.
2. Fournier, P.E., et al., *Comparative genomics of multidrug resistance in Acinetobacter baumannii*. PLoS Genet, 2006. **2**(1): p. e7.
3. Dijkshoorn, L., A. Nemec, and H. Seifert, *An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii*. Nat Rev Microbiol, 2007. **5**(12): p. 939-51.
4. van den Broek, P.J., et al., *Epidemiology of multiple Acinetobacter outbreaks in The Netherlands during the period 1999-2001*. Clin Microbiol Infect, 2006. **12**(9): p. 837-43.
5. Jawad, A., et al., *Survival of Acinetobacter baumannii on dry surfaces: comparison of outbreak and sporadic isolates*. J Clin Microbiol, 1998. **36**(7): p. 1938-41.
6. Peleg, A.Y., H. Seifert, and D.L. Paterson, *Acinetobacter baumannii: emergence of a successful pathogen*. Clin Microbiol Rev, 2008. **21**(3): p. 538-82.
7. Tomaras, A.P., et al., *Attachment to and biofilm formation on abiotic surfaces by Acinetobacter baumannii: involvement of a novel chaperone-usher pili assembly system*. Microbiology, 2003. **149**(Pt 12): p. 3473-84.
8. Varposhti, M., F. Entezari, and M.M. Feizabadi, *Synergistic interactions in mixed-species biofilms of pathogenic bacteria from the respiratory tract*. Revista Da Sociedade Brasileira De Medicina Tropical, 2014. **47**(5): p. 649-652.
9. Perez, F., et al., *Global challenge of multidrug-resistant Acinetobacter baumannii*. Antimicrob Agents Chemother, 2007. **51**(10): p. 3471-84.
10. Sader, H.S., T.R. Fritsche, and R.N. Jones, *Potency and spectrum trends for cefepime tested against 65746 clinical bacterial isolates collected in North American medical*

centers: results from the SENTRY Antimicrobial Surveillance Program (1998-2003). Diagn Microbiol Infect Dis, 2005. **52**(3): p. 265-73.

11. Centers for Disease, C. and Prevention, *Acinetobacter baumannii infections among patients at military medical facilities treating injured U.S. service members, 2002-2004*. MMWR Morb Mortal Wkly Rep, 2004. **53**(45): p. 1063-6.
12. Stull, T.L., J.J. LiPuma, and T.D. Edlind, *A broad-spectrum probe for molecular epidemiology of bacteria: ribosomal RNA*. J Infect Dis, 1988. **157**(2): p. 280-6.
13. Maiden, M.C., et al., *Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms*. Proc Natl Acad Sci U S A, 1998. **95**(6): p. 3140-5.
14. Snitkin, E.S., et al., *Genome-wide recombination drives diversification of epidemic strains of Acinetobacter baumannii*. Proc Natl Acad Sci U S A, 2011. **108**(33): p. 13758-63.
15. Dijkshoorn, L., et al., *Comparison of outbreak and nonoutbreak Acinetobacter baumannii strains by genotypic and phenotypic methods*. J Clin Microbiol, 1996. **34**(6): p. 1519-25.
16. van Dessel, H., et al., *Identification of a new geographically widespread multiresistant Acinetobacter baumannii clone from European hospitals*. Res Microbiol, 2004. **155**(2): p. 105-12.
17. Brisse, S., et al., *Molecular surveillance of European quinolone-resistant clinical isolates of Pseudomonas aeruginosa and Acinetobacter spp. using automated ribotyping*. J Clin Microbiol, 2000. **38**(10): p. 3636-45.
18. Vos, P., et al., *AFLP: a new technique for DNA fingerprinting*. Nucleic Acids Res, 1995. **23**(21): p. 4407-14.
19. Eijkelkamp, B.A., et al., *Adherence and motility characteristics of clinical Acinetobacter baumannii isolates*. FEMS Microbiol Lett, 2011. **323**(1): p. 44-51.

20. Cooper, J.E. and E.J. Feil, *Multilocus sequence typing--what is resolved?* Trends Microbiol, 2004. **12**(8): p. 373-7.

21. Antunes, L.C., P. Visca, and K.J. Towner, *Acinetobacter baumannii: evolution of a global pathogen*. Pathog Dis, 2014. **71**(3): p. 292-301.

22. Bartual, S.G., et al., *Development of a multilocus sequence typing scheme for characterization of clinical isolates of Acinetobacter baumannii*. J Clin Microbiol, 2005. **43**(9): p. 4382-90.

23. Diancourt, L., et al., *The population structure of Acinetobacter baumannii: expanding multiresistant clones from an ancestral susceptible genetic pool*. PLoS One, 2010. **5**(4): p. e10034.

24. Chen, W., *Comment on: AbaR4 replaces AbaR3 in a carbapenem-resistant Acinetobacter baumannii isolate belonging to global clone 1 from an Australian hospital*. J Antimicrob Chemother, 2012. **67**(2): p. 512; author reply 513-4.

25. Lewis, T., et al., *High-throughput whole-genome sequencing to dissect the epidemiology of Acinetobacter baumannii isolates from a hospital outbreak*. J Hosp Infect, 2010. **75**(1): p. 37-41.

26. Wright, M.S., et al., *Erratum for "New Insights into Dissemination and Variation of the Health Care-Associated Pathogen Acinetobacter baumannii from Genomic Analysis"*. MBio, 2015. **6**(3): p. e00585.

27. Pogue, J.M., et al., *Carbapenem-resistant Acinetobacter baumannii: epidemiology, surveillance and management*. Expert Rev Anti Infect Ther, 2013. **11**(4): p. 383-93.

28. Kim, U.J., et al., *Update on the Epidemiology, Treatment, and Outcomes of Carbapenem-resistant Acinetobacter infections*. Chonnam Med J, 2014. **50**(2): p. 37-44.

29. Hidron, A.I., et al., *NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to*

the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infect Control Hosp Epidemiol, 2008. **29**(11): p. 996-1011.

30. Reddy, T., et al., *Trends in antimicrobial resistance of Acinetobacter baumannii isolates from a metropolitan Detroit health system*. Antimicrob Agents Chemother, 2010. **54**(5): p. 2235-8.
31. Zhu, L., et al., *Complete genome analysis of three Acinetobacter baumannii clinical isolates in China for insight into the diversification of drug resistance elements*. PLoS One, 2013. **8**(6): p. e66584.
32. Di Nocera, P.P., et al., *Genome organization of epidemic Acinetobacter baumannii strains*. BMC Microbiol, 2011. **11**: p. 224.
33. Imperi, F., et al., *The genomics of Acinetobacter baumannii: insights into genome plasticity, antimicrobial resistance and pathogenicity*. IUBMB Life, 2011. **63**(12): p. 1068-74.
34. Adams, M.D., et al., *Comparative genome sequence analysis of multidrug-resistant Acinetobacter baumannii*. J Bacteriol, 2008. **190**(24): p. 8053-64.
35. Mathee, K., et al., *Dynamics of Pseudomonas aeruginosa genome evolution*. Proc Natl Acad Sci U S A, 2008. **105**(8): p. 3100-5.
36. Ogier, J.C., et al., *Units of plasticity in bacterial genomes: new insight from the comparative genomics of two bacteria interacting with invertebrates, Photorhabdus and Xenorhabdus*. BMC Genomics, 2010. **11**: p. 568.
37. Alekshun, M.N. and S.B. Levy, *Molecular mechanisms of antibacterial multidrug resistance*. Cell, 2007. **128**(6): p. 1037-50.
38. Philippon, A., R. Labia, and G. Jacoby, *Extended-Spectrum Beta-Lactamases*. Antimicrobial Agents and Chemotherapy, 1989. **33**(8): p. 1131-1136.
39. Walsh, C.T., et al., *Bacterial resistance to vancomycin: five genes and one missing hydrogen bond tell the story*. Chem Biol, 1996. **3**(1): p. 21-8.

40. Munita, J.M. and C.A. Arias, *Mechanisms of Antibiotic Resistance*. Microbiol Spectr, 2016. **4**(2).

41. Higgins, C.F., *Multiple molecular mechanisms for multidrug resistance transporters*. Nature, 2007. **446**(7137): p. 749-57.

42. Houang, E.T., et al., *Epidemiology of rifampin ADP-ribosyltransferase (arr-2) and metallo-beta-lactamase (blaIMP-4) gene cassettes in class 1 integrons in Acinetobacter strains isolated from blood cultures in 1997 to 2000*. Antimicrob Agents Chemother, 2003. **47**(4): p. 1382-90.

43. Vila, J., et al., *Mutation in the gyrA gene of quinolone-resistant clinical isolates of Acinetobacter baumannii*. Antimicrob Agents Chemother, 1995. **39**(5): p. 1201-3.

44. Piddock, L.J., *Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria*. Clin Microbiol Rev, 2006. **19**(2): p. 382-402.

45. Paulsen, I.T., *Multidrug efflux pumps and resistance: regulation and evolution*. Curr Opin Microbiol, 2003. **6**(5): p. 446-51.

46. Li, L., et al., *Rapid multiplexed phenotypic screening identifies drug resistance functions for three novel efflux pumps in Acinetobacter baumannii*. J Antimicrob Chemother, 2016. **71**(5): p. 1223-32.

47. Tseng, T.T., et al., *The RND permease superfamily: an ancient, ubiquitous and diverse family that includes human disease and development proteins*. J Mol Microbiol Biotechnol, 1999. **1**(1): p. 107-25.

48. Magnet, S., P. Courvalin, and T. Lambert, *Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in Acinetobacter baumannii strain BM4454*. Antimicrob Agents Chemother, 2001. **45**(12): p. 3375-80.

49. Marchand, I., et al., *Expression of the RND-type efflux pump AdeABC in Acinetobacter baumannii is regulated by the AdeRS two-component system*. Antimicrob Agents Chemother, 2004. **48**(9): p. 3298-304.

50. Coyne, S., et al., *Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in Acinetobacter baumannii*. Antimicrob Agents Chemother, 2010. **54**(10): p. 4389-93.

51. Damier-Piolle, L., et al., *AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in Acinetobacter baumannii*. Antimicrob Agents Chemother, 2008. **52**(2): p. 557-62.

52. Rosenfeld, N., et al., *Expression of the resistance-nodulation-cell division pump AdeIJK in Acinetobacter baumannii is regulated by AdeN, a TetR-type regulator*. Antimicrob Agents Chemother, 2012. **56**(5): p. 2504-10.

53. Pao, S.S., I.T. Paulsen, and M.H. Saier, Jr., *Major facilitator superfamily*. Microbiol Mol Biol Rev, 1998. **62**(1): p. 1-34.

54. Saier, M.H., Jr., et al., *Evolutionary origins of multidrug and drug-specific efflux pumps in bacteria*. FASEB J, 1998. **12**(3): p. 265-74.

55. Law, C.J., P.C. Maloney, and D.N. Wang, *Ins and outs of major facilitator superfamily antiporters*. Annu Rev Microbiol, 2008. **62**: p. 289-305.

56. Reddy, V.S., et al., *The major facilitator superfamily (MFS) revisited*. FEBS J, 2012. **279**(11): p. 2022-35.

57. Roca, I., et al., *CraA, a major facilitator superfamily efflux pump associated with chloramphenicol resistance in Acinetobacter baumannii*. Antimicrob Agents Chemother, 2009. **53**(9): p. 4013-4.

58. Rajamohan, G., V.B. Srinivasan, and W.A. Gebreyes, *Molecular and functional characterization of a novel efflux pump, AmvA, mediating antimicrobial and disinfectant resistance in Acinetobacter baumannii*. J Antimicrob Chemother, 2010. **65**(9): p. 1919-25.

59. Paulsen, I.T., et al., *The SMR family: a novel family of multidrug efflux proteins involved with the efflux of lipophilic drugs*. Mol Microbiol, 1996. **19**(6): p. 1167-75.

60. Chung, Y.J. and M.H. Saier, Jr., *SMR-type multidrug resistance pumps*. Curr Opin Drug Discov Devel, 2001. **4**(2): p. 237-45.

61. Srinivasan, V.B., G. Rajamohan, and 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, 2009. **53**(12): p. 5312-6.

62. Higgins, C.F., *ABC transporters: from microorganisms to man*. Annu Rev Cell Biol, 1992. **8**: p. 67-113.

63. Rees, D.C., E. Johnson, and O. Lewinson, *ABC transporters: the power to change*. Nat Rev Mol Cell Biol, 2009. **10**(3): p. 218-27.

64. Broehan, G., et al., *Functional analysis of the ATP-binding cassette (ABC) transporter gene family of Tribolium castaneum*. BMC Genomics, 2013. **14**: p. 6.

65. Brown, M.H., I.T. Paulsen, and R.A. Skurray, *The multidrug efflux protein NorM is a prototype of a new family of transporters*. Mol Microbiol, 1999. **31**(1): p. 394-5.

66. Omote, H., et al., *The MATE proteins as fundamental transporters of metabolic and xenobiotic organic cations*. Trends Pharmacol Sci, 2006. **27**(11): p. 587-93.

67. Otsuka, M., et al., *Identification of essential amino acid residues of the NorM Na+/multidrug antiporter in Vibrio parahaemolyticus*. J Bacteriol, 2005. **187**(5): p. 1552-8.

68. Su, X.Z., et al., *AbeM, an H+-coupled Acinetobacter baumannii multidrug efflux pump belonging to the MATE family of transporters*. Antimicrob Agents Chemother, 2005. **49**(10): p. 4362-4.

69. McDonnell, G. and A.D. Russell, *Antiseptics and disinfectants: activity, action, and resistance*. Clin Microbiol Rev, 1999. **12**(1): p. 147-79.

70. Brooks, S.E., et al., *Chlorhexidine resistance in antibiotic-resistant bacteria isolated from the surfaces of dispensers of soap containing chlorhexidine*. Infection Control and Hospital Epidemiology, 2002. **23**(11): p. 692-695.

71. Hassan, K.A., et al., *Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins*. Proc Natl Acad Sci U S A, 2013. **110**(50): p. 20254-9.

72. Finn, R.D., et al., *Pfam: the protein families database*. Nucleic Acids Research, 2014. **42**(D1): p. D222-D230.

73. Coyne, S., P. Courvalin, and B. Perichon, *Efflux-mediated antibiotic resistance in Acinetobacter spp*. Antimicrob Agents Chemother, 2011. **55**(3): p. 947-53.

74. Baranova, N. and H. Nikaido, *The baeSR two-component regulatory system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in Escherichia coli and increases its resistance to novobiocin and deoxycholate*. J Bacteriol, 2002. **184**(15): p. 4168-76.

75. Nishino, K., Y. Senda, and A. Yamaguchi, *CRP regulator modulates multidrug resistance of Escherichia coli by repressing the mdtEF multidrug efflux genes*. J Antibiot (Tokyo), 2008. **61**(3): p. 120-7.

76. Nishino, K., et al., *Role of the AraC-XylS family regulator YdeO in multi-drug resistance of Escherichia coli*. J Antibiot (Tokyo), 2009. **62**(5): p. 251-7.

77. Zhou, D. and R. Yang, *Global analysis of gene transcription regulation in prokaryotes*. Cell Mol Life Sci, 2006. **63**(19-20): p. 2260-90.

78. Cases, I. and V. de Lorenzo, *Promoters in the environment: transcriptional regulation in its natural context*. Nat Rev Microbiol, 2005. **3**(2): p. 105-18.

79. Aravind, L., et al., *The many faces of the helix-turn-helix domain: transcription regulation and beyond*. FEMS Microbiol Rev, 2005. **29**(2): p. 231-62.

80. Ramos, J.L., et al., *The TetR family of transcriptional repressors*. Microbiol Mol Biol Rev, 2005. **69**(2): p. 326-56.

81. Fillat, M.F., *The FUR (ferric uptake regulator) superfamily: diversity and versatility of key transcriptional regulators*. Arch Biochem Biophys, 2014. **546**: p. 41-52.

82. MacPherson, S., M. Larochelle, and B. Turcotte, *A fungal family of transcriptional regulators: the zinc cluster proteins*. Microbiol Mol Biol Rev, 2006. **70**(3): p. 583-604.

83. Zhang, Y. and L. Wang, *The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants*. BMC Evol Biol, 2005. **5**: p. 1.

84. Hoskisson, P.A. and S. Rigali, *Chapter 1: Variation in form and function the helix-turn-helix regulators of the GntR superfamily*. Adv Appl Microbiol, 2009. **69**: p. 1-22.

85. Mukhopadhyay, S. and P.K. Jackson, *The tubby family proteins*. Genome Biol, 2011. **12**(6): p. 225.

86. Maddocks, S.E. and P.C. Oyston, *Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins*. Microbiology, 2008. **154**(Pt 12): p. 3609-23.

87. Huffman, J.L. and R.G. Brennan, *Prokaryotic transcription regulators: more than just the helix-turn-helix motif*. Curr Opin Struct Biol, 2002. **12**(1): p. 98-106.

88. Picossi, S., B.R. Belitsky, and A.L. Sonenshein, *Molecular mechanism of the regulation of Bacillus subtilis gltAB expression by GltC*. J Mol Biol, 2007. **365**(5): p. 1298-313.

89. Devesse, L., et al., *Crystal structures of DntR inducer binding domains in complex with salicylate offer insights into the activation of LysR-type transcriptional regulators*. Mol Microbiol, 2011. **81**(2): p. 354-67.

90. Schell, M.A., *Molecular biology of the LysR family of transcriptional regulators*. Annu Rev Microbiol, 1993. **47**: p. 597-626.

91. Ezezika, O.C., et al., *Distinct effector-binding sites enable synergistic transcriptional activation by BenM, a LysR-type regulator*. J Mol Biol, 2007. **367**(3): p. 616-29.

92. Ruangprasert, A., et al., *Full-length structures of BenM and two variants reveal different oligomerization schemes for LysR-type transcriptional regulators*. J Mol Biol, 2010. **404**(4): p. 568-86.

93. Singhai, M., et al., *A study on device-related infections with special reference to biofilm production and antibiotic resistance*. J Glob Infect Dis, 2012. **4**(4): p. 193-8.

94. Vuotto, C., et al., *Antibiotic Resistance Related to Biofilm Formation in Klebsiella pneumoniae*. Pathogens, 2014. **3**(3): p. 743-58.

95. Săndulescu, O., et al., *Dual infection with Acinetobacter baumannii and Klebsiella pneumoniaein a patient with multiple comorbidities – case presentation*. BMC Infectious Diseases, 2014. **14**(7): p. P26.

96. Westerman, M. and R.G. Bennett, *Colonization of skilled-care facility residents with antimicrobial-resistant pathogens*. Journal of the American Geriatrics Society, 2001. **49**(12): p. 1735-1736.

97. Ho, P.L. and G. Hong Kong intensive care unit antimicrobial resistance study, *Carriage of methicillin-resistant Staphylococcus aureus, ceftazidime-resistant Gram-negative bacilli, and vancomycin-resistant enterococci before and after intensive care unit admission*. Crit Care Med, 2003. **31**(4): p. 1175-82.

98. Moons, P., C.W. Michiels, and A. Aertsen, *Bacterial interactions in biofilms*. Crit Rev Microbiol, 2009. **35**(3): p. 157-68.

99. Schink, B., *Synergistic interactions in the microbial world*. Antonie Van Leeuwenhoek, 2002. **81**(1-4): p. 257-61.

100. Lynd, L.R., et al., *Microbial cellulose utilization: fundamentals and biotechnology*. Microbiol Mol Biol Rev, 2002. **66**(3): p. 506-77, table of contents.

101. Jagmann, N., H.P. Brachvogel, and B. Philipp, *Parasitic growth of Pseudomonas aeruginosa in co-culture with the chitinolytic bacterium Aeromonas hydrophila*. Environ Microbiol, 2010. **12**(6): p. 1787-802.

102. Muralidharan, V., et al., *Hydrogen transfer between methanogens and fermentative heterotrophs in hyperthermophilic cocultures*. Biotechnol Bioeng, 1997. **56**(3): p. 268-78.

103. Huber, R., et al., *Thermotoga-Maritima Sp-Nov Represents a New Genus of Unique Extremely Thermophilic Eubacteria Growing up to 90-Degrees-C*. Archives of Microbiology, 1986. **144**(4): p. 324-333.

104. Bult, C.J., et al., *Complete genome sequence of the methanogenic archaeon, Methanococcus jannaschii*. Science, 1996. **273**(5278): p. 1058-73.

105. Morvan, B., et al., *Quantitative determination of H2-utilizing acetogenic and sulfate-reducing bacteria and methanogenic archaea from digestive tract of different mammals*. Curr Microbiol, 1996. **32**(3): p. 129-33.

106. Hibbing, M.E., et al., *Bacterial competition: surviving and thriving in the microbial jungle*. Nat Rev Microbiol, 2010. **8**(1): p. 15-25.

107. Fredrickson, A.G. and G. Stephanopoulos, *Microbial competition*. Science, 1981. **213**(4511): p. 972-9.

108. Nilsson, L., et al., *Growth inhibition of Listeria monocytogenes by a nonbacteriocinogenic Carnobacterium piscicola*. J Appl Microbiol, 2005. **98**(1): p. 172-83.

109. Elayaraja, S., et al., *Production, purification and characterization of bacteriocin from Lactobacillus murinus AU06 and its broad antibacterial spectrum*. Asian Pac J Trop Biomed, 2014. **4**(Suppl 1): p. S305-11.

110. Piard, J.C. and M. Desmazeaud, *Inhibiting Factors Produced by Lactic-Acid Bacteria .1. Oxygen Metabolites and Catabolism End-Products*. Lait, 1991. **71**(5): p. 525-541.

111. Piard, J.C. and M. Desmazeaud, *Inhibiting Factors Produced by Lactic-Acid Bacteria .2. Bacteriocins and Other Antibacterial Substances*. Lait, 1992. **72**(2): p. 113-142.

112. Wilson, B.A., S.M. Thomas, and M.F. Ho, *The Human Vaginal Microbiome*. Metagenomics of the Human Body, 2011: p. 91-115.

113. Kreth, J., et al., *Competition and coexistence between Streptococcus mutans and Streptococcus sanguinis in the dental biofilm*. J Bacteriol, 2005. **187**(21): p. 7193-203.

114. Takada, K., M. Hirasawa, and T. Ikeda, *Isolation and purification of bacteriocin from Prevotella intermedia (Bacteroides intermedius)*. J Periodontol, 1991. **62**(7): p. 439-44.

115. Riley, M.A. and J.E. Wertz, *Bacteriocins: evolution, ecology, and application*. Annu Rev Microbiol, 2002. **56**: p. 117-37.

116. Merritt, J., et al., *LuxS controls bacteriocin production in Streptococcus mutans through a novel regulatory component*. Mol Microbiol, 2005. **57**(4): p. 960-9.

117. Qi, F., et al., *Inactivation of the ciaH Gene in Streptococcus mutans diminishes mutacin production and competence development, alters sucrose-dependent biofilm formation, and reduces stress tolerance*. Infect Immun, 2004. **72**(8): p. 4895-9.

118. Kuramitsu, H.K., et al., *Interspecies interactions within oral microbial communities*. Microbiol Mol Biol Rev, 2007. **71**(4): p. 653-70.

119. Rice, L.B., *Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE*. J Infect Dis, 2008. **197**(8): p. 1079-81.

120. Wu, K.M., et al., *Genome sequencing and comparative analysis of Klebsiella pneumoniae NTUH-K2044, a strain causing liver abscess and meningitis*. J Bacteriol, 2009. **191**(14): p. 4492-501.

121. Domenico, P., et al., *Polysaccharide capsule-mediated resistance to opsonophagocytosis in Klebsiella pneumoniae*. Infect Immun, 1994. **62**(10): p. 4495-9.

122. Ko, W.C., et al., *Community-acquired Klebsiella pneumoniae bacteremia: global differences in clinical patterns*. Emerg Infect Dis, 2002. **8**(2): p. 160-6.

123. Carpenter, J.L., *Klebsiella pulmonary infections: occurrence at one medical center and review*. Rev Infect Dis, 1990. **12**(4): p. 672-82.

124. Fung, C.P., et al., *A global emerging disease of Klebsiella pneumoniae liver abscess: is serotype K1 an important factor for complicated endophthalmitis?* Gut, 2002. **50**(3): p. 420-4.

125. Qi, Y., et al., *ST11, the dominant clone of KPC-producing Klebsiella pneumoniae in China*. J Antimicrob Chemother, 2011. **66**(2): p. 307-12.

126. Miro, E., et al., *Spread of plasmids containing the bla(VIM-1) and bla(CTX-M) genes and the qnr determinant in Enterobacter cloacae, Klebsiella pneumoniae and Klebsiella oxytoca isolates*. J Antimicrob Chemother, 2010. **65**(4): p. 661-5.

127. Lery, L.M., et al., *Comparative analysis of Klebsiella pneumoniae genomes identifies a phospholipase D family protein as a novel virulence factor*. BMC Biol, 2014. **12**: p. 41.

128. Shon, A.S., R.P. Bajwa, and T.A. Russo, *Hypervirulent (hypermucoviscous) Klebsiella pneumoniae: a new and dangerous breed*. Virulence, 2013. **4**(2): p. 107-18.

129. Iredell, J., J. Brown, and K. Tagg, *Antibiotic resistance in Enterobacteriaceae: mechanisms and clinical implications*. Bmj-British Medical Journal, 2016. **352**.

130. Lewis, J.S., 2nd, et al., *First report of the emergence of CTX-M-type extended-spectrum beta-lactamases (ESBLs) as the predominant ESBL isolated in a U.S. health care system*. Antimicrob Agents Chemother, 2007. **51**(11): p. 4015-21.

131. van der Bij, A.K. and J.D. Pitout, *The role of international travel in the worldwide spread of multiresistant Enterobacteriaceae*. J Antimicrob Chemother, 2012. **67**(9): p. 2090-100.

132. Hennequin, C., et al., *Antibiotic resistance and plasmid transfer capacity in biofilm formed with a CTX-M-15-producing Klebsiella pneumoniae isolate*. J Antimicrob Chemother, 2012. **67**(9): p. 2123-30.

133. Munoz-Price, L.S., et al., *Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases*. Lancet Infect Dis, 2013. **13**(9): p. 785-96.

134. Dortet, L., L. Poirel, and P. Nordmann, *Rapid detection of extended-spectrum-beta-lactamase-producing enterobacteriaceae from urine samples by use of the ESBL NDP test*. J Clin Microbiol, 2014. **52**(10): p. 3701-6.

135. Kumar, V., et al., *Comparative genomics of Klebsiella pneumoniae strains with different antibiotic resistance profiles*. Antimicrob Agents Chemother, 2011. **55**(9): p. 4267-76.

136. El Fertas-Aissani, R., et al., *Virulence profiles and antibiotic susceptibility patterns of Klebsiella pneumoniae strains isolated from different clinical specimens*. Pathol Biol (Paris), 2013. **61**(5): p. 209-16.

137. Storberg, V., *ESBL-producing Enterobacteriaceae in Africa - a non-systematic literature review of research published 2008-2012*. Infect Ecol Epidemiol, 2014. **4**.

138. Nordmann, P., G. Cuzon, and T. Naas, *The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria*. Lancet Infect Dis, 2009. **9**(4): p. 228-36.

139. Sisto, A., et al., *Carbapenem non-susceptible Klebsiella pneumoniae from Micronet network hospitals, Italy, 2009 to 2012*. Euro Surveill, 2012. **17**(33).

140. Kitchel, B., et al., *Molecular epidemiology of KPC-producing Klebsiella pneumoniae isolates in the United States: clonal expansion of multilocus sequence type 258*. Antimicrob Agents Chemother, 2009. **53**(8): p. 3365-70.

141. Chmelnitsky, I., et al., *Unique genes identified in the epidemic extremely drug-resistant KPC-producing Klebsiella pneumoniae sequence type 258*. J Antimicrob Chemother, 2013. **68**(1): p. 74-83.

142. Fallon, E.O., A. Pop-Vicas, and E.D. Agata, *The Emerging Threat of Multidrug-Resistant Gram-Negative Organisms in Long-Term Care Facilities*. Journals of Gerontology Series a-Biological Sciences and Medical Sciences, 2009. **64**(1): p. 138-141.

143. D'Agata, E.M., *Rapidly rising prevalence of nosocomial multidrug-resistant, Gram-negative bacilli: a 9-year surveillance study*. Infect Control Hosp Epidemiol, 2004. **25**(10): p. 842-6.

144. Schwaber, M.J., et al., *Clinical and economic impact of bacteremia with extended-spectrum-beta-lactamase-producing Enterobacteriaceae*. Antimicrobial Agents and Chemotherapy, 2006. **50**(4): p. 1257-1262.

145. Maragakis, L.L., et al., *Incidence and prevalence of multidrug-resistant Acinetobacter using targeted active surveillance cultures*. Jama-Journal of the American Medical Association, 2008. **299**(21): p. 2513-2514.

146. Marchaim, D., et al., *"Swimming in resistance": Co-colonization with carbapenem-resistant Enterobacteriaceae and Acinetobacter baumannii or Pseudomonas aeruginosa*. Am J Infect Control, 2012. **40**(9): p. 830-5.

147. Hobley, L., et al., *Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes*. FEMS Microbiol Rev, 2015. **39**(5): p. 649-69.

148. Harding, J.L. and M.M. Reynolds, *Combating medical device fouling*. Trends Biotechnol, 2014. **32**(3): p. 140-6.

149. Drescher, K., et al., *Biofilm streamers cause catastrophic disruption of flow with consequences for environmental and medical systems*. Proc Natl Acad Sci U S A, 2013. **110**(11): p. 4345-50.

150. Burmolle, M., et al., *Interactions in multispecies biofilms: do they actually matter?* Trends Microbiol, 2014. **22**(2): p. 84-91.

151. Banks, M.K. and J.D. Bryers, *Bacterial species dominance within a binary culture biofilm*. Appl Environ Microbiol, 1991. **57**(7): p. 1974-9.

152. Raskin, L., B.E. Rittmann, and D.A. Stahl, *Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms*. Appl Environ Microbiol, 1996. **62**(10): p. 3847-57.

153. Nadell, C.D., J.B. Xavier, and K.R. Foster, *The sociobiology of biofilms*. FEMS Microbiol Rev, 2009. **33**(1): p. 206-24.

154. Nadell, C.D., et al., *Cutting through the complexity of cell collectives*. Proc Biol Sci, 2013. **280**(1755): p. 20122770.

155. West, S.A., et al., *The social lives of microbes*. Annual Review of Ecology Evolution and Systematics, 2007. **38**: p. 53-77.

156. Absalon, C., K. Van Dellen, and P.I. Watnick, *A communal bacterial adhesin anchors biofilm and bystander cells to surfaces*. PLoS Pathog, 2011. **7**(8): p. e1002210.

157. Visca, P., F. Imperi, and I.L. Lamont, *Pyoverdine siderophores: from biogenesis to biosignificance*. Trends Microbiol, 2007. **15**(1): p. 22-30.

158. Allison, D.G., *The biofilm matrix*. Biofouling, 2003. **19**(2): p. 139-50.

159. Allison, S.D., *Cheaters, diffusion and nutrients constrain decomposition by microbial enzymes in spatially structured environments*. Ecology Letters, 2005. **8**(6): p. 626-635.

160. Drescher, K., et al., *Solutions to the public goods dilemma in bacterial biofilms*. Curr Biol, 2014. **24**(1): p. 50-5.

161. Nadell, C.D., K. Drescher, and K.R. Foster, *Spatial structure, cooperation and competition in biofilms*. Nat Rev Microbiol, 2016. **14**(9): p. 589-600.

162. Rendueles, O. and J.M. Ghigo, *Mechanisms of Competition in Biofilm Communities*. Microbiology Spectrum, 2015. **3**(3).

163. Hayes, C.S., S.K. Aoki, and D.A. Low, *Bacterial Contact-Dependent Delivery Systems*. Annual Review of Genetics, Vol 44, 2010. **44**: p. 71-90.

164. Russell, A.B., S.B. Peterson, and J.D. Mougous, *Type VI secretion system effectors: poisons with a purpose*. Nature Reviews Microbiology, 2014. **12**(2): p. 137-148.

165. Ho, B.T., T.G. Dong, and J.J. Mekalanos, *A View to a Kill: The Bacterial Type VI Secretion System*. Cell Host & Microbe, 2014. **15**(1): p. 9-21.

166. Schluter, J., et al., *Adhesion as a weapon in microbial competition*. Isme Journal, 2015. **9**(1): p. 139-149.

167. Kim, W., et al., *Importance of positioning for microbial evolution*. Proceedings of the National Academy of Sciences of the United States of America, 2014. **111**(16): p. E1639-E1647.

168. Brown, S.P., R.F. Inglis, and F. Taddei, *Evolutionary ecology of microbial wars: within-host competition and (incidental) virulence*. Evolutionary Applications, 2009. **2**(1): p. 32-39.

169. Inglis, R.F., et al., *Spite and virulence in the bacterium Pseudomonas aeruginosa*. Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(14): p. 5703-5707.

170. Lion, S. and M. van Baalen, *Self-structuring in spatial evolutionary ecology*. Ecology Letters, 2008. **11**(3): p. 277-295.

171. Hall-Stoodley, L., J.W. Costerton, and P. Stoodley, *Bacterial biofilms: from the natural environment to infectious diseases*. Nat Rev Microbiol, 2004. **2**(2): p. 95-108.

172. Gaddy, J.A. and L.A. Actis, *Regulation of Acinetobacter baumannii biofilm formation*. Future Microbiol, 2009. **4**(3): p. 273-8.

173. Charnot-Katsikas, A., et al., *Two cases of necrotizing fasciitis due to Acinetobacter baumannii*. J Clin Microbiol, 2009. **47**(1): p. 258-63.

174. Hoyle, B.D. and J.W. Costerton, *Bacterial resistance to antibiotics: the role of biofilms*. Prog Drug Res, 1991. **37**: p. 91-105.

175. Cerqueira, G.M. and A.Y. Peleg, *Insights into Acinetobacter baumannii pathogenicity*. IUBMB Life, 2011. **63**(12): p. 1055-60.

176. Kaplan, J.B., *Antibiotic-induced biofilm formation*. Int J Artif Organs, 2011. **34**(9): p. 737-51.

177. Qi, L., et al., *Relationship between Antibiotic Resistance, Biofilm Formation, and Biofilm-Specific Resistance in Acinetobacter baumannii*. Front Microbiol, 2016. **7**: p. 483.

178. Wagner, C. and M. Hensel, *Adhesive mechanisms of Salmonella enterica*. Adv Exp Med Biol, 2011. **715**: p. 17-34.

179. Gaddy, J.A., A.P. Tomaras, and L.A. Actis, *The Acinetobacter baumannii 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells*. Infect Immun, 2009. **77**(8): p. 3150-60.

180. Agladze, K., X. Wang, and T. Romeo, *Spatial periodicity of Escherichia coli K-12 biofilm microstructure initiates during a reversible, polar attachment phase of development and requires the polysaccharide adhesin PGA*. J Bacteriol, 2005. **187**(24): p. 8237-46.

181. Rumbo-Feal, S., et al., *Whole transcriptome analysis of Acinetobacter baumannii assessed by RNA-sequencing reveals different mRNA expression profiles in biofilm compared to planktonic cells*. PLoS One, 2013. **8**(8): p. e72968.

182. LeChevallier, M.W., C.D. Cawthon, and R.G. Lee, *Factors promoting survival of bacteria in chlorinated water supplies*. Appl Environ Microbiol, 1988. **54**(3): p. 649-54.

183. Reid, G., et al., *Bacterial biofilm formation in the urinary bladder of spinal cord injured patients*. Paraplegia, 1992. **30**(10): p. 711-7.

184. Yang, D. and Z. Zhang, *Biofilm-forming Klebsiella pneumoniae strains have greater likelihood of producing extended-spectrum beta-lactamases*. J Hosp Infect, 2008. **68**(4): p. 369-71.

185. Childers, B.M., et al., *MrkD1P from Klebsiella pneumoniae strain IA565 allows for coexistence with Pseudomonas aeruginosa and protection from protease-mediated biofilm detachment*. Infect Immun, 2013. **81**(11): p. 4112-20.

186. Siebel, M.A. and W.G. Characklis, *Observations of binary population biofilms*. Biotechnol Bioeng, 1991. **37**(8): p. 778-89.

187. Skillman, L.C., I.W. Sutherland, and M.V. Jones, *The role of exopolysaccharides in dual species biofilm development*. J Appl Microbiol, 1998. **85 Suppl 1**: p. 13S-18S.

188. Langstraat, J., M. Bohse, and S. Clegg, *Type 3 fimbrial shaft (MrkA) of Klebsiella pneumoniae, but not the fimbrial adhesin (MrkD), facilitates biofilm formation*. Infect Immun, 2001. **69**(9): p. 5805-12.

189. Di Martino, P., et al., *Klebsiella pneumoniae type 3 pili facilitate adherence and biofilm formation on abiotic surfaces*. Res Microbiol, 2003. **154**(1): p. 9-16.

190. Schroll, C., et al., *Role of type 1 and type 3 fimbriae in Klebsiella pneumoniae biofilm formation*. BMC Microbiol, 2010. **10**: p. 179.

191. Jagnow, J. and S. Clegg, *Klebsiella pneumoniae MrkD-mediated biofilm formation on extracellular matrix- and collagen-coated surfaces*. Microbiology, 2003. **149**(Pt 9): p. 2397-405.

192. Balestrino, D., et al., *The characterization of functions involved in the establishment and maturation of Klebsiella pneumoniae in vitro biofilm reveals dual roles for surface exopolysaccharides*. Environ Microbiol, 2008. **10**(3): p. 685-701.

193. Li, Y.H. and X. Tian, *Quorum sensing and bacterial social interactions in biofilms*. Sensors (Basel), 2012. **12**(3): p. 2519-38.

194. Ng, W.L. and B.L. Bassler, *Bacterial quorum-sensing network architectures*. Annu Rev Genet, 2009. **43**: p. 197-222.

195. Nadell, C.D. and B.L. Bassler, *A fitness trade-off between local competition and dispersal in Vibrio cholerae biofilms*. Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(34): p. 14181-14185.

196. Schluter, J., et al., *The Evolution of Quorum Sensing as a Mechanism to Infer Kinship*. Plos Computational Biology, 2016. **12**(4).

197. Fontaine, L., et al., *Quorum-sensing regulation of the production of blp bacteriocins in Streptococcus thermophilus*. Journal of Bacteriology, 2007. **189**(20): p. 7195-7205.

198. Risoen, P.A., et al., *Functional analysis of promoters involved in quorum sensing-based regulation of bacteriocin production in Lactobacillus*. Molecular Microbiology, 2000. **37**(3): p. 619-628.

199. Cornforth, D.M. and K.R. Foster, *Competition sensing: the social side of bacterial stress responses*. Nature Reviews Microbiology, 2013. **11**(4): p. 285-293.

200. LeRoux, M., S.B.P. Eterson, and J.D. Mougous, *Bacterial danger sensing*. Journal of Molecular Biology, 2015. **427**(23): p. 3744-3753.

201. Korgaonkar, A.K. and M. Whiteley, *Pseudomonas aeruginosa Enhances Production of an Antimicrobial in Response to N-Acetylglucosamine and Peptidoglycan*. Journal of Bacteriology, 2011. **193**(4): p. 909-917.

202. Niu, C., et al., *Isolation and characterization of an autoinducer synthase from Acinetobacter baumannii*. Journal of Bacteriology, 2008. **190**(9): p. 3386-3392.

203. Bhargava, N., P. Sharma, and N. Capalash, *N-acyl homoserine lactone mediated interspecies interactions between A. baumannii and P. aeruginosa*. Biofouling, 2012. **28**(8): p. 813-822.

204. Yin, W.F., et al., *N-Acyl Homoserine Lactone Production by Klebsiella pneumoniae Isolated from Human Tongue Surface*. Sensors, 2012. **12**(3): p. 3472-3483.

205. Russo, T.A., et al., *Hypervirulent K. Pneumoniae Secretes More and More Active Iron-Acquisition Molecules than "Classical" K. Pneumoniae Thereby Enhancing its Virulence*. Plos One, 2011. **6**(10).

Chapter 2: Homologs of the *Acinetobacter baumannii* AceI transporter represent a new family of bacterial multidrug efflux systems

OBSERVATION

Homologs of the *Acinetobacter baumannii* AceI Transporter Represent a New Family of Bacterial Multidrug Efflux Systems

Karl A. Hassan,[a] Qi Liu,[a] Peter J. F. Henderson,[b] Ian T. Paulsen[a]

Department of Chemistry and Biomolecular Sciences, Macquarie University, Sydney, NSW, Australia[a]; Astbury Centre for Structural Molecular Biology, School of Biomedical Sciences, University of Leeds, Leeds, United Kingdom[b]

ABSTRACT **Multidrug efflux systems are a major cause of resistance to antimicrobials in bacteria, including those pathogenic to humans, animals, and plants. These proteins are ubiquitous in these pathogens, and five families of bacterial multidrug efflux systems have been identified to date. By using transcriptomic and biochemical analyses, we recently identified the novel AceI (*Acinetobacter* chlorhexidine efflux) protein from *Acinetobacter baumannii* that conferred resistance to the biocide chlorhexidine, via an active efflux mechanism. Proteins homologous to AceI are encoded in the genomes of many other bacterial species and are particularly prominent within proteobacterial lineages. In this study, we expressed 23 homologs of AceI and examined their resistance and/or transport profiles. MIC analyses demonstrated that, like AceI, many of the homologs conferred resistance to chlorhexidine. Many of the AceI homologs conferred resistance to additional biocides, including benzalkonium, dequalinium, proflavine, and acriflavine. We conducted fluorimetric transport assays using the AceI homolog from *Vibrio parahaemolyticus* and confirmed that resistance to both proflavine and acriflavine was mediated by an active efflux mechanism. These results show that this group of AceI homologs represent a new family of bacterial multidrug efflux pumps, which we have designated the proteobacterial antimicrobial compound efflux (PACE) family of transport proteins.**

IMPORTANCE **Bacterial multidrug efflux pumps are an important class of resistance determinants that can be found in every bacterial genome sequenced to date. These transport proteins have important protective functions for the bacterial cell but are a significant problem in the clinical setting, since a single efflux system can mediate resistance to many structurally and mechanistically diverse antibiotics and biocides. In this study, we demonstrate that proteins related to the *Acinetobacter baumannii* AceI transporter are a new class of multidrug efflux systems which are very common in *Proteobacteria*: the proteobacterial antimicrobial compound efflux (PACE) family. This is the first new family of multidrug efflux pumps to be described in 15 years.**

Received 15 October 2014 **Accepted** 23 December 2014 **Published** 10 February 2015

Citation Hassan KA, Liu Q, Henderson PJF, Paulsen IT. 2015. Homologs of the *Acinetobacter baumannii* AceI transporter represent a new family of bacterial multidrug efflux systems. mBio 6(1):e01982-14. doi:10.1128/mBio.01982-14.

Editor Karen Bush, Indiana University Bloomington

Address correspondence to Ian Paulsen, ian.paulsen@mq.edu.au, or Karl A. Hassan, karl.hassan@mq.edu.au.

Multidrug efflux is a ubiquitous mechanism of drug resistance in bacterial pathogens that is mediated by integral membrane transport proteins. These proteins are typically very promiscuous, recognizing a range of antimicrobial substrates that differ in both structure and valency. To date, five distinct families of transport proteins have been shown to include multidrug efflux systems: the major facilitator superfamily, the resistance/nodulation/division superfamily, the ATP-binding cassette superfamily, the multidrug and toxic compound extrusion family, and the small multidrug resistance family.

Recently, we identified the *aceI* (*Acinetobacter* chlorhexidine efflux) gene in *Acinetobacter baumannii*, which is involved in adaptive resistance to the widely used biocide chlorhexidine (1). This gene was overexpressed more than 10-fold in response to a subinhibitory shock of chlorhexidine in *A. baumannii* ATCC 17978. The *aceI* gene encodes a membrane protein that is approximately 150 amino acid residues in length and contains two tandem bacterial transmembrane pair (BTP; Pfam accession number PF05232) domains (2). Heterologous expression of *aceI* increased *Escherichia coli* resistance to chlorhexidine (1) and, conversely, deletion of *aceI* from the *A. baumannii* genome increased its susceptibility to chlorhexidine (3). The AceI protein was shown to interact directly with chlorhexidine and to mediate its efflux via an energy-dependent mechanism (1). However, resistance to other antimicrobial compounds was not observed (1).

Genes that encode BTP domain proteins homologous to *aceI* are carried by diverse bacterial species but are particularly common among proteobacterial lineages. Similar to *A. baumannii*, genes encoding BTP domain proteins were upregulated in the human pathogens *Pseudomonas aeruginosa* PAO1 and *Burkholderia cenocepacia* J2315 in response to chlorhexidine and were able to mediate resistance to this biocide (1). Furthermore, related BTP domain protein genes from the soil bacterium *Acinetobacter baylyi* ADP1 and the plant commensal bacterium *Pseudomonas protegens* Pf-5 were also shown to mediate resistance to chlorhexidine when expressed in *E. coli* (1). Deletion of this gene from *A. baylyi* ADP1 increased its susceptibility to chlorhexidine (1).

In addition to *aceI*, the *A. baumannii* genome harbors a second gene that encodes a BTP family protein, A1S_1503, that does not confer chlorhexidine resistance and whose expression is not in-

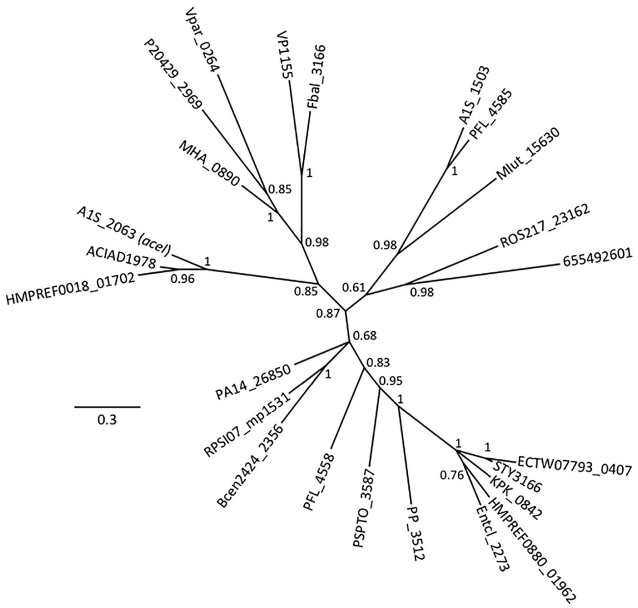


FIG 1 Tree showing the phylogenetic relationships of BTP family proteins included in this study. The tree was generated using MrBayes 3.2.1 (14) from a ClustalX2 alignment of protein sequences obtained from the National Center for Biotechnology Information database. Interior node values are clade credibility values (posterior probabilities) generated and assigned by MrBayes.

duced by chlorhexidine. Similarly, *P. protegens* harbors a second gene encoding a BTP domain protein that appears to be nonfunctional with respect to chlorhexidine resistance (1), and the *P. aeruginosa* and *B. cenocepacia* genomes carry one or two BTP domain protein genes that are not induced by chlorhexidine (4, 5).

Here, we sought to identify alternative drug substrates for BTP domain proteins. We demonstrate that, in addition to chlorhexidine, many BTP domain proteins are able to mediate resistance to other biocides, as well as fluorescent dyes. The protein from *Vibrio parahaemolyticus* VP1155 provided particularly strong resistance to biocides and dyes and mediated rapid transport of acriflavine and proflavine. These results indicate that BTP domain proteins represent a new family of transport proteins that includes multidrug efflux systems, which we have designated the proteobacterial antimicrobial compound efflux (PACE) family.

BTP protein gene cloning and expression. At the time of writing, the Pfam database (version 27.0) listed close to 800 proteins that contain BTP domains from more than 600 bacterial species (2). The majority (95%) of these proteins were predicted to have the same tandem BTP domain architecture as AceI and were encoded by *Proteobacteria*, particularly the gamma, beta, and alpha subdivisions (although this may be biased by the species for which genome sequence data are available). In addition to *Proteobacteria*, the genomes of several *Veillonella* and *Micrococcus* species (*Firmicutes* and *Actinobacteria*, respectively) also carried BTP domain protein genes. We have not, however, detected these genes in the genomes of archaeal or eukaryotic organisms.

In this study, we examined the drug resistance/transport capabilities of 24 BTP domain proteins, including AceI. These proteins were selected to encompass the full spectrum of currently sampled phylogenetic diversity within this group (Fig. 1) and included 18 gammaproteobacterial proteins, 3 betaproteobacterial proteins, and 1 representative protein from each of *Alphaproteobacteria*, *Firmicutes*, and *Actinobacteria* (Table 1). Seven of the genes encoding these proteins were previously cloned into the *E. coli* pTTQ18 expression vector via conventional methods (1). The remaining 17 were synthetically designed *E. coli* codon-optimized sequences and were synthesized in single gBlock gene fragments (Integrated DNA Technologies) and then cloned into the pTTQ18 plasmid vector (6). With the exception of Vpar_0264, ROS217_23162 and MHA_0890, the proteins under investigation were expressed at levels detectable in Western blot assays of whole-cell lysates (see Fig. S1 in the supplemental material). This level of expression success (87.5%) is in line with our previous experiences using this expression system for the heterologous production of transport systems (7–9).

Chlorhexidine resistance mediated by AceI homologs. Previously, we demonstrated that BTP domain proteins homologous to the *A. baumannii* AceI protein from *A. baylyi* ADP1 (ACIAD1978), *P. aeruginosa* PA14 (PA14_26850), *P. protegens*

TABLE 1 Drug resistance conferred by BTP family proteins[a]

Organism	Gene or protein (locus tag)	MIC or MIC range (μg/ml)[b]				
		CH	DQ	BK	PF	AF
NA (negative control)	NA[c] (vector only)	0.195–0.39	50	0.39–0.78	6.25	3.125
Acinetobacter baumannii ATCC 17978	A1S_2063 (*aceI*)	**1.56**	12.5-25	0.39–0.78	6.25	3.125
Acinetobacter radioresistens SH164	HMPREF0018_01702	**0.78**	25	0.39–0.78	6.25	3.125
Acinetobacter baylyi ADP1	ACIAD1978	**0.78**	50	0.78	6.25	3.125
Mannheimia haemolytica PHL213	MHA_0890	0.39	50	0.78	6.25	3.125
Pseudoalteromonas sp. BSi20429	P20429_2969	0.195	50	0.78	6.25	**6.25**
Veillonella parvula DSM 2008	Vpar_0264	0.195	50	0.39–0.78	6.25	3.125
Vibrio parahaemolyticus RIMD 2210633	VP1155	**1.56**	50	**3.125**	**25**	**12.5**
Ferrimonas balearica DSM 9799	Fbal_3166	**0.78–1.56**	50	0.78	3.125	**6.25**
Micrococcus luteus NCTC 2665	Mlut_15630	0.195	50	0.39–0.78	6.25	3.125
Acinetobacter baumannii ATCC 17978	A1S_1503	0.195	25	0.78	6.25	**6.25**
Pseudomonas protegens Pf-5	PFL_4585	0.195	**100**	**1.56**	3.125–6.25	3.125
Tepidiphilus margaritifer DSM 15129	655492601[d]	0.195	50–100	0.78–1.56	1.56–3.125	3.125
Roseovarius sp. 217	ROS217_23162[e]	0.195	50	0.78	6.25	3.125
Pseudomonas aeruginosa PA14	PA14_26850	**0.78**	**100**	**1.56**	6.25	3.125
Ralstonia solanacearum PSI07	RPS107_mp1531	0.195–0.39	50–100	**1.56–3.125**	3.125	**6.25**
Burkholderia cenocepacia HI2424	Bcen2424_2356	**1.56**	25	**1.56–3.125**	**12.5–25**	**6.25**
Pseudomonas protegens Pf-5	PFL_4558	**1.56**	12.5-25	0.39–0.78	3.125–6.25	3.125
Pseudomonas syringae pv. Tomato strain DC3000	PSPTO_3587	**0.78**	50	0.78	**12.5–25**	**6.25**
Pseudomonas putida KT2440	PP_3512	**0.78**	50	0.78–1.56	6.25	3.125
Enterobacter cloacae SCF1	Entcl_2273	0.39	25	**1.56**	6.25	3.125
Yokenella regensburgei ATCC 43003	HMPREF0880_01962	**0.78**	50	0.78–1.56	3.125–6.25	3.125
Klebsiella pneumoniae 342	KPK_0842	**0.78**	25	0.78–1.56	**12.5**	3.125
Salmonella enterica subsp. *enterica* serovar Typhi strain ct18	STY3166	0.195	25–50	0.39	6.25	3.125
Escherichia coli TW07793	ECTW07793_0407	0.39–0.78	50	0.78	6.25–12.5	**6.25**

[a] None of the cloned BTP family genes conferred reproducible resistance to tetracycline, chloramphenicol, tetraphenylphosphonium, ethidium, Hoechst 33342, pyronin Y, acridine yellow, or 4′,6-diamidino-2-phenylindole.
[b] The values given are from at least two independent biological replicates, and those indicating reproducible increases in resistance are shown in boldface. Abbreviations: CH, chlorhexidine; DQ, dequalinium; BK, benzalkonium; PF, proflavine; AF, acriflavine.
[c] NA, not applicable.
[d] The GenBank protein ID is given for the *Tepidiphilus margaritifer* DSM 15129 protein.
[e] The cloned *Roseovarius* sp. 217 gene ROS217_23162 contains a single base change that results in a serine-to-phenylalanine mutation at position 139 (C-terminal tail).

Pf-5 (PFL_4558), and *B. cenocepacia* HI2424 (Bcen2424_2356) were able to confer resistance to chlorhexidine, whereas the phylogenetically distinct (Fig. 1) proteins from *A. baumannii* (A1S_1503) and *P. protegens* (PFL_4585) did not confer resistance (1). Chlorhexidine MIC analyses were conducted as previously described (8) to gauge the level of resistance provided by the 17 newly cloned BTP domain protein homologs. These assays were conducted in medium containing 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce basal levels of expression. Among the 24 cloned genes, we saw reproducible increases in chlorhexidine resistance from 12 genes (Table 1). This result confirmed that chlorhexidine is a common substrate of this group of transporters.

BTP domain proteins are multidrug efflux systems. To explore the possibility that BTP domain proteins represent a new family of multidrug efflux transporters, we tested their capacities to confer resistance to a range of additional antimicrobial compounds in MIC analyses. These compounds included: the biocides dequalinium, tetraphenylphosphonium, and benzalkonium, the antibiotics tetracycline and chloramphenicol, and a number of fluorescent antimicrobial dyes that are common substrates of multidrug efflux systems, such as proflavine, acriflavine, ethidium, Hoechst 33342, pyronin Y, acridine yellow, and 4′,6-diamidino-2-phenylindole (Table 1).

Resistance to a variety of antimicrobials was common, particularly among the *Pseudomonas* and betaproteobacterial genes (Table 1). Six BTP domain protein genes conferred reproducible resistance to the biocide benzalkonium. Additionally, two *Pseudomonas* proteins, PA14_26850 and PFL_4585, mediated resistance to dequalinium. Notably, PFL_4585 is a paralog of the chlorhexidine resistance protein PFL_4558, demonstrating that both BTP domain proteins encoded by *P. protegens* Pf-5 mediate resistance to selected biocides. Nine of the BTP domain protein genes also conferred resistance to one or both of the DNA-intercalating antimicrobial dyes acriflavine and proflavine (Table 1). Only 6 genes out of the 24 examined in this study did not provide resistance to any of the antimicrobials tested. Of these, three were not expressed at levels detectable by Western blotting (see Fig. S1 in the supplemental material). The *V. parahaemolyticus* gene VP1155 provided the highest and most consistent increases in resistance to the greatest number of compounds, with at least 4-fold increases in resistance to chlorhexidine, benzalkonium, proflavine, and acriflavine. To determine whether resistance mediated by these proteins was dependent on a TolC-like outer membrane, we examined resistance mediated by AceI and VP1155 in a TolC-inactivated background (10). Both of these proteins mediated resistance in this mutant strain.

To explore further the substrate recognition profile of the VP1155 protein, we conducted Biolog OmniLog Phenotype microarray (PM) experiments (11). The resistance levels of *E. coli* BL21 carrying pTTQ18-VP1155 were compared to those of *E. coli* BL21 carrying pTTQ18 for 240 different antimicrobials in the

PM11-20 plate series, as previously described (1, 11). These tests confirmed that VP1155 provides resistance to the substrates identified by conventional MIC assays: chlorhexidine, proflavine, and acriflavine (see Fig. S2 in the supplemental material; benzalkonium is not included in the PM11-20 panel of compounds). Furthermore, the Biolog PM tests indicated that VP1155 also provides resistance to 9-aminoacridine, domiphen bromide, 3,5-diamino-1,2,4-triazole (guanazole), and plumbagin (see Fig. S2).

Fluorimetric transport assays demonstrate efflux mediated by VP1155. The observations of resistance to the DNA-intercalating antimicrobial dyes proflavine and acriflavine from several of the BTP domain protein genes presented the opportunity to assay directly the efflux of these substrates in real-time fluorimetric transport assays. We applied these assays to cells expressing VP1155, exploiting the capacity of this protein to mediate resistance to both dyes. The assays were conducted in the *E. coli* triple deletion mutant strain BW25113 (Δ*acrB* Δ*emrE* Δ*mdfA*::*kan*), which is defective in the three major *E. coli* multidrug efflux system genes (12) and provides a sensitive background for these assays. Cells carrying the pTTQ18-VP1155 expression plasmid were assayed both pre- and postinduction of VP1155 expression by using 0.2 mM IPTG (see Fig. S3 in the supplemental material). Cells carrying "empty" pTTQ18 treated with IPTG were also included as a negative control. The transport assays were conducted essentially as described previously (13), except that cells were grown in glycerol-supplemented medium and reenergized by using glycerol to initiate transport from substrate-loaded cells.

The fluorescence intensity of both proflavine and acriflavine is lower when intercalated into DNA, so efflux from the cell was characterized by an increase in fluorescence over time. In our transport experiments we observed a rapid increase in fluorescence in cells that expressed the VP1155 protein but not in control cells lacking this protein (Fig. 2; see also Fig. S3 in the supplemental material). These results provide additional evidence that efflux is the mechanism of resistance operating in this group of proteins.

Conclusions. In this study, we examined a large panel of genes encoding proteins related to the AceI chlorhexidine efflux system for their ability to confer resistance to a set of 12 different biocides, antibiotics, and antimicrobial dyes. To facilitate this broad survey of phylogenetically diverse proteins, we adopted a synthetic cloning approach; the majority of genes were codon optimized for expression in *E. coli* and chemically synthesized for cloning into our expression system. Of the 24 transport proteins studied, 21 were expressed at levels detectable in Western blot assays of whole-cell lysates, and 18 conferred resistance to one or more antimicrobial compounds.

Our results demonstrate that this group of proteins is a new family of bacterial multidrug efflux systems, which we have designated the proteobacterial antimicrobial compound efflux (PACE) family. The PACE family is only the sixth family of bacterial multidrug efflux systems to have been described and the first new family for more than 15 years. Multidrug efflux systems encoded by nosocomial pathogens are particularly problematic. Antimicrobial pressures selecting for increased expression of multidrug exporters can promote resistance to not only the selecting compound but also to a swath of otherwise-effective compounds.

Using a radiolabeled substrate, we previously demonstrated that the AceI protein within the PACE family is able to mediate the active efflux of chlorhexidine. In this work, we have identified fluorescent substrates for several members of the PACE family that facilitate the development of rapid fluorimetric efflux assays. These assays will be highly valuable in future studies to define the molecular transport mechanism operating in members of this family, including the mode of energization, which is likely to involve an electrochemical gradient.

Notably, PACE family proteins are encoded in the core genomes of many proteobacterial species that are separated by hundreds of millions of years of evolution. Given that the substrates we have now defined for these efflux systems—chlorhexidine, de-

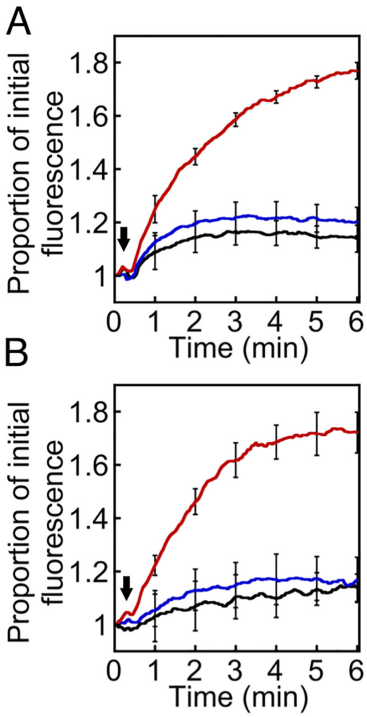


FIG 2 Acriflavine and proflavine efflux mediated by VP1155. *E. coli* BW25113 Δ*acrB* Δ*emrE* Δ*mdfA*::kan cells carrying pTTQ18 only (black line) or pTTQ18-VP1155 (blue [noninduced] and red [induced]) were grown in Luria-Bertani medium containing 0.5% glycerol to an optical density at 600 nm of 0.6. Samples of pTTQ18-VP1155 cells were taken and assayed as noninduced controls (blue lines). The cultures were then grown for a further 1 h, after which 0.2 mM IPTG was added to induce expression directed by the Ptac promoter in pTTQ18. The cells were washed in assay buffer (HEPES, pH 7.0) and loaded with 20 μM acriflavine (A) or proflavine (B) in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). The loaded cells were again washed and suspended in assay buffer (37°C). Transport was initiated with the addition of 1% glycerol at the point marked with an arrow. The assays were performed in biological triplicates, and the error bars show the standard errors of the means at 1-min intervals. Expression of the RGSH6-tagged VP1155 protein in the samples was assessed by Western blotting (see Fig. S3 in the supplemental material) and was positively correlated with efflux.

qualinium, benzalkonium, proflavine, and acriflavine—are synthetic biocides that have only been widely used within the last century, it seems unlikely that these compounds are the physiological substrates of these transporters. Nonetheless, as with other multidrug efflux systems, the substrate promiscuity of PACE efflux systems is likely to have enhanced the success of proteobacterial pathogens in clinical settings.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01982-14/-/DCSupplemental.

Figure S1, TIF file, 1.4 MB.
Figure S2, TIF file, 1.8 MB.
Figure S3, TIF file, 0.6 MB.

ACKNOWLEDGMENTS

This work was supported by a project grant from the Australian National Health and Medical Research Council (1060895) to I.T.P., K.A.H., and P.J.F.H. and the award of a Leverhulme Trust emeritus fellowship to P.J.F.H.

We thank Shimon Schuldiner from the Hebrew University of Jerusalem for the gift of *E. coli* strain BW25113 Δ*acrB* Δ*emrE* Δ*mdfA*::kan.

REFERENCES

1. **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.
2. **Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J, Sonnhammer EL, Tate J, Punta M.** 2014. Pfam: the protein families database. Nucleic Acids Res **42:**D222–D230. http://dx.doi.org/10.1093/nar/gkt1223.
3. **Tucker AT, Nowicki EM, Boll JM, Knauf GA, Burdis NC, Trent MS, Davies BW.** 2014. Defining gene-phenotype relationships in *Acinetobacter baumannii* through one-step chromosomal gene inactivation. mBio **5:**e01313-14. http://dx.doi.org/10.1128/mBio.01313-14.
4. **Coenye T, Van Acker H, Peeters E, Sass A, Buroni S, Riccardi G, Mahenthiralingam E.** 2011. Molecular mechanisms of chlorhexidine tolerance in *Burkholderia cenocepacia* biofilms. Antimicrob Agents Chemother **55:**1912–1919. http://dx.doi.org/10.1128/AAC.01571-10.
5. **Nde CW, Jang HJ, Toghrol F, Bentley WE.** 2009. Global transcriptomic response of *Pseudomonas aeruginosa* to chlorhexidine diacetate. Environ Sci Technol **43:**8406–8415. http://dx.doi.org/10.1021/es9015475.
6. **Stark MJ.** 1987. Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli*. Gene **51:**255–267. http://dx.doi.org/10.1016/0378-1119(87)90314-3.
7. **Saidijam M, Bettaney KE, Szakonyi G, Psakis G, Shibayama K, Suzuki S, Clough JL, Blessie V, Abu-Bakr A, Baumberg S, Meuller J, Hoyle CK, Palmer SL, Butaye P, Walravens K, Patching SG, O'Reilly J, Rutherford NG, Bill RM, Roper DI, Phillips-Jones MK, Henderson PJ.** 2005. Active membrane transport and receptor proteins from bacteria. Biochem Soc Trans **33:**867–872. http://dx.doi.org/10.1042/BST0330867.
8. **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.
9. **Hassan KA, Xu Z, Watkins RE, Brennan RG, Skurray RA, Brown MH.** 2009. Optimized production and analysis of the staphylococcal multidrug efflux protein QacA. Protein Expr Purif **64:**118–124. http://dx.doi.org/10.1016/j.pep.2008.11.009.
10. **Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol **2:**2006.0008. http://dx.doi.org/10.1038/msb4100050.
11. **Mackie AM, Hassan KA, Paulsen IT, Tetu SG.** 2014. Biolog phenotype microarrays for phenotypic characterization of microbial cells. Methods Mol Biol **1096:**123–130. http://dx.doi.org/10.1007/978-1-62703-712-9_10.
12. **Tal N, 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. http://dx.doi.org/10.1073/pnas.0902400106.
13. **Turner RJ, Taylor DE, Weiner JH.** 1997. Expression of *Escherichia coli* TehA gives resistance to antiseptics and disinfectants similar to that conferred by multidrug resistance efflux pumps. Antimicrob Agents Chemother **41:**440–444.
14. **Ronquist F, Huelsenbeck JP.** 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. BioInformatics **19:**1572–1574. http://dx.doi.org/10.1093/bioinformatics/btg180.

Chapter 3: Regulation of the *aceI* multidrug efflux pump gene in *Acinetobacter baumannii*

Regulation of the *aceI* multidrug efflux pump gene in *Acinetobacter baumannii*

Qi Liu, Karl A. Hassan, Heather Clift, Hasinika K. A. Hewawasam Gamage, Liping Li, Bridget C. Mabbutt and Ian T. Paulsen*

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

*Corresponding author: Prof. Ian Paulsen, Department of Chemistry and Biomolecular Sciences, Macquarie University, North Ryde, NSW, 2109, Australia. Ph: +61 2 98508152; Fax: +61 2 98508313; Email: ian.paulsen@mq.edu.au.

J Antimicrob Chemother
doi:10.1093/jac/dky034

Journal of Antimicrobial Chemotherapy

Regulation of the *aceI* multidrug efflux pump gene in *Acinetobacter baumannii*

Qi Liu, Karl A. Hassan, Heather E. Ashwood, Hasinika K. A. H. Gamage, Liping Li, Bridget C. Mabbutt and Ian T. Paulsen*

Department of Chemistry and Biomolecular Sciences, Macquarie University, North Ryde, NSW, Australia

*Corresponding author. Tel: +61-2-98508152; Fax: +61-2-98508313; Email: ian.paulsen@mq.edu.au

Received 11 October 2017; returned 28 November 2017; revised 22 December 2017; accepted 15 January 2018

Objectives: To investigate the function of AceR, a putative transcriptional regulator of the chlorhexidine efflux pump gene *aceI* in *Acinetobacter baumannii*.

Methods: Chlorhexidine susceptibility and chlorhexidine induction of *aceI* gene expression were determined by MIC and quantitative real-time PCR, respectively, in *A. baumannii* WT and Δ*aceR* mutant strains. Recombinant AceR was prepared as both a full-length protein and as a truncated protein, AceR (86–299), i.e. AceRt, which has the DNA-binding domain deleted. The binding interaction of the purified AceR protein and its putative operator region was investigated by electrophoretic mobility shift assays and DNase I footprinting assays. The binding of AceRt with its putative ligand chlorhexidine was examined using surface plasmon resonance and tryptophan fluorescence quenching assays.

Results: MIC determination assays indicated that the Δ*aceI* and Δ*aceR* mutant strains both showed lower resistance to chlorhexidine than the parental strain. Chlorhexidine-induced expression of *aceI* was abolished in a Δ*aceR* background. Electrophoretic mobility shift assays and DNase I footprinting assays demonstrated chlorhexidine-stimulated binding of AceR with two sites upstream of the putative *aceI* promoter. Surface plasmon resonance and tryptophan fluorescence quenching assays suggested that the purified ligand-binding domain of the AceR protein was able to bind with chlorhexidine with high affinity.

Conclusions: This study provides strong evidence that AceR is an activator of *aceI* gene expression when challenged with chlorhexidine. This study is the first characterization, to our knowledge, of a regulator controlling expression of a PACE family multidrug efflux pump.

Introduction

Acinetobacter baumannii has emerged as a major hospital-acquired opportunistic human pathogen, responsible for a range of infections including those of the respiratory tract, urinary tract and blood.[1] The success of *A. baumannii* in the hospital setting stems at least partly from its resistance to almost all commonly used antibiotics. Many of the resistance determinants carried by *A. baumannii* have been acquired horizontally. Indeed, *A. baumannii* strain AYE carries an 86 kb drug-resistance island, the largest known drug-resistance island to have been identified in a bacterial genome.[2] In addition to these acquired drug-resistance factors, *A. baumannii* carries intrinsic resistance genes in its core genome, including a large number of putative drug efflux pumps. Five families of efflux pumps have been extensively characterized in bacteria:[3–5] major facilitator superfamily; resistance nodulation-cell division superfamily; small multidrug resistance family; ATP-binding cassette superfamily; and multidrug and toxic compound extrusion family. Representatives of all these families have been identified in *A. baumannii*.[6] Recently, we have defined a sixth family of multidrug efflux pump, the proteobacterial antimicrobial compound efflux (PACE) family, exemplified by the AceI protein from *A. baumannii*.[7,8] Members of this family confer resistance to biocides including chlorhexidine, benzalkonium, acriflavine and proflavine.

Multidrug efflux pumps are frequently encoded adjacent to a divergently transcribed regulatory protein that controls expression of the pump gene in response to its substrates. For example, expression of the major facilitator superfamily efflux pump gene *qacA* in *Staphylococcus aureus* is regulated by a divergently encoded transcriptional regulator, QacR, a member of the TetR family of repressors.[9] Another example is the LysR-type transcriptional regulator (LTTR) family protein AdeL, which is encoded opposite to the *adeFGH* operon, and regulates expression of these genes, which encode a resistance nodulation-cell division efflux system in *A. baumannii*.[10]

Table 1. Bacterial strains and plasmids used in this study

	Reference/source
Strain	
A. baumannii ATCC 17978[a]	33
A. baumannii AB5075-UW	34
AB5075-Δ*aceI* (ABUW1673_204: T26)	34
AB5075-Δ*aceR* (ABUW1672_127: T26)	34
E. coli DH5α	
Plasmid	
pTTQ18$_{RGSH6}$	19,20
pTTQ18$_{RGSH6}$-*aceR* (amp^R)	this work
pTTQ18$_{RGSH6}$-*aceRt* (amp^R)	this work
pWH1266	21,22
pWH1266-*aceR* (Tet^R)	this work

[a]GenBank accession no. NC009085.

The *aceI* gene in *A. baumannii*, which confers chlorhexidine resistance, is located adjacent to a divergently transcribed regulator gene encoding an LTTR family protein. LTTRs are the largest family of prokaryotic transcriptional regulators.[11] LTTRs can function as activators or repressors of single or operonic genes or as global regulators, controlling expression of large numbers of genes across the genome.[11] LTTRs regulate gene products involved across diverse processes, including metabolism, cell division, virulence, motility, toxin production, attachment and secretion.[11,12]

LTTR proteins are organized with an N-terminal DNA-binding domain (DBD) connected via a helical linker to an effector-binding domain (EBD) at the C-terminus. Although LTTRs have well-conserved structures, they tend to display low sequence identity between family members possibly because of evolving distinct effector recognition. The DBD has the greatest sequence conservation, owing to its winged helix–turn–helix (HTH) motif and the linker helix.[13,14] The more diverse EBD comprises two α/β subdomains held together by hinge-like β-strands;[15,16] generally, effector molecules bind in the cleft between these two subdomains. Binding results in a conformational change that alters the affinity of the LTTR for the promoter region DNA to enable transcriptional regulation.[17,18]

The present study determines the regulation of the *aceI* transporter gene and demonstrates a divergently transcribed LysR family regulator, which we have named AceR, to be responsible for transcriptional activation of *aceI* expression in response to chlorhexidine.

Materials and methods

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *A. baumannii* strains were cultivated in LB or Mueller–Hinton broth, respectively, at 37°C with aeration. When necessary, media were supplemented with the antibiotics ampicillin (100 mg/L) or tetracycline (30 mg/L).

Both full-length (*aceR*) and truncated (*aceRt*; see description in the text below) genes were cloned into the plasmid vector pTTQ18$_{RGSH6}$ for overexpression in *E. coli* DH5α, as described previously.[6,19,20] The full-length *aceR* gene was additionally cloned into the plasmid pWH1266 for complementation of the *A. baumannii* AB5075-UW Δ*aceR* mutant strain, as previously described.[21,22] Primers utilized for cloning are listed in Table S1 (available as Supplementary data at *JAC* Online). Sanger sequencing was used to verify all cloned genes.

MIC determination assays

MIC determination assays for chlorhexidine resistance were conducted on *A. baumannii* strains in Mueller–Hinton broth using a conventional 2-fold serial dilution method, as previously described.[23] All MIC values were determined in triplicate.

Preparation of recombinant AceR and AceRt

The recombinant proteins AceR and a truncated variant AceRt were overexpressed as C-terminal His-tag fusion proteins in *E. coli* DH5α. Aliquots (4 mL) from overnight cultures grown at 37°C were added to growth medium (LB broth, 400 mL) and shaken at 37°C until cell density readings achieved $OD_{600} = 0.4$. Expression was induced with IPTG (1.6 mM) and the growth temperature lowered to 20°C. After 24 h, cells were centrifuged and pellets resuspended in Buffer A [20 mM Tris-HCl buffer (pH 8.0) with 10% glycerol and 400 mM NaCl]. Following addition of DNase I, cells were lysed using a French press (two passages, 20 000 psi). Unlysed cells and debris were removed by centrifugation (20 000 **g**, 30 min) and the recovered supernatant filtered by syringe (0.4 μm).

Purification of recombinant products was undertaken by Ni-NTA affinity chromatography. Filtrate (25 mL) was loaded (peristaltic pump) at 4°C onto a pre-packed column (1 mL; GE Healthcare) equilibrated in Buffer A with 5 mM imidazole. Following washing (2 times, 10 mL) with Buffer A with 20–60 mM imidazole, the protein product was eluted in Buffer A with 250 mM imidazole (5 mL). Fractions of eluate containing protein were pooled, dialysed overnight into Buffer B [50 mM HEPES buffer (pH 7.5) with 200 mM NaCl and 10% glycerol] and frozen. The purity of the recombinant product was established by SDS-PAGE and western blots with an anti-RGSH6-HRP antibody conjugate (Qiagen). Pure samples were desalted and concentrated to 400 mg/L prior to conducting binding assays [gel electrophoretic mobility shift assays (EMSAs), DNase I footprinting and protein-substrate binding].

RNA isolation

Mid-log cell cultures (5–10 mL, $OD_{600} = 0.6$) of each *A. baumannii* strain were treated with chlorhexidine (8 μM) for 30 min at 37°C, with shaking. Control cultures contained no chlorhexidine. Cell pellets were recovered (5000 **g**, 15 min) and lysed in QIAzol reagent (Qiagen). Total RNA was isolated using the RNeasy mini kit (Qiagen) and DNA removed with DNase I (TURBO DNA-free™ kit; Invitrogen). First-strand cDNA synthesis was facilitated using a reverse transcription kit (QuantiTect®; Qiagen) with 1000 ng of RNA input. All procedures followed the manufacturers' instructions.

Quantitative real-time PCR

All cDNA aliquots (1000 ng) were used in equal concentration in a quantitative real-time (qRT)–PCR reaction mix containing 5 μL of 2× GoTaq® qPCR Master Mix (Promega) and 300 nM of each primer (Table S1) and the reaction volume was made up to 10 μL with nuclease-free water. The reaction was conducted using a LightCycler 480 instrument (Roche) and the running conditions were 95°C for 2 min, 40× (95°C for 15 s, 55°C for 2 min and 72°C for 20 s) and 72°C for 10 min. All samples were analysed in triplicate and *rpoD* was used as the internal control. Relative quantification of gene expression was calculated using the $2^{-\Delta\Delta CT}$ approximation method.[24]

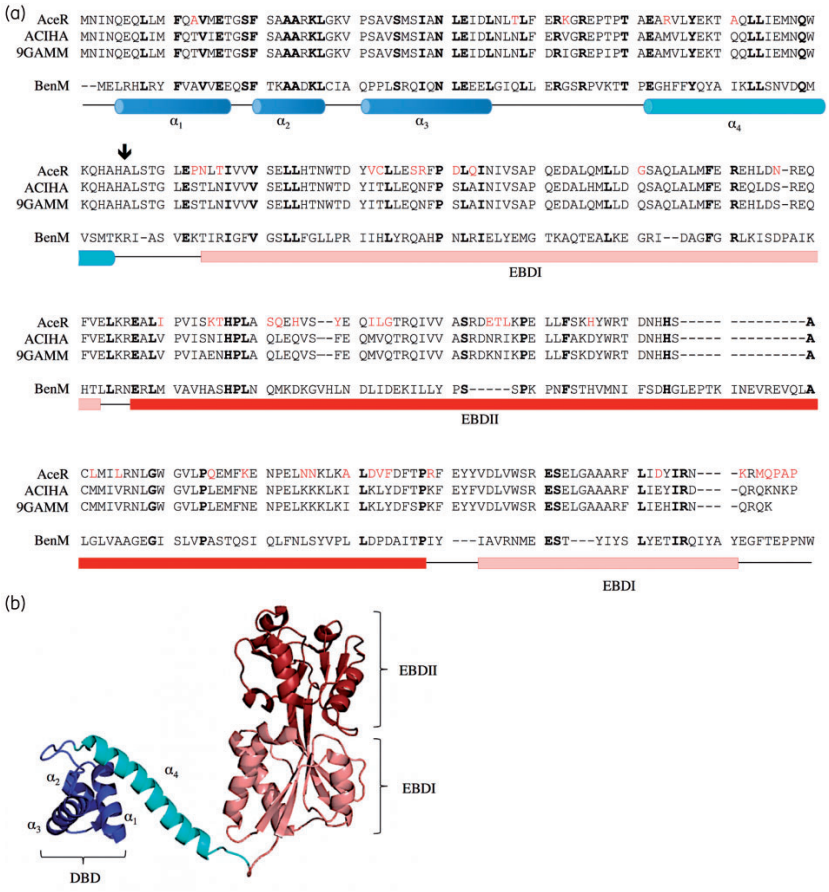


Figure 1. Proposed organization of the AceR sequence with respect to defined LTTR family member BenM. (a) Alignment of AceR with *Acinetobacter* spp. sequence representatives at the 80% identity level; N9F3E8_ACIHA from *Acinetobacter haemolyticus* CIP 64.3 T (ACIHA) and N8R3C8_9GAMM from *Acinetobacter* sp. CIP A165 (9GAMM). Residues in AceR differing from the consensus (of three sequences) are coloured red. Alignment includes the sequence of LTTR member BenM (from *Acinetobacter* sp. ADP1) whose three-dimensional structure is known (27% identity). Locations of the HTH motif (helices α_1–α_3), the linker helix (α_4) and the EBDs (subdomains EBDI and EBDII) are indicated (from crystal structure PDB 3K1N). The start residue for the AceRt construct employed in this study, in which the HTH motif is deleted, is indicated with an arrow. Orthologues identified from a BLASTP search against UniProtKB; sequences aligned with CLUSTAL.[35] (b) Ribbon view of the BenM crystal structure,[36] coloured so as to distinguish the DBD (HTH motif) (dark blue), linker helix (cyan), EBDI (pink) and EBDII (red).

Gel EMSAs

A DNA fragment containing the *aceR-aceI* intergenic region (242 bp), including the predicted *aceI* promoter was prepared by PCR amplification from the chromosome of *A. baumannii* ATCC 17978 (99% in sequence identity of *A. baumannii* AB5075-UW) using a pair of primers (Finteg and Rinteg; Table S1). The PCR products were purified using the Wizard SV gel and PCR cleanup kit (Promega). A digoxigenin gel shift kit (second generation; Roche) was used for labelling of the DNA fragments and detection of

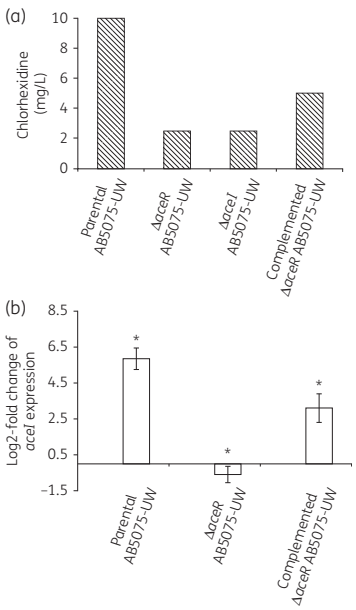


Figure 2. Effect of chlorhexidine on *A. baumannii* AB5075-UW strains. (a) Chlorhexidine susceptibility of *A. baumannii* parental AB5075-UW, AB5075-UW Δ*aceR* and AB5075-UW Δ*aceI* isogenic mutants and the AB5075-UW Δ*aceR* mutant complemented with the *aceR* gene cloned on the plasmid vector pWH1266. MICs were determined using a broth dilution method and the data shown are averaged from three biological replicates. (b) qRT–PCR assays of *aceI* gene expression following chlorhexidine induction in parental AB5075-UW, the AB5075-UW Δ*aceR* isogenic mutant and the AB5075-UW Δ*aceR* mutant complemented with the *aceR* gene cloned on the plasmid vector pWH1266. Change in expression of *aceI* gene transcription was measured as fold change normalized to *rpoD* gene expression and subsequently calculated as log2-fold change relative to the untreated cell culture. Values are averages of three independent qRT–PCR experiments. *P* values were calculated using the paired Student's *t*-test (*$P < 0.05$).

signals according to the manufacturer's instructions. Binding reactions were performed by incubating the labelled DNA fragment with 0–2 μM of the purified AceR with chlorhexidine (8 μM) for 30 min (25°C) in 10 μL of binding buffer [10 mM HEPES, pH 7.6, 0.5 mM EDTA, 5 mM $(NH_4)_2SO_4$, 0.5 mM DTT, 15 mM KCl]. After incubation, 5 μL of gel loading buffer [0.25× TBE buffer, 60% glycerol, 40% bromophenol blue, 0.2% (w/v)] was added and then the reactions were electrophoresed in a 5% native polyacrylamide gel at 100 V for 1.5 h in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). The gel was then detected and analysed according to the manufacturer's instructions. Additional EMSAs were conducted using smaller DNA fragments (32 and 37 bp) and AceR (0–6 μM) with chlorhexidine (8 μM).

DNase I footprinting assay

PCR primers (Table S1) with 5′-labelled 6-FAM (fluorescein amidite) were obtained from Integrated DNA Technologies. Labelled DNA fragments (300 ng) were prepared by PCR amplification from the chromosome of *A. baumannii* ATCC 17978 using the commercially synthesized primers (F122-fam and R122; R122-fam and F122). Both ends of the PCR product were separately labelled with 5′-labelled 6-FAM (only one 5′-labelled primer was used in each experiment) and used in separate footprinting experiments.

For DNase I footprinting, the labelled DNA fragment was incubated with the purified AceR protein (0, 4 and 8 μM) with chlorhexidine (8 μM) in 50 μL of incubation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 3 mM magnesium acetate, 0.1 mM EDTA, 0.1 mM DTT) for 30 min at room temperature. Subsequent DNA digestion was conducted by addition of 0.5 U RQ1 RNase free DNase I (Promega) to the mixture to a final volume of 100 μL. After digestion for 1 min, the digestion reaction was stopped by the addition of 100 μL of stop solution (200 mM NaCl, 20 mM EDTA and 1% SDS) followed by incubation at 65°C for 10 min. The final reaction mixtures were extracted with 200 μL of phenol/chloroform/isoamyl alcohol (25:24:1) and purified by ethanol precipitation. The purified samples were sent to the Australian Genome Research Facility for Genotyping-Fragment Separation, which then were analysed using Geneious 2 software (Biomatters).

Surface plasmon resonance

Interactions between AceRt and chlorhexidine were measured on a Biacore X100 system (GE Science) operating at a flow rate of 30 μL/min. Prior to immobilization, the purified AceRt protein (10 μM in Buffer B, see above) was diluted 1:100 into sodium acetate (10 mM, pH 4.0) and the CM5 chip activated with a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide (1:1) applied for 10 min. All measurements were performed at 25°C in Buffer B. Chlorhexidine solutions over the range 0.3–320 μM were applied for 30 s and disassociated for 120 s. Data were analysed and non-specific binding normalized using Biacore X100 evaluation software. GraphPad Prism 7 software was used for calculating the dissociation constant (K_d) of this interaction.

Tryptophan fluorescence quenching assays

Steady-state spectrophotofluorimetry was conducted on the purified protein using a spectrofluorimeter (PerkinElmer LS 55). The purified AceRt (5 μM in Buffer B) was analysed at room temperature. Tryptophan residues in AceRt were excited at 295 nm and fluorescence emissions were recorded at 330 nm. Microlitre additions of chlorhexidine in Buffer B were added to final concentrations from 0 to 114 μM and accounted for <13% of the final volume. Samples were mixed in a 1 cm × 0.2 cm quartz cell for 1 min after each addition before the fluorescence emission was monitored. All readings were corrected for buffer background emission and sample dilution. The relative fluorescence quenching (ΔI) was calculated as follows: $\Delta I = (1 - I/I_0) \times 100/100$, where I_0 is the intensity of fluorescence in the absence of quencher and I is the intensity of fluorescence upon addition of quencher. The standard deviation was calculated for the individual ΔI values from three independent experiments. K_d for the interaction was determined by non-linear regression using GraphPad Prism 7.

Results and discussion

AceR is required for chlorhexidine induction of aceI

This study focused on a putative LTTR, encoded by a gene we have named *aceR* (A1S_2064), which is divergently transcribed from the *aceI* multidrug efflux gene. A multiple sequence alignment of AceR with homologous LTTR proteins (Figure 1) suggested an extended

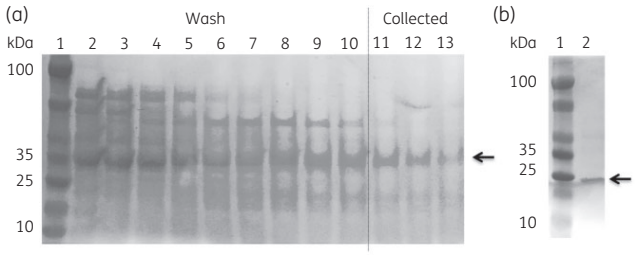


Figure 3. SDS-PAGE for immobilized metal affinity chromatography (IMAC)-purified AceR and AceRt used in this work. (a) Fractions taken during IMAC purification of recombinant AceR (arrow, 35 kDa) produced by *E. coli* DH5α cells carrying *aceR* constructs in vector pTTQ18$_{RGSH6}$. Lanes 2–10, eluates from washes by Buffer A with increasing imidazole gradient (20–60 mM, in 5 mM steps). Lanes 11–13, retained sample eluted with Buffer A and 250 mM imidazole. (b) AceRt sample (arrow, 25 kDa) following imidazole elution of IMAC column imidazole (as before). Gel stained with Coomassie brilliant blue.

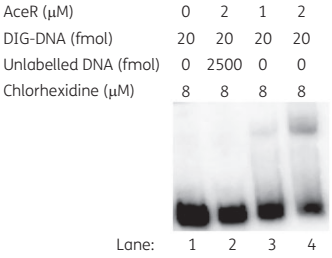


Figure 4. EMSAs showing AceR binding to the *aceI–aceR* intergenic region. Lanes 1–4, the concentration of AceR protein was 0, 2, 1 and 2 μM respectively; the concentration of DIG-labelled probe and chlorhexidine was constant at 20 fmol and 8 μM, respectively. Additionally, the sample in lane 2 includes 2500 fmol of unlabelled probe that was incubated with the AceR protein. Samples were electrophoresed on a 5% native polyacrylamide gel. DIG, digoxigenin.

N-terminal segment, thus the start codon for *aceR* is likely 105 bp upstream from the previously annotated start codon. This is supported by analysis of RNA-Seq transcriptomic data (Q. Liu, K. A. Hassan and I. T. Paulsen, unpublished data), which locates the *aceR* transcript start ~15–20 bp upstream of this new proposed start codon (Figure S1).

To investigate whether AceR plays a role in controlling the expression of *aceI*, we first compared chlorhexidine resistance levels in *A. baumannii* AB5075-UW and isogenic Δ*aceI* knockout and Δ*aceR* knockout mutants. Antimicrobial susceptibility assays (Figure 2a) showed both the Δ*aceI* and Δ*aceR* mutants to have an identical 4-fold decrease in chlorhexidine resistance relative to the parental strain. Complementation of the Δ*aceR*-inactivated mutant with the cloned *aceR* gene partially restored chlorhexidine resistance. These data suggest that AceR might function as an activator of *aceI* gene expression, since a knockout in a negative regulator would presumably lead to an increased MIC.

To investigate the role for AceR as an activator of *aceI* gene expression, we used qRT–PCR to examine the expression levels of *aceI* in the WT, Δ*aceR* mutant and complemented-Δ*aceR* mutant *A. baumannii* AB5075-UW strains with and without chlorhexidine induction. We found transcription of *aceI* in the WT strain to be induced 34-fold by chlorhexidine (Figure 2b). In contrast, *aceI* expression was not induced by chlorhexidine in the Δ*aceR* mutant strain. The chlorhexidine induction of *aceI* gene expression was partially restored in the complemented mutant strain. The most probable explanation that only partial complementation was observed in both the MIC and qRT–PCR data is that expression levels of *aceR* are likely different in the complementation experiments as the *aceR* gene was expressed from the pWH1266 plasmid rather than being reintroduced at the original locus in the *A. baumannii* Δ*aceR* mutant strain.

Preparation of the EBD of AceR

Our observations with *A. baumannii* WT and mutant strains suggested that AceR could activate expression of *aceI* in the presence of chlorhexidine. To investigate this at the protein level, recombinant AceR was prepared in both full-length and truncated forms with a C-terminal His$_6$-tag. AceR (86–299) (a 24.5 kDa protein, named here AceRt) was designed to remove the DBD and retain the EBD sequence only (Figure 1). Previous studies of other LTTRs have demonstrated EBDs to be stable domains that retain independent function.[11,25] Following purification as His$_6$-tagged fusion products, SDS-PAGE confirmed our preparations of AceR and AceRt as proteins of 35 and 25 kDa, respectively (Figure 3). In line with previous investigations of LTTRs (e.g. BenM and CatM[26,27]), solutions of full-length AceR showed lower stability than AceRt. Differential scanning fluorimetry showed that the full-length AceR (T_m = 53°C) had a lower melting temperature than that of AceRt (T_m = 60°C) and ~40% of the full-length AceR protein precipitated after removal of imidazole from the buffer.

AceR binds to the intergenic region between aceI and aceR

LTTRs frequently bind to multiple DNA sites within the intergenic region between their own coding gene and an adjacent divergently transcribed gene they regulate.[11] These binding sites are typically inverted repeats (T-N$_{11}$-A) located near the promoter

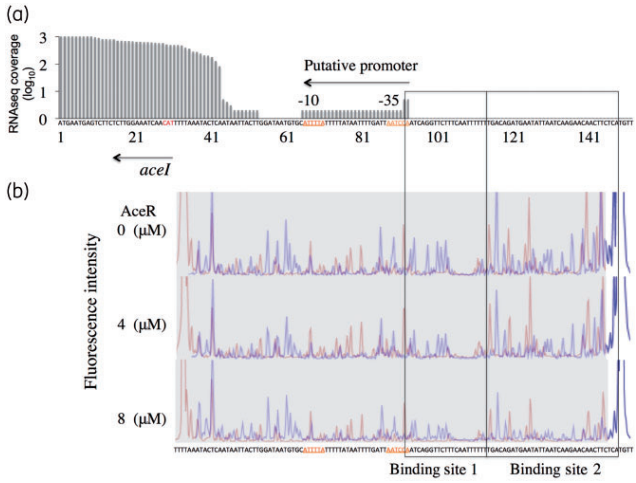


Figure 5. Identification of the AceR-binding site by a DNase I footprinting assay. (a) RNA-Seq transcriptomic data showing the transcription start site for the *aceI* transcript. *X*-axis shows the nucleotide sequence of *aceI* and its putative promoter (−10 and −35 boxes are indicated). *Y*-axis displays the relative sequence coverage from RNA-Seq data, showing that the *aceI* transcript starts 11 bp from the −10 box of the promoter. (b) DNase I footprinting with purified AceR. A DNA fragment (122 bp) of the intergenic region between *aceI* and *aceR* was PCR amplified and labelled with 5′-labelled 6-FAM. DNase I digestion reactions were prepared and analysed by capillary electrophoresis in an ABI 3730XL sequencer as described in the Materials and methods section. Three electropherograms show the reactions with the following increasing concentrations of AceR: 0, 4 and 8 μM. Electropherograms consist of overlapped blue and red nucleotide peaks, which correspond to experiments performed with DNA labelled on the *aceI* and *aceR* ends of the fragment, respectively. Protected regions are associated with decreasing peak intensity as AceR concentration increases.

region of the regulated gene, but can vary in both base pair composition and length.[11]

EMSAs were conducted using a 242 bp DNA segment encompassing the entire *aceI–aceR* intergenic region, plus 69 bp of the *aceI* gene and 14 bp of the *aceR* gene (Figure 4 and Figure S2). In the absence of AceR, a single DNA fragment of 242 bp is observed. With increasing addition of AceR, a higher molecular weight species becomes more abundant, which presumably represents an AceR-DNA complex. Specific competition with excess unlabelled probe DNA resulted in loss of the higher molecular weight species. Excess poly[d(A-T)] was used as a non-specific competitor in each reaction to prevent unspecific binding of protein to the DNA fragment. The EMSA data suggested that AceR specifically binds to the *aceI* and *aceR* intergenic region *in vitro*.

DNase I footprinting localizes the AceR binding site

DNase I footprinting was performed to localize further the AceR-binding sequence. Sequence coverage of the *aceI* and *aceR* transcripts in our RNA-Seq transcriptomic data (Q. Liu, K. A. Hassan and I. T. Paulsen, unpublished data) shows the likely transcriptional start site of the *aceI* transcript is 11 bp downstream of a putative *aceI* promoter. This promoter consists of a −10 box (TAAAAT), 17 bp spacing and then a relatively poor −35 box (TGGATT) (Figure 5a). DNase I footprinting was performed using a 122 bp DNA fragment upstream of the translation start of the *aceI* gene. Two regions experienced partial protection from DNase I digestion dependent on AceR concentration (Figure 5b). Binding site 2 includes an imperfect palindrome AGAACAACTTCT that resembles a typical LTTR-binding motif,[11] whereas binding site 1 includes a weak imperfect palindromic region AATCAGGTTCTTTCAATT that is adjacent to the likely −35 box of the *aceI* promoter.

To confirm whether AceR can bind to both sites within the *aceI–aceR* intergenic region, additional ESMAs were conducted using smaller (32 and 37 bp) DNA fragments centred on binding sites 1 and 2. AceR was inferred to bind the DNA fragments of the two binding sites in a concentration-dependent manner (Figure 6). The gel shift was abolished by competition with an excess of unlabelled specific DNA (i.e. the unlabelled putative binding site DNA). These results confirmed that AceR binds at least two distinct sites upstream of the predicted *aceI* promoter sequence, a property similar to many characterized LTTRs.[11,13] LTTRs are proposed to operate as a dimer of dimers. One dimer may first bind (in apo form) to one regulatory binding site. Subsequently, upon binding of the effector molecule, a second dimer may bind to a distinct but adjacent site to form a tetramer with the bound dimer.[28] Binding sites 1 and 2 may represent these regulatory and effector sites.

The chlorhexidine-induced binding of AceR to these sites may help to co-ordinate binding of the RNA polymerase to the *aceI* promoter. Expression of the *aceI* gene probably requires activation owing to the relatively poor −35 box consensus sequence. AceR may activate expression through interacting with the RNA polymerase holoenzyme, enhancing its binding to the promoter region.

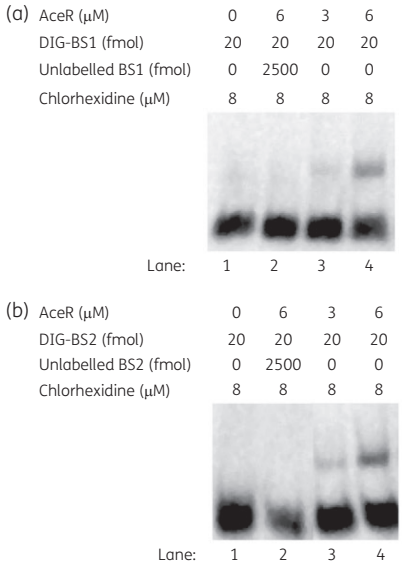


Figure 6. EMSAs showing AceR binding to two sites in the *aceI–aceR* intergenic region. Sequence of the DNA fragments for binding site 1 (a) and binding site 2 (b) are TGATTAATCC**AATCAGGTTCTTTCAATT**TTTT and GACAGATGAATATTAATCA**AGAACAACTTCT**CATGTT, respectively. DIG-labelled DNA fragments (binding sites 1 and 2, 20 fmol) as probes were commercially synthesized for the reactions in lanes 1–4 and incubated with an increasing concentration of AceR (0–6 μM), as indicated at the top of each panel. Samples in lane 2 additionally include 2500 fmol of unlabelled probe. BS1, binding site 1; BS2, binding site 2; DIG, digoxigenin.

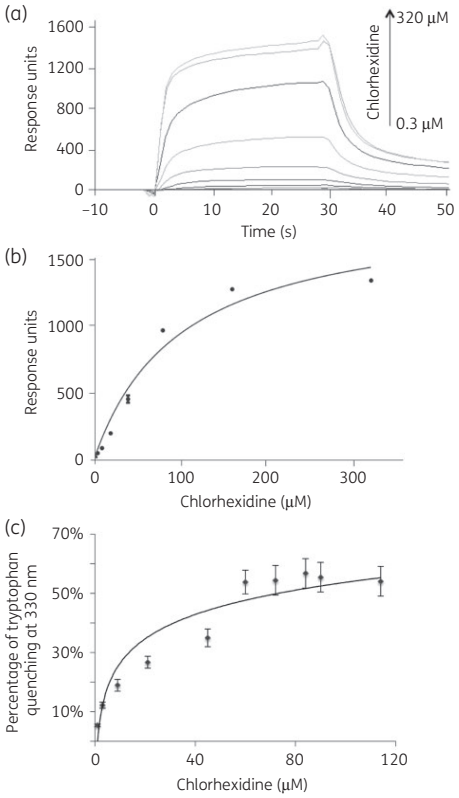


Figure 7. Binding affinity between AceRt and chlorhexidine using SPR and fluorescence quenching. (a) Sensorgrams and the saturation curve of the titration of AceRt with chlorhexidine were determined by SPR. A CM5 chip (Biacore) was coated with AceRt with 1400 response units and an increasing concentration of chlorhexidine was injected into the microfluidic channel. (b) Response units were extracted for each chlorhexidine concentration in the sensorgrams to plot an affinity curve against various chlorhexidine concentrations. (c) Tryptophan fluorescence quenching to monitor AceRt/chlorhexidine binding. Fluorescence spectra of AceRt (5 μM) upon addition of chlorhexidine were monitored at 330 nm.

EBD of AceR binds chlorhexidine

Direct monitoring of binding with chlorhexidine employed surface plasmon resonance (SPR) with the truncated AceRt protein, owing to its higher solubility than AceR. The sensorgrams showed AceRt binding responses to increase with chlorhexidine concentrations until saturation (Figure 7a). The overall K_d was calculated as 62.3 μM (Figure 7b). The binding stoichiometry of AceRt/chlorhexidine based on these results was 1:1.

To support the SPR data, a tryptophan fluorescence quenching assay was performed. Fluorescence of four tryptophan residues in the AceRt protein was monitored during chlorhexidine titration (Figure S3). Chlorhexidine quenches AceRt tryptophan fluorescence in a saturable manner with a K_d of 30.5 μM (Figure 7c). This dissociation parameter is in line with the K_d derived from our SPR assays (above). These K_d values are similar, but slightly lower than that observed for the AceI transporter (1.6∼5.8 μM).[8] Other LTTRs have affinity in the μM range for their specific inducing ligand.[17,29–31] Examples include PcpR from *Sphingobium chorophenolicum* that binds pentachlorophenol with a K_d of 70 μM[29] and CbbR from *Xanthobacter flavus* that binds NADPH with a K_d of 75 μM.[30]

Conclusions

The AceI efflux pump is the first characterized prototype of the PACE family of multidrug efflux pumps.[7,8] In this paper, we provide several lines of evidence that the novel LTTR protein AceR is an activator of *aceI* gene expression. We were also able to demonstrate direct interactions between the C-terminal domain of AceR with chlorhexidine, a known substrate of the AceI transporter.[7] Based on these data, we propose a molecular model for the regulation of the *aceI* gene (Figure 8). In this model, AceR binds to chlorhexidine inducing a conformational change, which results in two AceR dimers binding to at least two DNA sites localized upstream

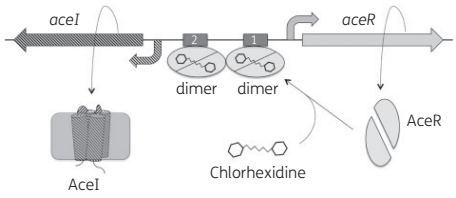


Figure 8. Proposed model of AceR transcriptional regulation of *aceI* gene expression. Following binding to chlorhexidine, two AceR dimers are proposed to bind to regulatory sites within the intergenic region between the *aceI* and *aceR* genes. AceR binding close to the *aceI* promoter results in activation of *aceI* transcription, presumably by strengthening the interaction between the RNA polymerase holoenzyme and the *aceI* promoter.

of the −35 box of the *aceI* promoter. This consequently activates expression of the *aceI* gene through enhancing binding of the RNA polymerase holoenzyme to the *aceI* promoter.

While this is the first characterization of a regulator of a PACE family efflux pump, various other bacterial efflux pump transporters have been shown to be regulated via a divergently encoded regulator that can recognize the substrate of the cognate transporter.[10,32] We believe that AceR will provide a good model system for studying the regulation of PACE family efflux pumps, which are frequently encoded adjacent to an LTTR gene. Investigating the ligands of the putative LTTR regulators of these PACE family transporters may provide valuable insight into the real physiological substrates of their efflux pumps.

Funding

Q. L. was supported by a Macquarie University International Research Excellence Scholarship, H. E. A. was supported by an Australia Research Training Programme Scholarship and I. T. P. was supported by an Australian Research Council Laureate Fellowship (FL140100021). Additional support for the work was provided by grants 1060895 and 1120298 from the National Health and Medical Research Council of Australia.

Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1–S3 are available as Supplementary data at *JAC* Online.

References

1 Dijkshoorn L, Nemec A, Seifert H. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nat Rev Microbiol* 2007; **5**: 939–51.

2 Adams MD, Goglin K, Hujer KM *et al*. Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*. *J Bacteriol* 2008; **190**: 8053–64.

3 Paulsen IT. Multidrug efflux pumps and resistance: regulation and evolution. *Curr Opin Microbiol* 2003; **6**: 446–51.

4 Piddock LJ. Multidrug-resistance efflux pumps—not just for resistance. *Nat Rev Microbiol* 2006; **4**: 629–36.

5 Li XZ, Plésiat P, Nikaido H. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clin Microbiol Rev* 2015; **28**: 337–418.

6 Li L, Hassan KA, Paulsen IT *et al*. Rapid multiplexed phenotypic screening identifies drug resistance functions for three novel efflux pumps in *Acinetobacter baumannii*. *J Antimicrob Chemother* 2016; **71**: 1223–32.

7 Hassan KA, Liu Q, Paulsen IT *et al*. Homologs of the *Acinetobacter baumannii* AceI transporter represent a new family of bacterial multidrug efflux systems. *MBio* 2015; **6**: e01982-14.

8 Hassan KA, Jackson SM, Paulsen IT *et al*. Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. *Proc Natl Acad Sci USA* 2013; **110**: 20254–9.

9 Grkovic S, Brown MH, Schumacher MA *et al*. The staphylococcal QacR multidrug regulator binds a correctly spaced operator as a pair of dimers. *J Bacteriol* 2001; **183**: 7102–9.

10 Coyne S, Rosenfeld N, Lambert T *et al*. Overexpression of resistance-nodulation-cell division pump AdeFGH confers multidrug resistance in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2010; **54**: 4389–93.

11 Maddocks SE, Oyston PC. Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. *Microbiology* 2008; **154**: 3609–23.

12 Russell DA, Byrne GA, O'Connell EP *et al*. The LysR-type transcriptional regulator VirR is required for expression of the virulence gene *vapA* of *Rhodococcus equi* ATCC 33701. *J Bacteriol* 2004; **186**: 5576–84.

13 Schell MA. Molecular biology of the LysR family of transcriptional regulators. *Annu Rev Microbiol* 1993; **47**: 597–626.

14 Aravind L, Balaji S, Iyer LM *et al*. The many faces of the helix-turn-helix domain: transcription regulation and beyond. *FEMS Microbiol Rev* 2005; **29**: 231–62.

15 Ezezika OC, Haddad S, Neidle EL *et al*. Oligomerization of BenM, a LysR-type transcriptional regulator: structural basis for the aggregation of proteins in this family. *Acta Crystallogr F Struct Biol Cryst Commun* 2007; **63**: 361–8.

16 Choi H, Kim S, Ryu SE *et al*. Structural basis of the redox switch in the OxyR transcription factor. *Cell* 2001; **105**: 103–13.

17 Devesse L, Smirnova I, Dian C *et al*. Crystal structures of DntR inducer binding domains in complex with salicylate offer insights into the activation of LysR-type transcriptional regulators. *Mol Microbiol* 2011; **81**: 354–67.

18 Bundy BM, Collier LS, Neidle EL *et al*. Synergistic transcriptional activation by one regulatory protein in response to two metabolites. *Proc Natl Acad Sci USA* 2002; **99**: 7693–8.

19 Hassan KA, Brzoska AJ, Paulsen IT *et al*. Roles of DHA2 family transporters in drug resistance and iron homeostasis in *Acinetobacter* spp. *J Mol Microbiol Biotechnol* 2011; **20**: 116–24.

20 Stark MJ. Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli*. *Gene* 1987; **51**: 255–67.

21 Jacobs AC, Hood I, Boyd KL *et al*. Inactivation of phospholipase D diminishes *Acinetobacter baumannii* pathogenesis. *Infect Immun* 2010; **78**: 1952–62.

22 Hunger M, Schmucker R, Kishan V *et al*. Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter calcoaceticus* and its use for *Escherichia coli* shuttle plasmids. *Gene* 1990; **87**: 45–51.

23 Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc* 2008; **3**: 163–75.

24 Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 2001; **25**: 402–8.

25 Lönneborg R, Brzezinski P. Factors that influence the response of the LysR type transcriptional regulators to aromatic compounds. *BMC Biochem* 2011; **12**: 49.

26 Clark T, Haddad S, Momany C *et al*. Crystallization of the effector-binding domains of BenM and CatM, LysR-type transcriptional regulators from *Acinetobacter* sp. ADP1. *Acta Crystallogr D Biol Crystallogr* 2004; **60**: 105–8.

27 Ezezika OC, Haddad S, Clark TJ *et al*. Distinct effector-binding sites enable synergistic transcriptional activation by BenM, a LysR-type regulator. *J Mol Biol* 2007; **367**: 616–29.

28 Tropel D, van der Meer JR. Bacterial transcriptional regulators for degradation pathways of aromatic compounds. *Microbiol Mol Biol Rev* 2004; **68**: 474–500.

29 Hayes RP, Moural TW, Lewis KM *et al*. Structures of the inducer-binding domain of pentachlorophenol-degrading gene regulator PcpR from *Sphingobium chlorophenolicum*. *Int J Mol Sci* 2014; **15**: 20736–52.

30 van Keulen G, Girbal L, Meijer WG *et al*. The LysR-type transcriptional regulator CbbR controlling autotrophic CO_2 fixation by *Xanthobacter flavus* is an NADPH sensor. *J Bacteriol* 1998; **180**: 1411–7.

31 Mandal RS, Ta A, Sinha R *et al*. Ribavirin suppresses bacterial virulence by targeting LysR-type transcriptional regulators. *Sci Rep* 2016; **6**: 39454.

32 Chen S, Wang H, Zhong Z *et al*. LysR family activator-regulated major facilitator superfamily transporters are involved in *Vibrio cholerae* antimicrobial compound resistance and intestinal colonisation. *Int J Antimicrob Agents* 2013; **41**: 188–92.

33 Smith MG, Gianoulis TA, Pukatzki S *et al*. New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes Dev* 2007; **21**: 601–14.

34 Gallagher LA, Ramage E, Weiss EJ *et al*. Resources for genetic and genomic analysis of emerging pathogen *Acinetobacter baumannii*. *J Bacteriol* 2015; **197**: 2027–35.

35 Goujon M, McWilliam H, Valentin F *et al*. A new bioinformatics analysis tools framework at EMBL-EBI. *Nucleic Acids Res* 2010; **38**: W695–9.

36 Ruangprasert A, Craven SH, Neidle EL *et al*. Full-length structures of BenM and two variants reveal different oligomerization schemes for LysR-type transcriptional regulators. *J Mol Biol* 2010; **404**: 568–86.

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).

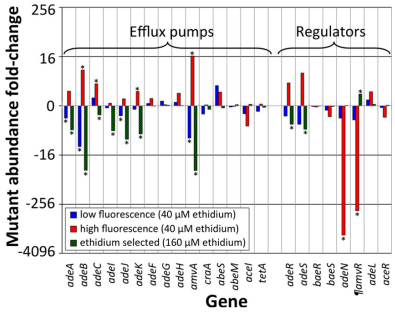


FIG 1 Selection of *A. baumannii* mutants carrying insertions in genes encoding the characterized efflux pumps AdeABC (12), AdeIJK (10), AdeFGH (20), AmvA (13, 14), CraA (21), AbeS (22), AbeM (23), and AceI (24, 25) and regulators AdeRS and BaeRS, which control expression of *adeABC* (15, 26); AdeN, 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).

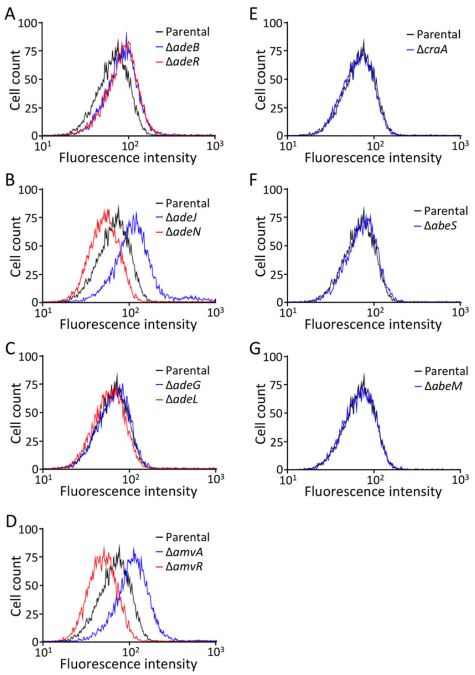


FIG 2 Flow cytometric analysis of *Acinetobacter baumannii* AB5075-UW parental strain (black), inactivated efflux pump mutants (blue), and inactivated efflux regulatory mutants (red). The fluorescence profile of the parental population is shown in all panels and is overlaid with the profiles of Δ*adeB* 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.

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-

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: Interactions between co-cultures of clinical isolates of *Acinetobacter baumannii* and *Klebsiella pneumoniae*

Abstract

The vast majority of bacteria on the planet live in polymicrobial communities, and likely engage in numerous interactions with neighbouring bacteria. However, most organisms have been primarily studied in the laboratory as pure cultures. In this study, we investigated molecular and physiological interactions between strains of the opportunistic pathogens *Acinetobacter baumannii* and *Klebsiella pneumoniae* that were co-isolated from a single respiratory infection in a patient.

Interactions between the strains were highly dependent on the culture conditions. *K. pneumoniae* predominates when grown planktonically in MH media, while both organisms co-exist planktonically in synthetic lung mimicking media, and *A. baumannii* predominates when grown in a biofilm. Biolog phenotype microarray analysis shows the two bacteria have very different carbon catabolite capabilities, suggesting that they pursue very different ecological strategies. Cross-feeding experiments in solid and liquid media show that *A. baumannii* can utilise metabolites secreted by *K. pneumoniae* as a sole carbon source for growth. RNA-Seq transcriptomics of the two strains co-cultured in a biofilm identified transcriptional changes involved with bacterial surface structures, amino acid catabolism and transport, and biofilm formation. The cross-feeding and biofilm experiments suggest that the predominance of *A. baumannii* in mature stage biofilms may be due to an ability to use lactate and/or ethanol secreted by *K. pneumoniae* when other nutrients have been exhausted.

We believe this represents the first view of interactions between two bacteria that have been co-isolated from a single patient, and that likely actually interacted with each other in a real world setting.

Introduction

Acintobacter baumannii and *Klebsiella pneumoniae* are opportunistic human pathogens involved in a range of infections such as respiratory, urinary tract and, blood infections, especially in immune-compromised patients. Both of these pathogens pose significant clinical problems due to their high level of multidrug resistance. *A. baumannii* and *K. pneumoniae* have been classified as members of the ESKAPE pathogens, the six top priority dangerous multidrug resistant microorganisms (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniea, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) by the Infectious Diseases Society of America [1].

Infections caused by co-colonization of multidrug resistance (MDR) bacteria are associated with remarkable morbidity and mortality, and continue to increase in frequency [2-5]. Co-colonization with various antimicrobial resistant bacteria has been well documented with prevalence percentages range from 1-11% [2, 4]. Rates of co-infection with MDR *Acinetobacter* spp. and extended spectrum β-lactamase producing microorganisms can go up to 38% of patients [6].

There is only limited literature regarding the prevalence, epidemiological characteristics, and clinical outcomes of intensive care unit (ICU) patients co-infected by *A. baumannii* and *K. pneumoniae*. Dual-infections by *A. baumannii* and *K. pneumoniae* have been shown to be a frequent event, involving 40% of 86 patients who tested positive for carbapenem-resistant Enterobacteriaceae (CRE) in an ICU in one study [7]. Co-infected patients have been described to be older and to have more severe underlying chronic diseases than individuals with single infections [7, 8].

Most of the bacteria are in dense and diverse biofilm communities, which can be surface-bound or free-floating and are usually represent spatially structured communities encased within a polymer matrix [9, 10]. Bacterial biofilms are increasingly recognised as the cause of remarkable persistence of pathogenesis in many infections such as respiratory infections [11, 12]. Bacterial interspecies interactions, such as cooperation(s) or competition(s), are commonly found in polymicrobial biofilms [13].

Synergistic interactions in microbial biofilm communities have been previously demonstrated. For example, *Acinetobacter sp* strain 6 was able to cross-feed *Pseudomonas putida* when grown on benzyl alcohol as the sole carbon source. The *Acinetobacter* strain metabolized benzyl alcohol and partially secreted benzoate, which *P. putida* was able to use as a carbon source [14]. Relationships within a biofilm community can also be competitive. For example, clinical studies have found that the abundance of *Streptococcus oligofermentans* and *Streptococcus mutans* in dental plaque are inversely correlated, suggestive of an antagonistic relationship. *S. mutans* secretes lactic acid which inhibits the growth of many oral bacteria, however, *S. oligofermentans* is able to degrade lactic acid to hydrogen peroxide, which inhibits the growth of *S. mutans* [15].

Mixed species biofilms can have clear spatial distribution patterns. For example, a dual-bacteria biofilm of *K. pneumoniae* and *P. aeruginosa* forms a distinctive organization with *P. aeruginosa* forms a base structure, while *K. pneumoniae* forms a tower-like structure on the top [16]. A similar structural arrangement has been observed with *P. aeruginosa* and *Agrobacterium tumefaciens* biofilms, except with *A. tumefaciens* forming the base structure [17].

We obtained clinical isolates of *A. baumannii* (AB6870155) and *K. pneumoniae* (KP6870155) that were co-isolated from a single respiratory infection in a patient injured during the Bali bombing in 2002. The aim of this study was to evaluate potential molecular and phenotypic interactions, both in planktonic cultures and in biofilms, underlying the co-existence of these two clinical isolates that came from a co-infection from a single patient.

Materials and Methods

Bacteria strains and growth conditions

AB6870155 and KP6870155 strains were co-isolated together from a lung infection of the patient injured in the Bali bombing in 2002. All of cell culture incubations were performed at 37º C. Unless other wise stated, for all of the experiments performed in this study, cells were collected in mid-log phase after being subcultured from overnight cell cultures. Muller-Hinton (MH) medium and synthetic lung mimicking medium (SLMM) [18] were used as media in various experiments, unless otherwise indicated.

Genome sequencing and annotation

The genome sequencing of AB6870155 was performed by using Illumina Mate Pair Sequencing with 5~5.9 kb randomly sheared fragments library. For KP6870155, the genome sequence was obtained using ¼ plate 454 Life Sciences (Roche) high-density pyrosequencing method. Sequencing reactions were performed at the Ramaciotti Centre for Gene Function Analysis (Sydney, Australia). The sequenced fragments were assembled *de novo* into large contigs by Mimicking Intelligent Read Assembly 3 [19] for AB6870155 and Roche Newbler software at the Ramaciotti Centre for KP6870155. Automated genome functional annotation was performed using the RAST Serve [20].

Comparative genomics

Complete genome sequences and annotations of major previously sequenced *A. baumannii* (ATCC17978, AB307, ACICU, AYE, AB0057, SDF, MDR-ZJ06, BJAB07104, BJAB07105, BJAB0868, D1279779, MTR_TJ, TCDC_AB0715, TYTH_1, ZW85_1) and *K. pneumoniae* strains (KP342, HS11286, KCTC2242, MGH78578, NTUH_K2044, KP1084, KP_CG43, KP_JM45, KP_rhinoscleromatis_SB3432) were downloaded from the NCBI database (ftp://ftp.ncbi.nlm.nih.gov.GenBank/genomes/Bacteria/), and used in comparative genomics analyses. The BLASTP algorithm [21] was used to identify orthologs in different *A. baumannii* and *K. pneumoniae* isolates, respectively, with a cut-off of $1e^{-5}$ and over 80% coverage in length.

Cell enumeration in mono- and mixed planktonic cultures

Aliquots (50 µl) of each the two strains from the overnight cultures, were added to fresh growth medium (MH medium, 5 ml) with shaking till cell density readings reached OD_{600}: 0.6. Then, the AB6870155 cell culture was diluted in 5: 3 in fresh MH medium. Subsequently, both of the KP6870155 and the diluted AB6870155 were serial diluted (1: 4 x 10^5) in fresh MH medium. Followed the dilution, the final diluted cell culture was plated on a LB plate and incubated for 12~15 hours (37 ºC). Cell enumeration and counting of colony forming units (CFUs) for each strain was performed with 3 replicates. The genome of AB6870155 does not contain genes that encoding β–galactosidase whereas the KP6870155 genome harbours several copies of this gene. The presence of β–galactosidase can be detected by X-gal/IPTG, which can form blue precipitant within cells resulted in blue colonies. Thus, plating the cells onto a LB agar plate containing X-gal/IPTG can differentiate the two co-isolated strains by different colours.

Co-culturing

Overnight cell cultures (100 µl) of the two microorganisms were subcultured into fresh MH medium (10 ml), respectively, until the cell density achieved OD_{600}: 0.6. Subsequently, the subcultured cells were diluted as described in above section, to obtain similar colony-forming units per millilitre (CFUs/ml) of the both strains. Aliquots of the diluted cells of each strain were separately cultured and co-cultured together in fresh MH or SLMM (1:50), followed by incubation with shaking at 37 ºC. The separately cultured and co-cultured cells (20 µl) were collected every hour for the first 6 hours, the 21st hour, the 23rd hour and the 50th hour, respectively. All the cell culture collections were serial diluted (1: 4 x 10^6), respectively. Eventually, 20 µl per drop of the final diluted cell culture was placed on the LB containing X-gal/IPTG plates and dried in RT followed by incubation for 12~15 hours. Counting of CFUs for each strain was determined with 3 replicates.

Biofilm quantitation assays in microtitire trays and in flow-cells

Biofilm formation assays were performed according to previous described method [9], with some alterations. Approximate equal amount of CFUs of the two strains were grown in 96-well polystyrene microtitre trays (100 µl/well) as individual cultures and as a mixed culture (half the amount cells of the each individual strain) for overnight incubation with shaking (100 rpm). Adherence of the resulting biofilm was washed twice with 100 µl PBS (phosphate buffered saline), fixing with 100 µl of ethanol (100%) for 1 min, and staining with 100 µl crystal violet (0.4% in 12% ethanol) for 10 minutes. Quantification of the biofilm was carried out by measuring the amount of crystal violent retained in the wells (at an absorbance of 595 nm) with a Fluorostar Omega spectrometer (BMG Labtech, Offenburg, Germany). The results represent the average of three independent experiments.

Biofilms were cultivated in flow-cell chambers as described previously [22] with some modifications. Approximate same amount of CFUs of the two strains were inoculated

individually or mixed together to the flow-cell chambers (Biocentrum-DTU, individual channel dimensions: 1 x 4 x 40 mm), covered with a microscope glass coverslip, which served as a substratum for biofilm attachment. After inoculation of the bacterial suspension (500 µl) into the flow chamber, cells were allowed to incubate for 1 hour at 37 ºC, after which MH medium was pumped into the flow-cell at a constant rate of 0.07 ml/min using an IPC-12 high precision peristaltic pump (Ismatec).

Biofilms were prepared for laser scanning confocal microscopy (LSCM) after 3 days of growth by staining with the Live/Dead BacLight bacterial viability probe (Molecular Probes®) prepared according to the manufacturer's protocol. A working concentration (500 µl) of the probe was injected gently into the flow-cell chambers to minimize disruption of the biofilms. The biofilms were stained for 15 minutes after which the excess dye was removed by restarting the flow of the growth medium and flushing for 30 minutes.

Microscopic observation and images acquisition of biofilms were performed by using an FV1000 confocal microscope (Olympus) equipped with argon and helium-neon lasers providing 488 and 543 nm excitation wavelengths, respectively. 510 nm to 530 nm interference and 610 nm long pass filters were used for emission. The captured biofilm images were further processed using Imaris software (Bitplane).

Biolog phenotype microarray assays for carbon source utilisation

Biolog phenotype microarrays (PMs) were performed as per the manufacturer's instruction [23], to identify compounds that could serve as sole carbon source for AB6870155 and KP6870155 (PM1-2; 190 compounds). PMs employ tetrazolium dye which reduction by bacteria respiratory enzymes serves as an indicator of active metabolism. Reduction of the dye causes formation of a purple colour that is recorded by a camera at defined time intervals, providing quantitative and kinetic information about response of cells in the PM plates.

After inoculation of the cells into the PM plates, plates were subsequently incubated in an Omilog inculbator/reader (Biolog) aerobically for 48 h at 37 ºC and monitored automatically in every 15 minutes for colour changes in the each well. Readings were recorded for 48 hours, and data was analysed with the Omnilog-PM software, which generated a time-course curve for colour formation. An average height threshold of 101 arbitrary omnilog units (AOUs) was chosen to identify the carbon sources used by the strains.

Cross-feeding on solid media

The two co-isolated organisms were grown on a Luria Bertani agar plate for overnight, respectively. Then, the cell colonies were removed using a sterile swab and washed by M9 salts solution for three times (Sigma-Aldrich®). The washed cell suspensions were diluted in M9 salts solution to achieve the cell density of OD_{600}: 0.2, which were subsequently streaked on a Petri dish plate containing solid M9 minimal medium (200ml/L sterile 5 x M9 salts solution, 12 g/L agar, 2 ml/L 1 M $MgSO_4$, 100 µl/L $CaCl_2$) with 20 mM of a single carbon source (sucrose or glycerol or mannose or maltose or serine or galactoase or tween 80 or ornithine or glucose-6-phosphate). Finally, bacterial growth was assessed after 24 hours incubation.

Cross-feeding experiments using Millicell hanging cell culture inserts

Aliquots (50 µl) of the co-isolated bacteria from the overnight cultures, were inoculated into fresh MH medium with shaking to achieve the cell density of OD_{600}: 0.6, respectively. The KP6870155 then was inoculated into Millicell hanging insert containing a sole carbon source. The insert only allowed the passage of small-secreted metabolites but not the bacterial cells. The AB6870155 was directly inoculated into 6-well plates which containing the single carbon that cannot be used by it and subsequently combined with inserts containing the KP6870155. The assembled 6 well plates were incubated for 24 hours with shaking. Fluorostar Omega

spectrometer (BMG labtech) was used to determine the growth of AB6870155. Each carbon source was assessed in triplicates.

Transcriptomic analysis of mono- and co-cultures

Cells of the two bacteria strains were collected at 24 hours as individually and as mixed biofilm in 6-well microtiter plates followed by washing with PBS buffer twice. Total RNA were extracted using an RNeasy® Mini Kit (Qiagen®). RNAs was quantified at 260 nm and its quality was assessed on an electrophoresis agarose gel under denaturing condition. Ribosomal RNA in the total RNA samples was then depleted by Ribo-Zero rRNA removal kit (illumina®) prior to sequencing according to the manufacture's instruction. rRNA-free RNA samples library preparation and Illumina NextSeq-500 sequencing were conducted in the Ramaciotti Centre for Gene Function Analysis (Sydney, Australia). Illumina NextSeq-500 sequencing generated 10 million paired end reads (75 bp read length) for each RNA sample.

AB6870155 sequence reads were first mapped to the *A. baumannii*-AB0057 reference genome using EDGE-pro with Bowtie2 incorporated [24]. Read counts data was normalized and differential expression (DE) analysis conducted using the DESeq R package [25]. Thus, read counts of samples were normalized for sequencing depth and distortion caused by highly differentially expressed genes. A negative binomial model was used to test the significance of differential expression between two conditions. A cutoff of FDR (False Discovery Rate) of less than 0.05 and a log2 fold change >1.0 was used to determine significantly differentially expressed genes.

Results and discussion

Comparative genomics of the co-isolated strains

Draft genome sequences of the AB6870155 and KP6870155 strains were assembled, and summary statistics is provided (Table S5.1, supplementary data). The gene content of these two strains was comparatively analysed with publicly available complete genomes of *A. baumannii* and *K. pneumoniae* strains, respectively (Table S5.2 and Table S5.3, supplementary data). Phylogenetic analyses placed AB6870155 within the International Clone I (IC1) clonal complex among strains, such as AB0057 (Figure 5.1a). The KP6870155 genome was the closest in sequence to that of a nosocomial infectious strain KCTC2242 (Figure 5.1b).

Regions of genome plasticity in the sequenced strains

Comparative genomics of *A. baumannii* and *K. pneumoniae* identified regions of DNA unique to each strain, and not found in closely related strains, which have been termed as regions of genome plasticity (RGPs) [26], that have likely been acquired by recent lateral gene transfer. AB6870155 was found to include 29 RGPs containing 410 genes, and the biggest one, AbaR-155, is 64 kb in length (Figure S5.2, supplementary data). The AbaR-155, consists of 20 genes conferring multidrug and metal resistance and is highly similar to AbaR3 island found in AB0057 [27]. KP6870155 contains 473 genes in 38 RGPs. The biggest RGP is approximately 33 kb in size and encodes proteins involved in capsular polysaccharide (CPS) biosynthesis (Figure S5.3, supplementary data). Other RGPs carry a type IV secretion system (T4SS) gene cluster (17 kb) and a pilus biogenesis gene cluster (5.3 kb). The CPS, the T4SS and the pilus all likely play a role in virulence of this strain [28-30]. There was no evidence for any recent lateral gene transfer events between the AB6870155 and KP6870155 strains.

Co-growth of AB6870155 and KP6870155 in a planktonic culture

As a first step to investigate interactions between AB6870155 and KP6870155 strains, their respective growth rates were determined in monoculture and in mixed cultures to identify differences and examine whether the strains could influence each other's growth. The initial growth rate of KP6870155 was much higher in the first 6 hours of incubation than AB6870155 when grown planktonically as monocultures in liquid Mueller Hinton (MH) media, however they produced similar number of colony forming units (CFUs) at the 21 and 50-hour in MH media. In mixed cultures, the growth rate of KP6870155 was similar to that under monoculture and co-culture conditions (Figure 5.2a). However, the growth rate of AB6870155 was much slower in the mixed culture in the first 6 hours incubation (0.5×10^8 CFUs/ml) and no cells were detectable at 21 hours compared to monoculture growth (1.3×10^9 CFUs/ml). Those results suggest that KP6870155 could completely outcompete AB6870155 when they were co-cultured planktonically in MH media.

Possible explanations for the observed competition between the co-cultured strains in MH media may be multi-factorial. Potential factors that may cause the observed competition include changes in the physio-chemical environment, such as pH, production of bacteriocins or other antibacterial toxins, and nutrient depletion [31, 32]. It has been shown that *A. baumannii* can produce a mildly alkaline environment in the presence of other organisms [33]. However, pH changes for the two organisms after co-culture was negligible. Jameson Effect, non-specific competition between species for nutrients, could also contribute to growth suspension of microorganisms [34, 35]. In other words, KP6870155 that achieved maximum population first exhausted available growth substrates, which in turn possibly suppressed the growth of AB6870155.

To examine the growth of the two organisms under conditions that are more similar to the respiratory tract, synthetic lung mimicking medium (SLMM) was used. As expected, AB6870155 and KP6870155 planktonic monocultures grew slower in SLMM compared to their growth in the more nutrient-rich MH media. In contrast to the MH data, AB6870155 and KP6870155 had virtually equal observed CFUs/ml at 6 and 23 hours when they were co-cultured together in SLMM (Figure 5.2b). This suggests that suppression of AB6870155 growth by KP6870155 may only occur in rich laboratory media such as the MH broth.

Carbon source utilisation profiles of the co-isolated strains

Heterotrophic organisms typically encounter limited amounts of complex mixtures of carbon sources that are often present at low concentration in their native environments. Therefore, microbial cells have developed multiple different strategies to utilise a wide array of different substrates as carbon sources [36].

Biolog phenotype microarrays (PMs) were used to examine the utilisation of 190 different sole carbon sources (PM01-02) (Figure S5.5, supplementary data) by AB6870155 and KP6870155. The PMs data showed that KP6870155 was able to utilise 105 carbon sources whereas AB6870155 could only use 58 carbon compounds (Figure 5.3; Figure S5.4). AB6870155 and KP6870155 shared the capacity to use 35 carbon sources out of 190. KP6870155 could respire on 70 substrates that AB6870155 cannot, including a wide variety of mono- and disaccharide sugars, whereas KP6870155 could not respire on 23 substrates, primarily organic acids that the AB6870155 can use as sole carbon sources (Figure 5.3; Figure S5.4). Those data revealed that these two strains have very different metabolic fingerprints, suggesting that they pursue very different ecological strategies, and hence may live within different microenvironments in the human respiratory tract at the same time physiologically complementing each other.

One possible explanation for the observed differences in metabolic capacity is that KP6870155 has a larger genome, which includes more metabolic genes or operons, such as the *dha* genes for glycerol degradation, that are absent in the genome of AB6870155. Additionally, KP6870155 also has a much greater predicted transporter gene repertoire (>290 genes) compared with AB6870155 (130 genes). The expanded transporter gene content in KP6870155 presumably plays a significant role in its enhanced substrate utilisation profile compared with AB6870155.

Cross-feeding between AB6870155 and KP6870155

Various forms of cross-feeding, which involve an exchange of nutrients among species of microbes, are common in microbial communities [37, 38]. The reciprocal altruism and metabolic wastes or by-products exchange among species are the two known evolutionary origins [39]. The distinctive metabolic profiles of these two strains obtained from the PMs analysis (Figure 5.3), provide a theoretical opportunity for the two organisms to cooperate with each other in carbon source utilisation. Therefore, we investigated the capacity of AB6870155 and KP6870155 to cross-feed each other.

Based on the PMs data, six carbon sources (sucrose, maltose, glycerol, mannose, galactose, serine) that could only be utilised by KP6870155, and three carbon sources (tricarballylic acid, sorbic acid, ornithine) that could only be utilised by AB6870155, were chosen for cross-feeding experiments on solid media. AB6870155 was not able to grow on either the sucrose or maltose agar plate, but when streaked on an agar plate containing either of the substrates along with KP6870155, growth of AB6870155 was observed at the closest edge to the KP6870155 streak (Figure 5.4). This suggests that a metabolic end product secreted by KP6870155 diffused into the agar, and was directly used as a growth substrate by AB6870155. No growth was observed for KP6870155 when it was cultured either alone or

together with AB6870155 on tricarballylic acid, sorbic acid, or ornithine as sole carbon sources.

To investigate the possibility that AB6870155 is able to utilize a metabolite secreted by KP6870155, cross-feeding assays in liquid media were conducted using Millicell hanging cell culture inserts (Millipore) (Figure 5.5a) within 6 and 96-well plates. This inserts prevent direct bacteria cell-cell contact, but allow exchange of secreted metabolites. Liquid media cross-feeding assays showed that growth of AB6870155 was supported in minimal media with sucrose or maltose as a sole carbon source only in the presence of KP6870155. Additionally, the other four carbon sources tested in the liquid media cross-feeding system (glycerol, mannose, serine, galactose) also showed growth of AB6870155 when co-cultured with KP6870155 (Figure 5.5b). This strongly supports the possibility that cross-feeding of AB6870155 is due to utilisation of a secreted metabolite produced from degradation of the selected sole carbon sources by KP6870155. Bioinformatic analysis of the metabolite degradation pathways present in both organisms, suggested this secreted metabolite could be ethanol or lactate. Ethanol and lactate are both metabolic end-products likely secreted by KP6870155, and can serve as a sole carbon source for AB6870155 (our unpublished data). Ethanol has been previously demonstrated as a diffusible factor produced by *Saccharomyces cerevisiae* that enhances the growth of *A. baumannii* when these two organisms are co-cultured [40].

Interactions between the co-isolated strains in biofilms

In natural environments microorganisms predominantly exist as biofilms as opposed to planktonic or free-floating cells [41, 42]. Previous studies described that both *A. baumannii* and *K. pneumoniae* can form biofilms with other organisms [16, 43]. Since AB6870155 and KP6870155 were isolated from a dual respiratory tract infection from a single individual, they were likely co-existing within a biofilm in that patient's respiratory tract. Hence, we

investigated the biofilm-forming capabilities of these two strains, alone and together. A static biofilm assay in plastic 96-well trays demonstrated that both of the strains could form biofilms individually, and that the biofilms were thicker and more stable when the two strains were co-cultured together, regardless of the growth media used (Figure 5.6). When biofilm formation was observed in a flow-cell continuous system, it was also shown that the co-cultured biofilm was thicker than biofilms formed by individual strains (Figure 5.7). These two assays suggest that synergistic interactions may occur in biofilms consisting of AB6870155 and KP6870155. It has been reported that interspecies cooperation and metabolic interactions are likely to develop among microorganisms in biofilms, including mutualistic and commensal relationships [16, 44]. Our cross feeding experiments between AB6870155 and KP6870155 suggest a potential mechanism for metabolic interaction when they are in close proximity.

Transcriptomics of *Acinetobacter baumannii* in response to *Klebsiella pneumoniae* in biofilms

Previous studies have shown that gene expression patterns in bacteria can alter when expose to another bacterial species. To investigate the interaction between AB6870155 and KP6870155 in a biofilm setting, we conducted RNA-Seq transcriptomics of the two strains co-grown in a biofilm in SLMM compared to each strain grown individually as monospecies biofilms.

A total of 19,965,896 (± 778,294) sequence reads were generated from the co-isolated RNA from each of the three biological replicates of the co-grown *A. baumannii* and *K. pneumoniae* strains. Approximately 12.8% (± 3.1%) of the reads were mapped to the *K. pneumoniae* KP6870155 genome while 70.4% (± 1.7%) of the reads aligned to the *A. baumannii* AB6870155 genome. The sequence data suggested that AB6870155 comprised a large proportion of the biofilm, which is supported by the static biofilm data (Figure 5.6b), which

showed that AB6870155 grows to a higher cell density than KP6870155 when grown as a biofilm in SLMM.

Bioinformatic analysis of the RNA-Seq results revealed that 49 genes showed significantly increased expression in AB6870155 when co-grown with KP6870155 compared with an AB6870155 monoculture, and 69 genes showed significantly decreased gene expression (Figure 5.8, $p \leq 0.05$ and $\log_2$ fold-change cut-off $1 \leq$ to ≤ -1). Similarly, there were 47 and 89 genes that showed significantly increased and decreased transcript abundance, respectively, in KP6870155 co-cultured with AB6870155 compared with KP6870155 alone (Figure 5.9).

Differentially expressed genes in *Acinetobacter baumannii* when co-grown with *Klebsiella pneumoniae* in a biofilm

The AB6870155 gene with the highest (33-fold) increase in expression when co-grown with KP6870155 was *aceI*, which encodes an efflux protein recently characterized to transport chlorhexidine [45, 46] and polyamines [unpublished data]. Genes encoding, γ-aminobutyraldehyde dehydrogenase and 4-aminobutyrate aminotransferase, enzymes in the pathway for degradation of the polyamine putrescine also showed at least 10-fold increased expression when co-grown with KP6870155. This suggests lowering intracellular concentrations of putrescine in *A. baumannii* may be an important factor in the interaction between the two bacteria in a biofilm. Putrescine has been shown to be essential for biofilm formation in some bacteria, e.g., *Yersinia pestis* [47], it also been implicated in *Pseudomonas fluorescens* SS101-protozoa interactions [48], and putrescine secretion has been observed following osmotic stress in *E. coli* [49, 50].

A nearly 10-fold increased expression of the chaperone-encoding genes *dnaJ*, *dnaK*, *groEL*, *groES* was observed when AB6870155 is co-grown with KP6870155 (Figure 8). These molecular chaperones are central to the tolerance to environmental stresses and involved in a

diverse set of cellular process including protein folding, protein translocation, and signal transduction [51]. Studies in various bacteria have implicated the DnaK and GroEL complexes as playing an important role in biofilm formation [51, 52].

The chaperone-usher pilus assembly genes *csuA/B*, *csuA* and *cusB* genes had increased expression in AB6870155 co-grown with KP6870155, with the largest increase (11-fold) observed for the *csuA/B* gene encoding the major pilus subunit. The *A. baumannii* chaperone usher pilus system has been shown to be important for the attachment to solid surfaces and biofilm formation [53].

Amino acid catabolism and efflux genes showed decreased expression in AB6870155 co-grown with KP6870155. Genes encoding histidine utilization (Hut) pathway proteins had 4 to 16 –fold decreased expression, and genes encoding N-succinyltransferase and succinylorinithine transamine enzymes, involved in arginine degradation, showed 12-fold and 16-fold decreased expression, respectively. The *argO* gene encoding a LysE family arginine exporter showed the greatest decrease in expression (>20-fold). Another LysE family amino acid efflux pump encoded by AB57_1624 also showed significantly decreased expression in AB6870155 co-grown with KP6870155.

A large cluster of phage genes (AB57_2008-2073) showed significantly decreased gene expression in AB6870155 co-grown with KP6870155, as did a family of DDE transposase gene scattered in the AB6870155. This suggests that expression of genes involved with mobile DNA is repressed by co-growth with KP6870155. Phage, transposon and integron integrase genes involved in DNA mobility typically show increased expression when cells are stressed or undergoing DNA damage [54-56]. This unexpected finding may suggest that AB6870155 is more stressed when grown as a monoculture than in co-culture. Many of the AB6870155 genes that show increased expression when co-grown with KP6870155 are

associated with biofilm formation, and this correlates with our observation that the two organisms grown together form a thicker biofilm than either alone.

Differentially expressed genes in *Klebsiella pneumoniae* when co-grown with *Acinetobacter baumannii* in a biofilm

The KP6870155 gene with the highest (25-fold) increase in expression when co-grown with AB6870155 encodes the enzyme lactaldehyde dehydrogenase, which converts lactaldehyde to L-lactate [57]. Conversely, the gene encoding L-lactate dehydrogenase, which converts L-lactate to pyruvate showed an almost 25-fold decrease in gene expression. Taken together, this suggests that KP6870155 may be producing and secreting increased levels of L-lactate when co-grown with AP6870155. Since we have demonstrated the ability of KP6870155 to cross-feed AB6870155 in solid and liquid media, it is tempting to speculate that increased production of L-lactate by KP6870155 could cross-feed AP6870155 in a biofilm setting. The increased biofilm density, and the large percentage of AB6870155 cells within the co-cultured biofilm we have observed in this study, could be due to AB6870155 being able to effectively utilise secreted L-lactate as a carbon source after other nutrients are depleted.

The KP6870155 gene encoding a lysine-arginine-ornithine binding protein was up-regulated more than 5-fold when co-cultured with AB6870155. Speculatively, increased import of these amino acids could be precursors for putrescine synthesis, which might be linked to the increased expression in AB6870155 of genes coding for putrescine breakdown or excretion.

A gene cluster (KPN2242_19395-19425) encoding type I fimbriae biogenesis proteins showed greatly decreased gene expression (the major pilin subunit FimA shows an almost 40-fold decrease in expression) when co-cultured with AB6870155. *K. pneumoniae* type I fimbriae appear to not play a role in biofilm formation and their expression is down-regulated

in biofilms, but they are an essential virulence factor in urinary tract infections in a mouse model system [30].

A cardiolipin synthase encoding gene showed 6.3-fold increased expression in KP6870155 when co-cultured with AB6870155. This enzyme catalyzes condensation of two phosphatidylglycerol molecules to yield cardiolipin, a minor lipid component of bacterial cell membranes. This may indicate that there are changes in membrane fluidity/structure in KP6870155 in co-cultured biofilms. Expression of a cluster of genes involved in the UDP-*N*-acetylmuramoyl-pentapeptide biosynthetic pathway for peptidoglycan synthesis all showed significantly decreased expression in KP6870155 when co-cultured with AB687015, suggesting there may be other cell surface alterations.

Multiple genes involved in tryptophan biosynthesis as well as a tryptophan uptake system showed significantly decreased expression, as did genes encoding the first two enzymatic steps in histidine degradation in KP6870155 when co-cultured with AB6870155. AB6870155 also showed decreased expression of histidine degradation genes (see above), suggesting this is a common response between the two bacteria in the biofilm.

Conclusions

We have investigated the molecular and physiological interactions between virulent strains of *A. baumannii* and *K. pneumoniae* co-isolated from a single respiratory tract infection. Interactions between the two strains appear to be complex, multifaceted and highly dependent on the culture conditions. For example, *K. pneumoniae* dominates the culture when grown planktonically in rich complex media, whereas *A. baumannii* is the dominant organism when the two are grown as a biofilm. The metabolic fingerprints of the two bacteria are very distinct, with KP6870155 able to utilize a much broader array of carbohydrates while AB6870155 could catabolize a broader range of organic acids. We present data in liquid and solid media suggesting that KP6870155 can cross-feed AB6870155, which may utilize lactate

or ethanol secreted as metabolic end products by KP6870155. This may be linked to the success of AB6870155 in mature biofilms.

RNA-Seq analysis revealed substantial differences in the transcriptomes of both bacteria when co-cultured in a biofilm. Both pathogens may be undergoing significant changes in surface structures, with increased expression of chaperone-usher pilus genes in AB6870155, while KP6870155 reduced expression of fimbriae biogenesis genes. Amino acid catabolism and transport appeared to be impacted in both organisms, and genes involved in putrescine catabolism and efflux showed greatly decreased expression in AB6870155. Lactate production in KP6870155 may be increased which potentially could lead to increased growth of AB6870155 in the biofilm.

There is a growing body of literature examining the interaction of organisms mixed cultures in recent years. Such studies have typically used standard reference strains. We believe our study is unique in providing a molecular and physiological view of interactions between two organisms originally isolated from a single infection.

Acknowledgements

None to acknowledge

Author Contributions

QL, KA, AP and IP conceived and designed this study; QL performed the experiments; QL, KA, IP and AP analysed data; QL, KA, IP wrote the manuscript with contribution from AP.

Funding

QL was supported by a Macquarie University International Research Excellence Scholarship and IP was supported by an Australian Research Council Laureate Fellowship (FL140100021). Additional support for the work was provided by grants 1060895 and 1120298 from the National Health and Medical Research Council of Australia.

Transparency declarations

None to declare

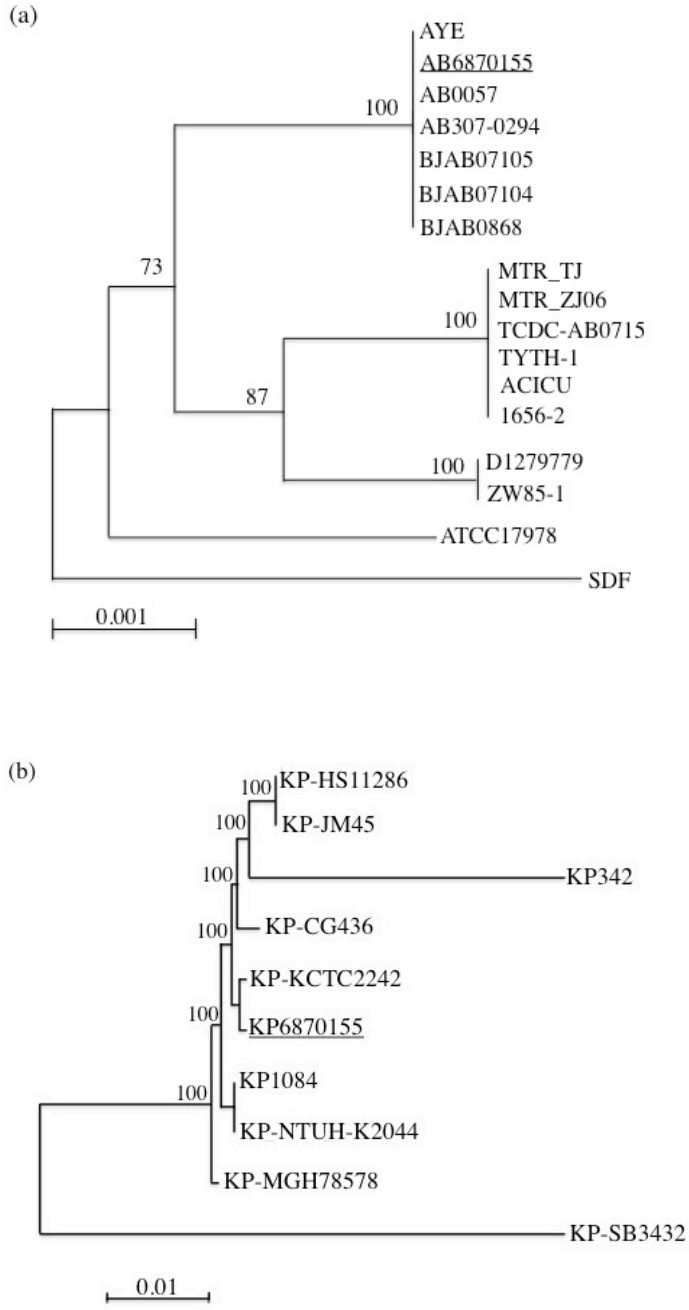


Figure 5.1. Phylogenetic relationships of *A. baumannii* and *K. pneumoniae*. The phylogenetic tree of eight sequenced *A. baumanni* strains were generated using four housekeeping genes (*gltA, pyrG, groEL, rpoB)* conserved across all of the strains. The phylogenetic tree of six sequenced *K. pneumoniae* genomes was conducted by using four 16S RNA genes *rrmB, rsmC, rrsH* and *armA*. The genes were aligned and concatenated using the Bio-Edit software [67], and the neighbour-joining tree was constructed using the MEGA5 program [71]. The numbers next to each node of the tree represent bootstrap values from 1,000 replicates. The scale unit is substitutions per site.

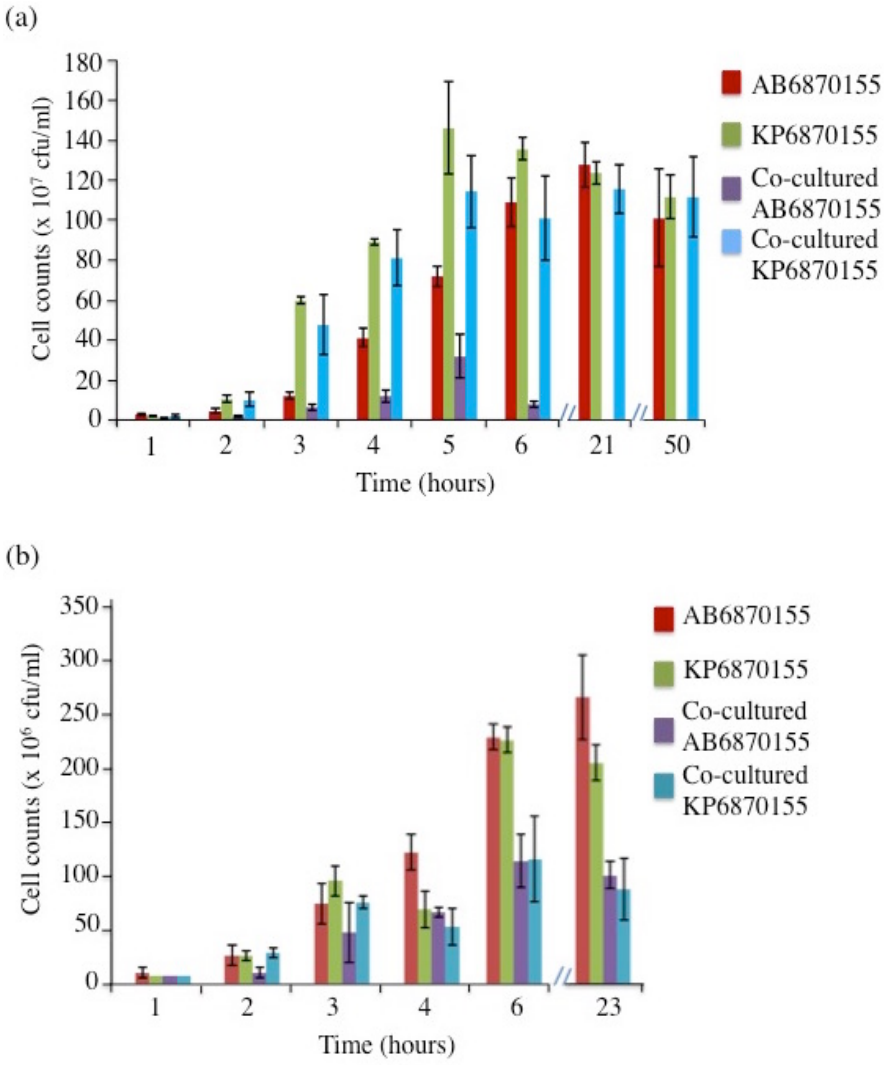


Figure 5.2. Co-culture growth between AB6870155 and KP6870155. (a) The two strains were individually cultured and co-cultured in MH; the bars represent cell counts within 50 hours. **(b)** The co-isolated strains were cultured separately and co-cultured in SLMM; the bars represent cell counts during 23 hours.

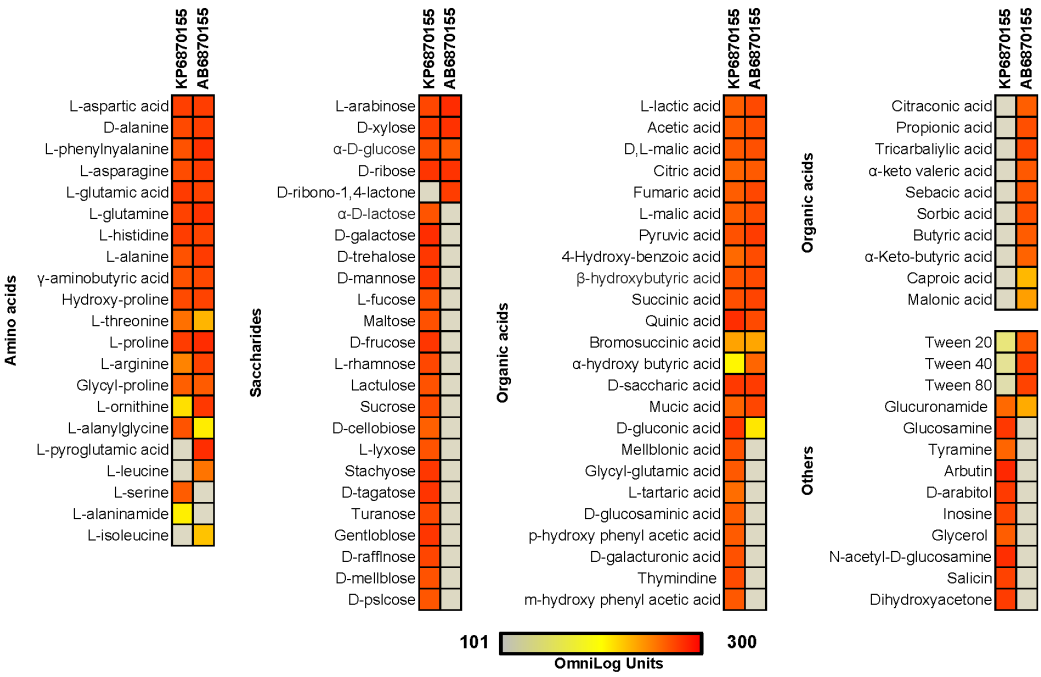


Figure 5.3. Heat map of carbon source utilization capabilities of KP6870155 and AB6870155. To represent the height of the kinetic curve relative to each compound, a colour scale was used ranging from grey (101 AOUs (arbitrary Omnilog units)), through yellow and orange to red (320 AOUs). A threshold of 101 AOUs was used to discriminate between utilized (> 101 AOUs) and non-utilized (< 101 AOUs) carbon sources (PM01-02).

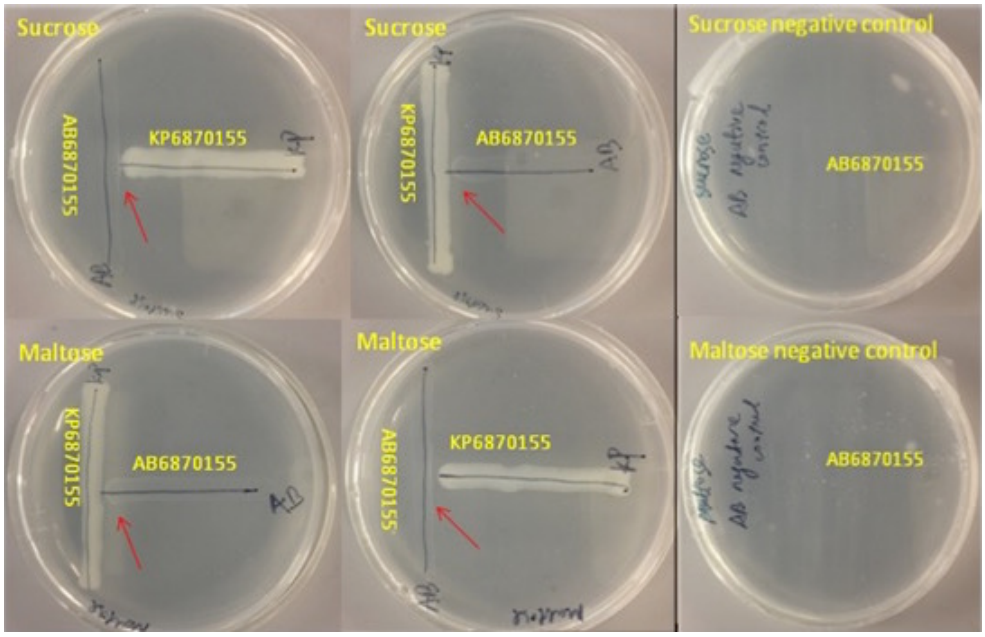


Figure 5.4. **Cross-feeding between AB6870155 and KP6870155 in utilization of sucrose and maltose as sole carbon source on 1.2% agar M9 minimal medium.** The two strains were inoculated adjacent to each other using a sterile loop. The media contains only a single carbon source, as indicated in yellow text. Red arrow points to the growth edge of AB6870155.

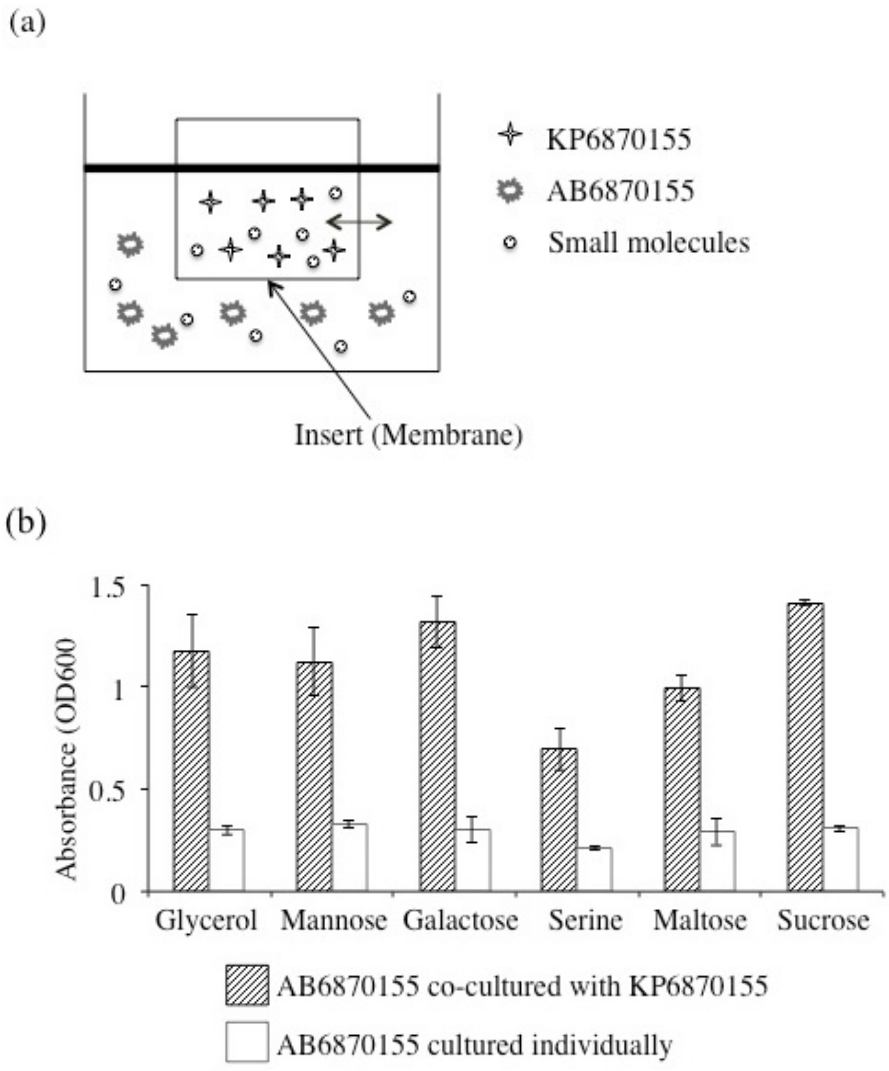


Figure 5.5. Cross-feeding of the two strains using Millicell hanging inserts. a) A schematic representation of the cross-feeding between the AB6870155 and the KP6870155 in a liquid system. b) Cross-feeding was monitored between the co-isolated strains when using those sole carbon sources (glycerol, mannose, galactose, serine, maltose, sucrose). Bars represent the growth of AB6870155 in the tested single carbon sources with or without KP6870155 measured as abourbance at 600 nm.

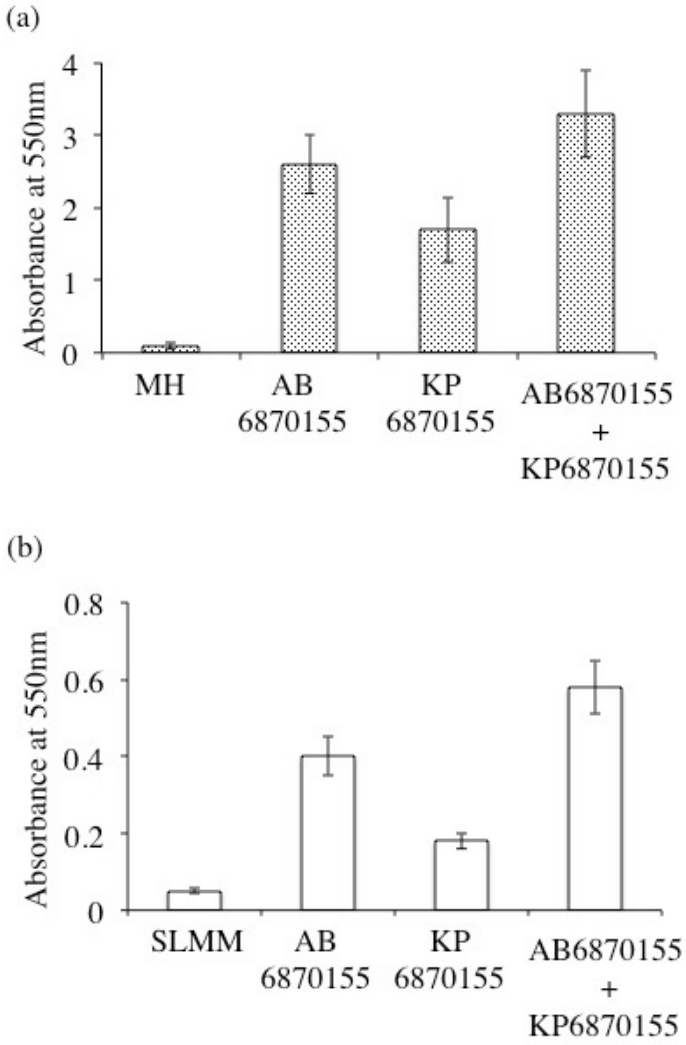


Figure 5.6. Quantitation of biofilm formation by in batch biofilm assays. Bars represent absorbances at 550nm of CV strained biofilms formed in cell cultured individually together as a co-cuturein MH (a) and SLMM (b) media.

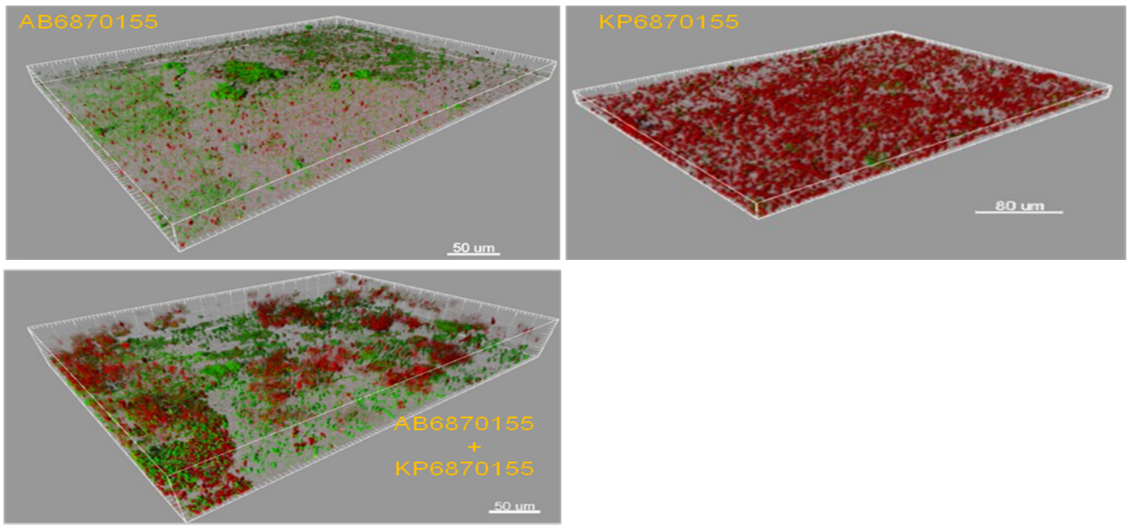


Figure 5.7. Confocal microscopy generated 3D images of the dual-species mixed biofilms grown in flow-cell chambers after 3 days of cultivation. Cells were stained using BcaLigh Live/Dead stain (Molecular Probes®); live cells are represented in green, whereas dead cells are shown in red.

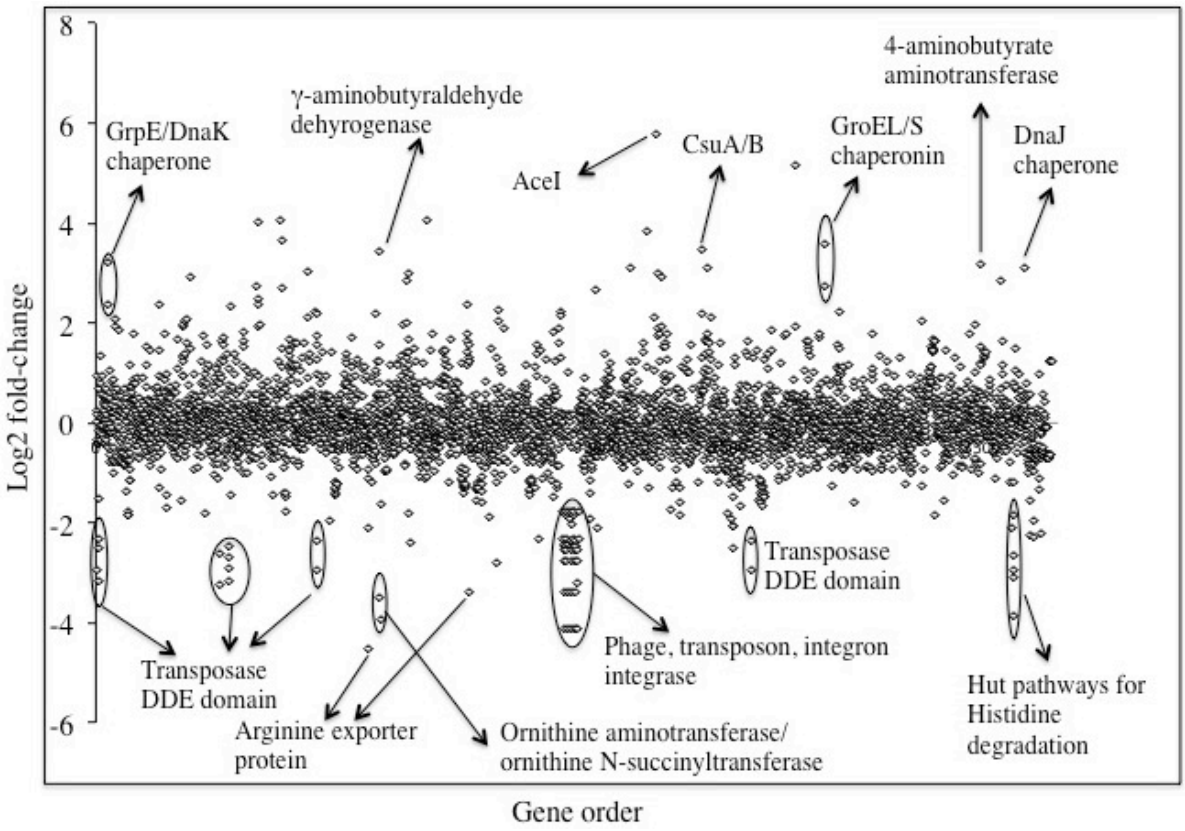


Figure 5.8. Global transcriptional response of AB6870155 to KP6870155 in mixed biofilms. Each point represents one annotated gene within the genome of AB6870155, with the X-axis showing the gene position, and the Y-axis showing the log2 fold change of gene transcript abundance in response to the presence of KP6870155 for 24 hours during biofilm formation. Genes or gene clusters of particular interest are labelled.

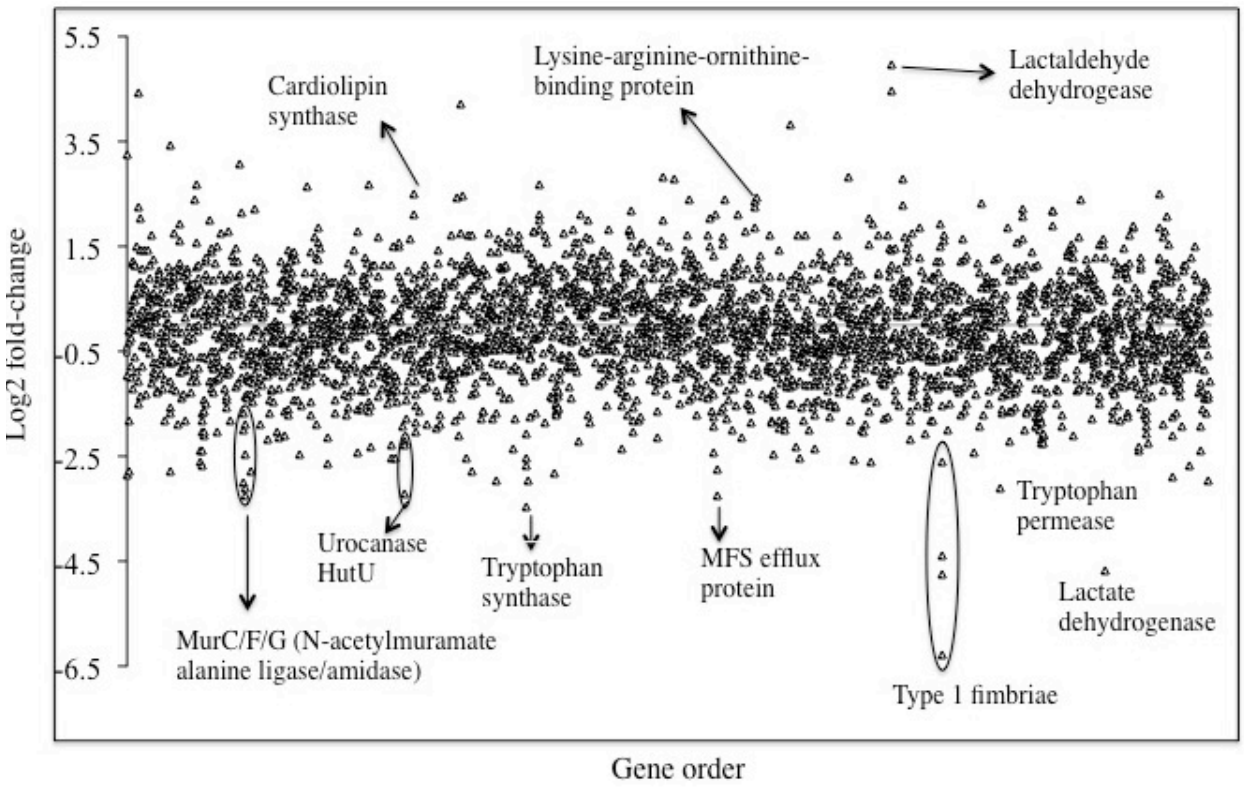


Figure 5.9. Global transcriptional response of KP6870155 to AB6870155 in mixed biofilms. Each point represents one annotated gene within the genome of KP6870155, with the X-axis showing the gene position, and the Y-axis showing the log2 fold change of gene transcript abundance in response to the presence of AB6870155 for 24 hours during biofilm formation. Genes or gene clusters of particular interest are labelled.

Supplementary data

Table S5.1. Genomic sequencing statistics of the AB6870155 and the KP6870155

Bacteria strains	Genome size (bp)	GC content (%)	tRNA number	Plasmid(s)
AB6870155	3,982,228	39.2	53	1
KP6870155	5,447,864	57.4	58	0

Table S5.2. Comparison of ORFs conserved in different *A. baumanii* strains

Strain	No. of genes conserved							
	AB6807155	**AB0057**	**AYE**	**ATCC17978**	**ACICU**	**SDF**	**MDR-ZJ06**	**AB307**
AB6807155	3655	3405	3211	2926	3095	2120	3082	3199
AB0057		3731	3607	2920	3059	2160	3062	3124
AYE			3649	2075	3022	2219	3038	3117
ATCC17978				3761	2939	1563	2065	2083
ACICU					3736	2195	3312	2962
SDF						2966	2245	2128
ABZJ							3752	2979
AB307								3451

Table S5.3. Comparison of ORFs conserved across different *K. pneumoniae* strains

Strain	No. of genes conserved					
	KP6807155	**KP342**	**KPHS11286**	**KCTC2242**	**MGH18578**	**NTUH_K2044**
KP6807155	5208	4331	4584	4396	4241	4423
KP342		5743	4297	4241	4149	4289
KPHS11286			5748	4389	4425	4456
KCTC2242				5124	4327	4557
MGH18578					5158	4349
NTUH_K2044						5259

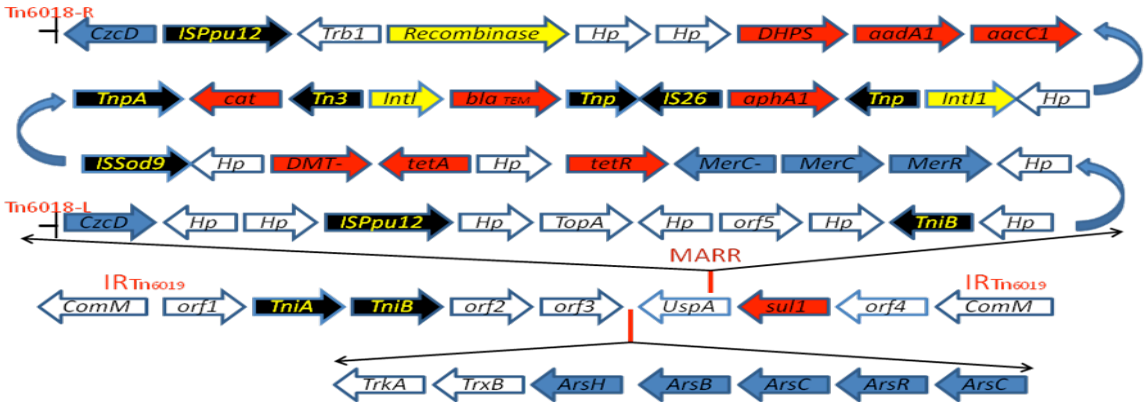


Figure S5.1. Gene structure of the resistance RGP, AbaR155, in AB6870155. AbaR155 is inserted into an ATPase gene (*comM*). Coloured arrows are used to indicate ORF categories: resistance to antibiotics in red, resistance to heavy metals or antiseptics in blue, transposases in black, integrases in yellow and other functions in white (Hp: hypothetical protein encoding genes).

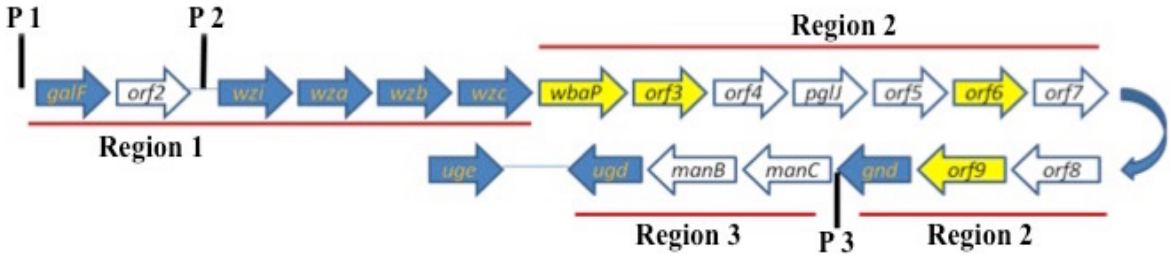


Figure S5.2. Capsular polysaccharide encoding gene cluster in KP6870155. ORFs are represented by arrows (blue for conserved genes, yellow for glycosyltransferases, and white for others). The polycistronic mRNAs are driven by promoters P1, P2, and P3. The polycistronic mRNA driven by P1 includes of *galF* and *orf2*. The polycistronic mRNA driven by P2 consists of genes of capsule repeat unit synthesis and polymerization as well as surface assembly. The transcriptional direction of *ugd* is opposite to that of *uge*

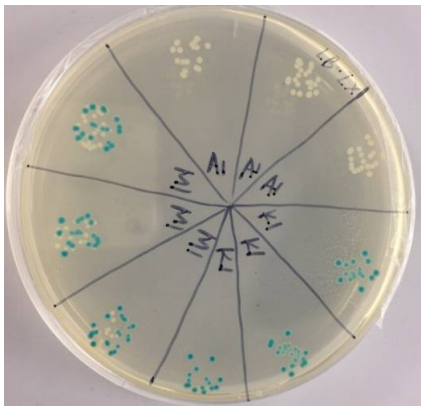


Figure S5.3. The co-isolated strains grown on Luria Bertani X-gal/IPTG agar plate. Blue and white colonies represent KP6870155 and AB6870155, respectively. The genome of AB6870155 does not contain genes that encoding β–galactosidase whereas the KP6870155 genome harbours several copies of this gene. The presence of β–galactosidase can be detected by X-gal/IPTG, which can form blue precipitant within cells resulted in blue colonies. Thus, plating the cells onto a LB agar plate containing X-gal/IPTG can differentiate the two co-isolated strains by different colours.

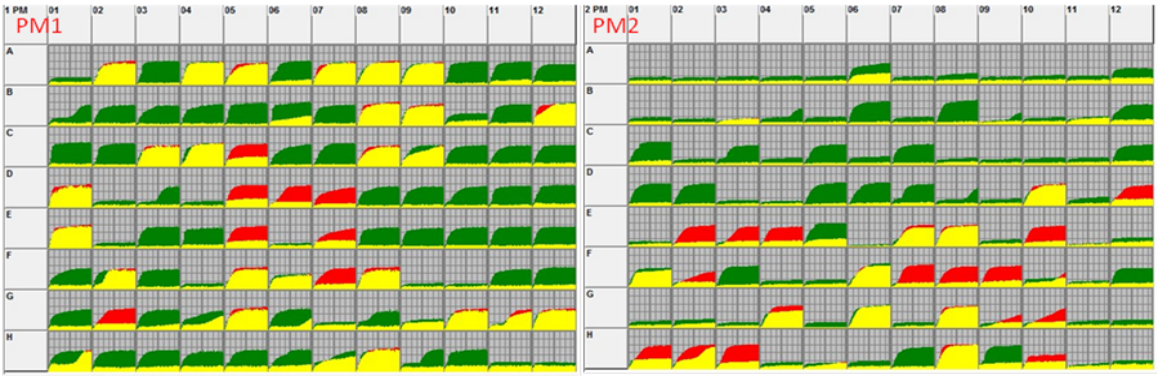


Figure S5.4. Comparison of carbon sources utilization (PM01-02) between the KP6870155 and the AB6870155. The Figure shows a utilization of 190 different carbon sources. Well A1 is a negative control. The metabolic activity was monitored every 15 minutes over 48 hours. The yellow kinetic curves indicate that both strains can utilize that carbon source, red kinetic curves represent the carbon sources can only be used by AB6870155, whereas green kinetic curves represent the carbon sources can only be used by KP6870155.

BIOLOG **Phenotype MicroArrays™**

PM1 MicroPlate™ Carbon Sources

A1 Negative Control	A2 L-Arabinose	A3 N-Acetyl-D-Glucosamine	A4 D-Saccharic Acid	A5 Succinic Acid	A6 D-Galactose	A7 L-Aspartic Acid	A8 L-Proline	A9 D-Alanine	A10 D-Trehalose	A11 D-Mannose	A12 Dulcitol
B1 D-Serine	B2 D-Sorbitol	B3 Glycerol	B4 L-Fucose	B5 D-Glucuronic Acid	B6 D-Gluconic Acid	B7 D,L-α-Glycerol-Phosphate	B8 D-Xylose	B9 L-Lactic Acid	B10 Formic Acid	B11 D-Mannitol	B12 L-Glutamic Acid
C1 D-Glucose-6-Phosphate	C2 D-Galactonic Acid-γ-Lactone	C3 D,L-Malic Acid	C4 D-Ribose	C5 Tween 20	C6 L-Rhamnose	C7 D-Fructose	C8 Acetic Acid	C9 α-D-Glucose	C10 Maltose	C11 D-Melibiose	C12 Thymidine
D-1 L-Asparagine	D2 D-Aspartic Acid	D3 D-Glucosaminic Acid	D4 1,2-Propanediol	D5 Tween 40	D6 α-Keto-Glutaric Acid	D7 α-Keto-Butyric Acid	D8 α-Methyl-D-Galactoside	D9 α-D-Lactose	D10 Lactulose	D11 Sucrose	D12 Uridine
E1 L-Glutamine	E2 M-Tartaric Acid	E3 D-Glucose-1-Phosphate	E4 D-Fructose-6-Phosphate	E5 Tween 80	E6 α-Hydroxy Glutaric Acid-γ-Lactone	E7 α-Hydroxy Butyric Acid	E8 β-Methyl-D-Glucoside	E9 Adonitol	E10 Maltotriose	E11 2-Deoxy Adenosine	E12 Adenosine
F1 Glycyl-L-Aspartic Acid	F2 Citric Acid	F3 M-Inositol	F4 D-Threonine	F5 Fumaric Acid	F6 Bromo Succinic Acid	F7 Propionic Acid	F8 Mucic Acid	F9 Glycolic Acid	F10 Glyoxylic Acid	F11 D-Cellobiose	F12 Inosine
G1 Glycyl-L-Glutamic Acid	G2 Tricarballylic Acid	G3 L-Serine	G4 L-Threonine	G5 L-Alanine	G6 L-Alanyl-Glycine	G7 Acetoacetic Acid	G8 N-Acetyl-β-D-Mannosamine	G9 Mono Methyl Succinate	G10 Methyl Pyruvate	G11 D-Malic Acid	G12 L-Malic Acid
H1 Glycyl-L-Proline	H2 p-Hydroxy Phenyl Acetic Acid	H3 m-Hydroxy Phenyl Acetic Acid	H4 Tyramine	H5 D-Psicose	H6 L-Lyxose	H7 Glucuronamide	H8 Pyruvic Acid	H9 L-Galactonic Acid-γ-Lactone	H10 D-Galacturonic Acid	H11 Phenylethyl-amine	H12 2-Aminoethanol

PM2A MicroPlate™ Carbon Sources

A1 Negative Control	A2 Chondroitin Sulfate C	A3 α-Cyclodextrin	A4 β-Cyclodextrin	A5 γ-Cyclodextrin	A6 Dextrin	A7 Gelatin	A8 Glycogen	A9 Inulin	A10 Laminarin	A11 Mannan	A12 Pectin
B1 N-Acetyl-D-Galactosamine	B2 N-Acetyl-Neuraminic Acid	B3 β-D-Allose	B4 Amygdalin	B5 D-Arabinose	B6 D-Arabitol	B7 L-Arabitol	B8 Arbutin	B9 2-Deoxy-D-Ribose	B10 I-Erythritol	B11 D-Fucose	B12 3-O-β-D-Galacto-pyranosyl-D-Arabinose
C1 Gentiobiose	C2 L-Glucose	C3 Lactitol	C4 D-Melezitose	C5 Maltitol	C6 α-Methyl-D-Glucoside	C7 β-Methyl-D-Galactoside	C8 3-Methyl Glucose	C9 β-Methyl-D-Glucuronic Acid	C10 α-Methyl-D-Mannoside	C11 β-Methyl-D-Xyloside	C12 Palatinose
D1 D-Raffinose	D2 Salicin	D3 Sedoheptulosan	D4 L-Sorbose	D5 Stachyose	D6 D-Tagatose	D7 Turanose	D8 Xylitol	D9 N-Acetyl-D-Glucosaminitol	D10 γ-Amino Butyric Acid	D11 δ-Amino Valeric Acid	D12 Butyric Acid
E1 Capric Acid	E2 Caproic Acid	E3 Citraconic Acid	E4 Citramalic Acid	E5 D-Glucosamine	E6 2-Hydroxy Benzoic Acid	E7 4-Hydroxy Benzoic Acid	E8 β-Hydroxy Butyric Acid	E9 γ-Hydroxy Butyric Acid	E10 α-Keto Valeric Acid	E11 Itaconic Acid	E12 5-Keto-D-Gluconic Acid
F1 D-Lactic Acid Methyl Ester	F2 Malonic Acid	F3 Melibionic Acid	F4 Oxalic Acid	F5 Oxalomalic Acid	F6 Quinic Acid	F7 D-Ribono-1,4-Lactone	F8 Sebacic Acid	F9 Sorbic Acid	F10 Succinamic Acid	F11 D-Tartaric Acid	F12 L-Tartaric Acid
G1 Acetamide	G2 L-Alaninamide	G3 N-Acetyl-L-Glutamic Acid	G4 L-Arginine	G5 Glycine	G6 L-Histidine	G7 L-Homoserine	G8 Hydroxy-L-Proline	G9 L-Isoleucine	G10 L-Leucine	G11 L-Lysine	G12 L-Methionine
H1 L-Ornithine	H2 L-Phenylalanine	H3 L-Pyroglutamic Acid	H4 L-Valine	H5 D,L-Carnitine	H6 Sec-Butylamine	H7 D,L-Octopamine	H8 Putrescine	H9 Dihydroxy Acetone	H10 2,3-Butanediol	H11 2,3-Butanone	H12 3-Hydroxy 2-Butanone

Figure S5.5. PM microplate carbon sources

Reference

1. Rice, L.B., *Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE.* J Infect Dis, 2008. **197**(8): p. 1079-81.
2. Trick, W.E., et al., *Colonization of skilled-care facility residents with antimicrobial-resistant pathogens.* Journal of the American Geriatrics Society, 2001. **49**(3): p. 270-276.
3. Pacio, G.A., et al., *Natural history of colonization with vancomycin-resistant enterococci, methicillin-resistant Staphylococcus aureus, and resistant gram-negative bacilli among long-term-care facility residents.* Infection Control and Hospital Epidemiology, 2003. **24**(4): p. 246-250.
4. Harris, A.D., et al., *Co-carriage rates of vancomycin-resistant Enterococcus and extended-spectrum beta-lactamase-producing bacteria among a cohort of intensive care unit patients: Implications for an active surveillance program.* Infection Control and Hospital Epidemiology, 2004. **25**(2): p. 105-108.
5. Donskey, C.J., *Antibiotic regimens and intestinal colonization with antibiotic-resistant gram-negative bacilli.* Clinical Infectious Diseases, 2006. **43**: p. S62-S69.
6. Maragakis, L.L., et al., *Incidence and prevalence of multidrug-resistant Acinetobacter using targeted active surveillance cultures.* Jama-Journal of the American Medical Association, 2008. **299**(21): p. 2513-2514.
7. Marchaim, D., et al., *"Swimming in resistance": Co-colonization with carbapenem-resistant Enterobacteriaceae and Acinetobacter baumannii or Pseudomonas aeruginosa.* Am J Infect Control, 2012. **40**(9): p. 830-5.
8. Charlson, M.E., et al., *A new method of classifying prognostic comorbidity in longitudinal studies: development and validation.* J Chronic Dis, 1987. **40**(5): p. 373-83.

9. Hall-Stoodley, L., J.W. Costerton, and P. Stoodley, *Bacterial biofilms: From the natural environment to infectious diseases.* Nature Reviews Microbiology, 2004. **2**(2): p. 95-108.

10. Hobley, L., et al., *Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes.* Fems Microbiology Reviews, 2015. **39**(5): p. 649-669.

11. Parsek, M.R. and P.K. Singh, *Bacterial biofilms: An emerging link to disease pathogenesis.* Annual Review of Microbiology, 2003. **57**: p. 677-701.

12. Penesyan, A., M. Gillings, and I.T. Paulsen, *Antibiotic Discovery: Combatting Bacterial Resistance in Cells and in Biofilm Communities.* Molecules, 2015. **20**(4): p. 5286-5298.

13. Burmolle, M., et al., *Interactions in multispecies biofilms: do they actually matter?* Trends in Microbiology, 2014. **22**(2): p. 84-91.

14. Hansen, S.K., et al., *Characterization of a Pseudomonas putida rough variant evolved in a mixed-species biofilm with Acinetobacter sp. strain C6.* J Bacteriol, 2007. **189**(13): p. 4932-43.

15. Tong, H., et al., *Streptococcus oligofermentans inhibits Streptococcus mutans through conversion of lactic acid into inhibitory H2O2: a possible counteroffensive strategy for interspecies competition.* Mol Microbiol, 2007. **63**(3): p. 872-80.

16. Stewart, P.S., et al., *Spatial distribution and coexistence of Klebsiella pneumoniae and Pseudomonas aeruginosa in biofilms.* Microbial Ecology, 1997. **33**(1): p. 2-10.

17. An, D.D., et al., *Quorum sensing and motility mediate interactions between Pseudomonas aeruginosa and Agrobacterium tumefaciens in biofilm cocultures.* Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(10): p. 3828-3833.

18. Palmer, K.L., L.M. Aye, and M. Whiteley, *Nutritional cues control Pseudomonas aeruginosa multicellular behavior in cystic fibrosis sputum.* J Bacteriol, 2007. **189**(22): p. 8079-87.

19. Chevreux, B., et al., *Using the miraEST assembler for reliable and automated mRNA transcript assembly and SNP detection in sequenced ESTs.* Genome Research, 2004. **14**(6): p. 1147-1159.

20. Aziz, R.K., et al., *The RAST server: Rapid annotations using subsystems technology.* Bmc Genomics, 2008. **9**.

21. Altschul, S.F., et al., *Basic Local Alignment Search Tool.* Journal of Molecular Biology, 1990. **215**(3): p. 403-410.

22. Christensen, B.B., et al., *Molecular tools for study of biofilm physiology.* Methods Enzymol, 1999. **310**: p. 20-42.

23. Bochner, B.R., P. Gadzinski, and E. Panomitros, *Phenotype MicroArrays for high-throughput phenotypic testing and assay of gene function.* Genome Research, 2001. **11**(7): p. 1246-1255.

24. Magoc, T., D. Wood, and S.L. Salzberg, *EDGE-pro: Estimated Degree of Gene Expression in Prokaryotic Genomes.* Evol Bioinform Online, 2013. **9**: p. 127-36.

25. Anders, S. and W. Huber, *Differential expression analysis for sequence count data.* Genome Biol, 2010. **11**(10): p. R106.

26. Mathee, K., et al., *Dynamics of Pseudomonas aeruginosa genome evolution.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(8): p. 3100-3105.

27. Adams, M.D., et al., *Comparative Genome Sequence Analysis of Multidrug-Resistant Acinetobacter baumannii.* Journal of Bacteriology, 2008. **190**(24): p. 8053-8064.

28. Fronzes, R., et al., *Structure of a Type IV Secretion System Core Complex.* Science, 2009. **323**(5911): p. 266-268.

29. Podschun, R. and U. Ullmann, *Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors.* Clin Microbiol Rev, 1998. **11**(4): p. 589-603.

30. Schroll, C., et al., *Role of type 1 and type 3 fimbriae in Klebsiella pneumoniae biofilm formation.* BMC Microbiol, 2010. **10**: p. 179.

31. Holzapfel, W.H., R. Geisen, and U. Schillinger, *Biological Preservation of Foods with Reference to Protective Cultures, Bacteriocins and Food-Grade Enzymes.* International Journal of Food Microbiology, 1995. **24**(3): p. 343-362.

32. Nilsson, L., et al., *The contribution of bacteriocin to inhibition of Listeria monocytogenes by Carnobacterium piscicola strains in cold-smoked salmon systems.* Journal of Applied Microbiology, 2004. **96**(1): p. 133-143.

33. Peleg, A.Y., et al., *Prokaryote-eukaryote interactions identified by using Caenorhabditis elegans.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(38): p. 14585-14590.

34. Delignette-Muller, M.L., et al., *Use of Bayesian modelling in risk assessment: Application to growth of Listeria monocytogenes and food flora in cold-smoked salmon.* International Journal of Food Microbiology, 2006. **106**(2): p. 195-208.

35. Mellefont, L.A., T.A. McMeekin, and T. Ross, *Effect of relative inoculum concentration on Listeria monocytogenes growth in co-culture.* International Journal of Food Microbiology, 2008. **121**(2): p. 157-168.

36. Chaudhuri, R.R., et al., *Complete Genome Sequence and Comparative Metabolic Profiling of the Prototypical Enteroaggregative Escherichia coli Strain 042.* Plos One, 2010. **5**(1).

37. Pernthaler, A., et al., *Diverse syntrophic partnerships from-deep-sea methane vents revealed by direct cell capture and metagenomics.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(19): p. 7052-7057.

38. Schink, B., *Synergistic interactions in the microbial world.* Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology, 2002. **81**(1-4): p. 257-261.

39. Bull, J.J. and W.R. Harcombe, *Population Dynamics Constrain the Cooperative Evolution of Cross-Feeding.* Plos One, 2009. **4**(1).

40. Smith, M.G., S.G. Des Etages, and M. Snyder, *Microbial synergy via an ethanol-triggered pathway.* Molecular and Cellular Biology, 2004. **24**(9): p. 3874-3884.

41. Douglas, L.J., *Candida biofilms and their role in infection.* Trends in Microbiology, 2003. **11**(1): p. 30-36.

42. El-Azizi, M.A., S.E. Starks, and N. Khardori, *Interactions of Candida albicans with other Candida spp. and bacteria in the biofilms.* Journal of Applied Microbiology, 2004. **96**(5): p. 1067-1073.

43. Dashiff, A., et al., *Predation of human pathogens by the predatory bacteria Micavibrio aeruginosavorus and Bdellovibrio bacteriovorus.* J Appl Microbiol, 2011. **110**(2): p. 431-44.

44. Romano, J.D. and R. Kolter, *Pseudomonas-saccharomyces interactions: Influence of fungal metabolism on bacterial physiology and survival.* Journal of Bacteriology, 2005. **187**(3): p. 940-948.

45. Hassan, K.A., et al., *Homologs of the Acinetobacter baumannii AceI Transporter Represent a New Family of Bacterial Multidrug Efflux Systems.* Mbio, 2015. **6**(1).

46. Hassan, K.A., et al., *Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(50): p. 20254-20259.

47. Patel, C.N., et al., *Polyamines are essential for the formation of plague biofilm.* J Bacteriol, 2006. **188**(7): p. 2355-63.

48. Song, C., et al., *Molecular and chemical dialogues in bacteria-protozoa interactions.* Sci Rep, 2015. **5**: p. 12837.

49. Record, M.T., et al., *Responses of E-coli to osmotic stress: Large changes in amounts of cytoplasmic solutes and water.* Trends in Biochemical Sciences, 1998. **23**(4): p. 143-148.

50. Munro, G.F., et al., *Dependence of the putrescine content of Escherichia coli on the osmotic strength of the medium.* J Biol Chem, 1972. **247**(4): p. 1272-80.

51. Lemos, J.A., Y. Luzardo, and R.A. Burne, *Physiologic effects of forced down-regulation of dnaK and groEL expression in Streptococcus mutans.* J Bacteriol, 2007. **189**(5): p. 1582-8.

52. Lemos, J.A., J. Abranches, and R.A. Burne, *Responses of cariogenic streptococci to environmental stresses.* Curr Issues Mol Biol, 2005. **7**(1): p. 95-107.

53. Tomaras, A.P., et al., *Attachment to and biofilm formation on abiotic surfaces by Acinetobacter baumannii: involvement of a novel chaperone-usher pili assembly system.* Microbiology, 2003. **149**(Pt 12): p. 3473-84.

54. Servant, G., et al., *Tye7 regulates yeast Ty1 retrotransposon sense and antisense transcription in response to adenylic nucleotides stress.* Nucleic Acids Research, 2012. **40**(12): p. 5271-5282.

55. Casacuberta, E. and J. Gonzalez, *The impact of transposable elements in environmental adaptation.* Molecular Ecology, 2013. **22**(6): p. 1503-1517.

56. Guerin, E., et al., *The SOS response controls integron recombination.* Science, 2009. **324**(5930): p. 1034.

57. Baldoma, L. and J. Aguilar, *Involvement of lactaldehyde dehydrogenase in several metabolic pathways of Escherichia coli K12.* J Biol Chem, 1987. **262**(29): p. 13991-6.

Chapter 6: Conclusions and future perspectives

6.1 Conclusions

A. baumannii is one of the six most important multidrug-resistant pathogens in clinical settings worldwide, according to the Infectious Society Disease of America [1]. It is responsible for a variety of infections, including ventilator-associated pneumonia and bloodstream infections, of which mortality rates can reach 35% [2]. Multidrug efflux systems in *A. baumannii* are an important mechanism of resistance to antimicrobials in this successful pathogen [3].

Five families of bacterial multidrug efflux systems, including the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS) superfamily, the multidrug and toxic compound extrusion (MATE) family, the resistance nodulation-cell division (RND) family and the small multidrug resistance (SMR) family, have been studied extensively across bacterial species including *A. baumannii* [4]. These transporter proteins play significant protective roles for the bacterial cell but are a serious problem in the clinical setting, because a single efflux system can mediate resistance to many structurally and mechanistically diverse antibiotics and biocides [3]. Recently, the novel AceI efflux protein, which confers resistance to the biocide chlorhexidine, was identified from *A. baumannii* by using transcriptomic and biochemical analyses [5].

6.1.1 Multiplicity and redundancy of multidrug transporter genes

There is a large and diverse group of multidrug transporters harboured in bacteria to against a wide variety of environmental toxins [6]. An increasing number of multidrug transporters in bacteria have been discovered to recognise a spectra of overlapped substrates [7]. For example, two multidrug transporters share 51% sequence identity, Bmr and Blt, protect *B. subtilis* cells from the same spectra of diverse drugs with only minor differences in the resistance levels [7].

This raises a question that why bacteria need such a large number of multidrug transporter encoding genes, and many of these multidrug efflux pumps have apparently overlapping substrate specificity in their economically organised genomes. One possible explanation would be these transporters actually transport distinct natural compounds, which have not been identified yet. Their interaction with toxins is probably opportunistic [8]. Baranova's study on Bmr and Blt in *B. subtilis* supported this explanation [7]. Another explanation might be that the physiological role of some of the multidrug transporters was probably not designed for drug export [9]. For example, mutations in genes encoding for transporters other than the major AcrAB-TolC multidrug efflux transporter have only a neglect effect on phenotype in *E. coli* [8]. Study has suggested that the overlapping substrates specificity of the transporters could serve as a back up role, which ensure coverage of a wide range of xenobiotics and provide fast responses to environmental stress [8]. This strategy opens a great opportunity for organisms to increase their evolvability by reducing constraints on change and allowing the accumulation of nonlethal variation.

6.1.2 AceI and its homologs represent a new family of multidrug efflux pumps

Data analyses from the Pfam database showed that proteins homologous to AceI are encoded in the genomes of many bacterial species but are particularly dominant within proteobacterial lineages. As described in Chapter 2, 23 homologs of AceI protein from 17 *γ-Proteobacteria*, 3 *β-Proteobacteria* and 1 representative from each of *α-Proteobacteria, Firmicutes*, and *Actinobacteria* were cloned and expressed in *E. coli*. Antimicrobial resistance profiles of all the expressed AceI-like proteins were examined.

Both MIC and Biolog Phenotype Microarray (PMs) experiments demonstrated that many of the homologs confer chlorhexidine resistance. Moreover, a large proportion of the AceI homologs confer resistance to additional biocides such as benzalkonium, dequalinium, proflavine, and acriflavine. In this study, only 6 genes out of the 24 examined did not confer resistance to any of the antimicrobials tested. Acriflavine and proflavine are fluorescent compounds, whose fluorescence is quenched when bound to nucleic acids, making them attractive candidates for whole cell fluorimteric transport assays. An AceI homolog from *Vibrio parahaemolyticus* was demonstrated to export acriflavine and proflavine using a fluorimetric assay.

Overall, this work presented in Chapter 2 showed a large panel of genes encoding AceI homologues confer resistance to a set of 12 different biocides, antibiotics, and antimicrobial dyes. Thus AceI and its homologs represent a new, sixth family of bacterial multidrug efflux systems, which was named the proteobacterial antimicrobial compound efflux (PACE) family. The PACE family represents the first new family of bacterial multidrug efflux described in the last 15 years

6.1.3 Expression of the *aceI* gene is regulated by a divergently transcribed LysR family regulator

Multidrug efflux pump gene expression is typically tightly controlled by cells, and are often regulated by divergently transcribed regulatory genes adjacent to the efflux pump gene [6, 10-12]. Multidrug efflux pump regulators can belong to one of several different protein families, including the LysR family transcriptional regulators (LTTRs), the largest known family of transcriptional regulators [13].

Since the discovery of the PACE family proteins, there have been no published studies on their genetic regulation. This opened up a unique opportunity for us to discover and characterize the first regulator of the PACE family. We found an LTTR encoded immediately adjacent to *aceI* gene which we named *aceR*, due to its proximity to *aceI*.

MIC results showed that *A. baumannii ΔaceI* and *ΔaceR* mutants showed decreased chlorhexidine resistance levels. Quantitative RT-PCR assays demonstrated that *aceI* expression was not induced by chlorhexidine in the Δ*aceR* mutant strain. Both the chlorhexidine resistance level and chlorhexidine-induced *aceI* expression in the *ΔaceR* mutant could be partially restored by complementation with the cloned *aceR* gene. This suggested that AceR might act as an activator of *aceI* gene expression.

It is common for an LTTR to bind to multiple DNA sites within the intergenic region between its own coding gene and the adjacent divergently transcribed gene. The mechanism of AceR-mediated regulation was further investigated by examining its binding with a DNA region upstream of the *aceI* gene. DNase I footprinting and EMSA experiments enabled the identification of two chlorhexidine-inducible AceR binding regions. Like many other LTTRs, each of the binding sites comprised an imperfect palindrome and was located near the -35 box of the promoter. The binding of AceR to these DNA sites could facilitate coordination of RNA polymerase binding to the *aceI* promoter in the presence of chlorhexidine as an inducer.

Both surface plasma resonance (SPR) and tryptophan fluorescence-quenching assays were performed to examine binding of chlorhexidine to the inducer-binding domain of AceR, which we have designated as AceRt. Similar to AceI [5], the K_d of the AceRt/chlorhexidine interaction was determined to be in the low μM range using both assays.

Taken together, the research presented in Chapter 3 provides evidence that AceR functions as a chlorhexidine-inducible transcriptional activator of *aceI*. A functional model of this regulatory system was proposed, which should provide a foundation for future work on the regulation of PACE family efflux pumps.

6.1.4 A new functional genomic method for the characterisation of efflux pumps in bacteria

A multitude of genes encoding putative antimicrobial efflux pumps have been found in sequenced bacterial genomes. However, only a tiny percentage of these have been experimentally demonstrated to mediate the efflux of antimicrobials. Identification of active multidrug efflux proteins within a large pool of potential efflux proteins can be challenging, and the regulators that regulate expression of these pumps can be even more difficult to identify using conventional methods. Chapter 4 of the thesis describes the development of a novel high-throughput screening method that combines fluorescence-activated cell sorting (FACS) and transposon-directed insertion sequencing (TraDIS), to identify genes encoding major efflux proteins and regulators in *A. baumannii.*

A population of over 100,000 random transposon mutants of *A. baumannii* were challenged with ethidium bromide, a common substrate of multidrug efflux pumps that is differentially fluorescent inside and outside the bacterial cytoplasm. We then physically enriched for mutants displaying aberrant accumulation of ethidium using FACS, and collected cells containing the highest and the lowest levels of ethidium fluorescence, respectively. The genomic locations of the transposon insertion sites within the collected cells were identified using TraDIS. Results from comparison of the insertion sites in the input mutant pool with these selected mutant pools indicated that the AdeABC, AdeIJK, and AmvA efflux pumps are

the major ethidium efflux systems in *A. baumannii*. We also identified genes that encode transcriptional repressors, such as *adeRS* and *adeN,* known to control expression of multidrug efflux systems from the most highly differentially selected genes in the flow-sorted samples. A novel regulator encoding gene, BAL062_01495, which is adjacent to and divergently transcribed from *amvA* was demonstrated to regulate expression of *amvA*. Additionally, this novel approach also identified many genes that are likely to be involved in cell division, morphology, and aggregation in *A. baumannii*.

This study provided the proof of concept that this combined technique involving FACS and TraDIS can identify genes encoding multidrug efflux pumps and their regulators. This novel approach should have a broad range of applications in microbiological research. The use of different fluorescent biocides or antibiotics should enable the characterization of drug targets and drug resistance mechanisms. The use of metal ion- or redox-sensitive fluorescent probes should enable the identification of genetic factors important in metal ion and redox cellular homeostasis. The use of fluorescent biosensors for small metabolites might be utilized in *in vitro* evolution studies for the identification of mutants with improved metabolic productivity.

6.1.5 Molecular interactions between *A. baumannii* and a co-infecting pathogen, *K. pneumonia*

In natural environments, bacteria often reside in biofilms as polymicrobial consortia, where the survival of each species within the community can be strongly dependent on interactions with other species [14]. Multidrug efflux pumps could play important roles in interactions among bacteria within polymicrobial biofilm communities. Previous studies showed that interspecies interactions could be essential for the survival of a microorganisms [15]. Quorum sensing (QS) systems have been reported to be among the most important intercellular communication mechanisms in bacteria especially in stress response and community behaviours [16]. A diverse variety of cellular functions are controlled by QS systems, such as

biosynthesis of antimicrobial peptides, metabolic switch, motility, polysaccharide synthesis and activation of many virulence factors [16]. Previous studies in *P. aeruginosa* and *E.coli* have demonstrated that multidrug efflux pumps were involved in the trafficking of autoinducers, which are chemical signalling molecules involved in QS communication of bacteria [17, 18].

Chapter 5 describes two bacterial strains *A. baumannii* (AB6870155) and *K. pneumoniae* (KP6870155) that were co-isolated from a single respiratory infection in one patient. This provided an opportunity to explore the interactions between these two pathogens and to see whether efflux pumps played any role in their interaction.

Co-culturing experiments with these two strains revealed large differences in their growth or survival depending on the culture conditions. *K. pneumoniae* predominates when grown planktonically in MH media, while both organisms co-exist planktonically in synthetic lung mimicking media, and *A. baumannii* predominates when grown in a biofilm.

These growth media-dependent differences led us to investigate the carbon source utilization capabilities of the two strains. PMs revealed *A. baumannii* and *K. pneumoniae* have very different carbon utilization profiles. The variations in carbon source consumption of the two microorganisms could provide an opportunity for the bacterial species to cooperate in their native environment, through synergistic cross-feeding interactions. Cross-feeding experiments in liquid and solid media, suggested that *A. baumannii* could be crossfed by metabolites secreted by *K. pneumoniae*. Moreover, co-cultured AB6870155 and KP6870155 were observed to form thicker biofilms than the two strains grown individually. Studies have shown that synergistic metabolic interactions can occur among bacterial species in biofilms [13, 19]. Such cross-feeding interactions might explain the observed thicker biofilms when the strains were grown together.

RNA-Seq trancriptomics of the two strains co-cultured in a biofilm revealed that many genes showed significant changes in gene expression compared to each strain grown singly. These included genes encoding chaperone-usher pilus biogenesis, amino acid catabolism and transport, and lactate production. The changes in expression level of these genes could play important roles in bacterial surface structures and growth, which might consequently contribute to the formation of the thicker mixed biofilms by the two strains.

Overall the results in Chapter 5 paint a complex picture of interactions between these two strains, and provides a unique angle for studying bacterial cross species interactions by utilizing two significant pathogens that have been isolated from a single infected patient.

6.2 Future directions

6.2.1 Exploration of the physiological substrates of the PACE efflux system

As described in Chapter 2, we have discovered a novel family of multidrug efflux pumps systems family, the PACE family. While PACE family proteins have been demonstrated to confer resistance to various biocides and whole cell transport assays have suggested this occurs via an efflux mechanism. An important future step would be to reconstitute a PACE family transporter in proteoliposomes and directly demonstrate substrate efflux. It would also be interesting to explore how different efflux proteins of the PACE family can recognize different substrates via structural and biochemical analyses.

Moreover, the defined substrates for the PACE family members are mainly biocides, which have only been synthesized in the last 50 to 150 years. However, the PACE family genes have been conserved in the core genomes of many proteobacterial species separated by hundreds of millions of years of evolution. It seems highly improbable that these synthetic compounds are the native substrates of the PACE family transporters.

Based on chemical informatic screening of a large number of natural compounds, we suspect that polyamines, highly abundant natural occurring compounds structurally similar to chlorhexidine, might be among the natural physiological substrates for the PACE family proteins. Polyamines are important natural organic compounds with two or more cationic amines, and are ubiquitously found in all living organisms [20]. The predominant polyamines in bacteria are putrescine, spermidine, spermine and cadaverine [21]. It was reported that exogenous polyamines are involved in a variety of functions including growth, drug susceptibility, virulence, biofilm formation, intercellular signalling, stress resistance, and RNA and protein synthesis [21].

Future experiments to further confirm polyamines as the physiological substrates of the PACE family transporters could include: I) to examine whether AceI confers resistance to polyamines by measurements and comparisons of MIC of polymines in both parental *A. baumannii* and Δ*aceI* mutant *A. baumannii* strains, II) to determine whether expression of *aceI* can be induced by polyamines, and III) to investigate whether polyamines can bind to AceR, the regulator of *aceI.* Additionally, other biochemical approaches can be used such as efflux transporter assays to determine whether polyamines can be transported by AceI via an efflux mechanism. Given the lack of clarity on the cellular role of polyamines, it might be interesting to conduct RNA-Seq experiments to investigate global transcriptomic level responses of polyamines in *A. baumannii.* This would likely also reveal whether the expression of other classes of multidrug efflux pump genes are induced polyamines, and hence potentially identify other polyamine efflux systems.

6.2.2 Crystal structure of the PACE family protein, AceI and its corresponding LTTR family protein, AceR

The LTTR regulator AceR was demonstrated to be involved in the activation of *aceI* expression and to bind to two DNA regions located upstream of the *aceI* promoter. Currently, no structure of a full-length LTTR in complex with DNA and its corresponding substrate has been reported. If the structures of AceR-DBD (DNA binding domain)-LH-DNA and AceR-EBD (effector binding domain)-chlorhexidine complexes could be determined, this would provide valuable insight into the mechanisms of DNA and chlorhexidine recognition by AceR.

While there are high resolution structures available for some multidrug efflux pumps, there are no crystal structures available for any members of the newly discovered PACE family. Therefore, if structures of AceI as well as AceI-chlorhexidine complexes could be determined, this would be a significant breakthrough in our understanding of these systems. It would also provide an interesting opportunity to explore functional similarities and differences between the AceI and AceR in their ability to bind chlorhexidine, and investigate how effector-induced conformational changes in the protein are achieved.

6.2.3 Insight into multispecies interactions in flow-cell biofilms

One of the limitations of the work presented in Chapter 5 was that within the timeline available it was not possible to ascertain the detailed architecture of the mixed biofilms formed by the two co-isolated microorganisms. This was due to the difficulties in fluorescently tagging the two strains, *A. baumannii* and *K. pneumoniae*, that are intrinsically highly resistant to virtually all currently used antibiotics. Therefore, it was difficult to find suitable selective markers that could be used to select, and maintain strains tagged with GFP or other fluorescent protein tags. We were able to make a plasmid-encoded GFP-tagged *K. pneumoniae* strain (data not shown), but it was unstable in biofilms, and we were not

successful in generating a tagged *A. baumannii* strain. Future work could focus on making chromosomally-integrated fluorescence markers for these strains, or fluorescent *in situ* hybridization (FISH) using species-specific probes can be considered [22]. The successful differential tagging of the two strains will enable us to distinguish their spatial arrangements in dual species biofilms by confocal microscopy.

Further investigation of the virulence of the two bacteria separately and when co-infected could be conducted using eukaryotic infection models, such as *Caenorhabditis elegans* and mouse virulence models. These eukaryotic hosts could provide the opportunity to examine both bacteria-bacteria as well as bacteria-host interactions *in vivo.*

Apart from the dual-species co-culturing, more complex polymicrobial communities can be subjects of investigation considering a very complex community structure of biofilms found in nature. Recently developed techniques such as nanoscale secondary ion mass spectrometry (nanoSIMS) [23], as well as metatranscriptomics [24], metaproteomics technologies [25], in combination with high-performance liquid chromatography and mass spectrometry, can aid in investigating these complex interactions. This will allow us to better understand the inner workings of multispecies microbial communities at both molecular and phenotypic levels.

6.2.4 Development of new therapies to combat infections caused by multidrug resistance (MDR) pathogens

Levels of antibiotic resistance in MDR pathogens have been continuously rising in the past several decades [26, 27]; however, existing antibiotics to treat these infections in patients are rapidly declining in effectiveness, resulting in a world public health crisis [28]. Our work on resistance and regulatory mechanisms of multidrug efflux systems, which are a major cause of resistance to antimicrobials in bacteria, could facilitate development of new therapies. The identification of important multidrug efflux pumps and regulators using TraDISort, could then

be screened against libraries of small molecules for potential inhibitors to act as multidrug efflux suppressors.

Since multidrug efflux pumps play significant roles in both bacterial intercellular interactions and biofilm formation. Efflux pumps involved in QS systems could be an alternative target for the development of effective antibiofilm drugs. Additionally, bacteria interspecies interactions in multispecies biofilms is a critical, but still poorly understood area. The study of bacteria-bacteria interactions within mixed species biofilms could identify new genes and regulatory systems critical for these interactions, which would have the potential to be targets for antimicrobial or antibiofilm drugs.

6.2.5 Reference

1. Boucher, H.W., et al., *Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America.* Clinical Infectious Diseases, 2009. **48**(1): p. 1-12.
2. Falagas, M.E. and P.I. Rafailidis, *Attributable mortality of Acinetobacter baumannii: no longer a controversial issue.* Critical Care, 2007. **11**(3).
3. Hassan, K.A., et al., *Homologs of the Acinetobacter baumannii AceI Transporter Represent a New Family of Bacterial Multidrug Efflux Systems.* Mbio, 2015. **6**(1).
4. Li, L.P., et al., *Rapid multiplexed phenotypic screening identifies drug resistance functions for three novel efflux pumps in Acinetobacter baumannii.* Journal of Antimicrobial Chemotherapy, 2016. **71**(5): p. 1223-1232.
5. Hassan, K.A., et al., *Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(50): p. 20254-20259.
6. Baranova, N. and H. Nikaido, *The baeSR two-component regulatory system activates transcription of the yegMNOB (mdtABCD) transporter gene cluster in Escherichia coli and increases its resistance to novobiocin and deoxycholate.* J Bacteriol, 2002. **184**(15): p. 4168-76.
7. Baranova, N.N., A. Danchin, and A.A. Neyfakh, *Mta, a global MerR-type regulator of the Bacillus subtilis multidrug-efflux transporters.* Mol Microbiol, 1999. **31**(5): p. 1549-59.
8. Tal, N. and S. Schuldiner, *A coordinated network of transporters with overlapping specificities provides a robust survival strategy.* Proc Natl Acad Sci U S A, 2009. **106**(22): p. 9051-6.
9. Krulwich, T.A., et al., *Do physiological roles foster persistence of drug/multidrug-efflux transporters? A case study.* Nat Rev Microbiol, 2005. **3**(7): p. 566-72.

10. Ahmed, M., et al., *A Protein That Activates Expression of a Multidrug Efflux Transporter Upon Binding the Transporter Substrates.* Journal of Biological Chemistry, 1994. **269**(45): p. 28506-28513.

11. Brooun, A., J.J. Tomashek, and K. Lewis, *Purification and ligand binding of EmrR, a regulator of a multidrug transporter.* Journal of Bacteriology, 1999. **181**(16): p. 5131-5133.

12. Grkovic, S., M.H. Brown, and R.A. Skurray, *Regulation of bacterial drug export systems.* Microbiology and Molecular Biology Reviews, 2002. **66**(4): p. 671-+.

13. Zaim, J. and A.M. Kierzek, *The structure of full-length LysR-type transcriptional regulators. Modeling of the full-length OxyR transcription factor dimer.* Nucleic Acids Research, 2003. **31**(5): p. 1444-1454.

14. Giaouris, E., et al., *Intra- and inter-species interactions within biofilms of important foodborne bacterial pathogens.* Front Microbiol, 2015. **6**: p. 841.

15. Leung, T.L.F. and R. Poulin, *Parasitism, Commensalism, and Mutualism: Exploring the Many Shades of Symbioses.* Vie Et Milieu-Life and Environment, 2008. **58**(2): p. 107-115.

16. Sun, J.J., Z.Q. Deng, and A.X. Yan, *Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations.* Biochemical and Biophysical Research Communications, 2014. **453**(2): p. 254-267.

17. Evans, K., et al., *Influence of the MexAB-OprM multidrug efflux system on quorum sensing in Pseudomonas aeruginosa.* Journal of Bacteriology, 1998. **180**(20): p. 5443-5447.

18. Rahmati, S., et al., *Control of the AcrAB multidrug efflux pump by quorum-sensing regulator SdiA.* Molecular Microbiology, 2002. **43**(3): p. 677-685.

19. Drescher, K., et al., *Solutions to the Public Goods Dilemma in Bacterial Biofilms.* Current Biology, 2014. **24**(1): p. 50-55.

20. Takahashi, T. and J. Kakehi, *Polyamines: ubiquitous polycations with unique roles in growth and stress responses.* Ann Bot, 2010. **105**(1): p. 1-6.

21. Shah, P. and E. Swlatlo, *A multifaceted role for polyamines in bacterial pathogens.* Molecular Microbiology, 2008. **68**(1): p. 4-16.

22. Zainal-Abidin, Z., et al., *Differential proteomic analysis of a polymicrobial biofilm.* J Proteome Res, 2012. **11**(9): p. 4449-64.

23. Kilburn, M.R., et al., *Application of nanoscale secondary ion mass spectrometry to plant cell research.* Plant Signal Behav, 2010. **5**(6): p. 760-2.

24. Martinez, X., et al., *MetaTrans: an open-source pipeline for metatranscriptomics.* Scientific Reports, 2016. **6**.

25. Hettich, R.L., et al., *Metaproteomics: harnessing the power of high performance mass spectrometry to identify the suite of proteins that control metabolic activities in microbial communities.* Anal Chem, 2013. **85**(9): p. 4203-14.

26. Michael, C.A., D. Dominey-Howes, and M. Labbate, *The antimicrobial resistance crisis: causes, consequences, and management.* Front Public Health, 2014. **2**: p. 145.

27. Viswanathan, V.K., *Off-label abuse of antibiotics by bacteria.* Gut Microbes, 2014. **5**(1): p. 3-4.

28. Zilahi, G., A. Artigas, and I. Martin-Loeches, *What's new in multidrug-resistant pathogens in the ICU?* Ann Intensive Care, 2016. **6**(1): p. 96.

Appendix

Appendix I: Biosafety approval letter

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

CRICOS Provider Number 00002J

Appendix II: Other co-author publications

ARTICLE IN PRESS

Research in Microbiology xxx (2018) 1–5

Contents lists available at ScienceDirect

Research in Microbiology

journal homepage: www.elsevier.com/locate/resmic

Pacing across the membrane: the novel PACE family of efflux pumps is widespread in Gram-negative pathogens

Karl A. Hassan [a, b, c, *], Qi Liu [c], Liam D.H. Elbourne [c], Irshad Ahmad [b], David Sharples [b], Varsha Naidu [c], Chak Lam Chan [b], Liping Li [c], Steven P.D. Harborne [b], Alaska Pokhrel [c], Vincent L.G. Postis [b, d], Adrian Goldman [b, e], Peter J.F. Henderson [b], Ian T. Paulsen [c]

[a] School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW, Australia
[b] School of BioMedical Sciences and Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, UK
[c] Department of Chemistry and Biomolecular Science, Macquarie University, North Ryde, NSW, Australia
[d] Biomedicine Research Group, Faculty of Health and Social Sciences, Leeds Beckett University, Leeds, UK
[e] Division of Biochemistry, Faculty of Biological and Environmental Sciences, University of Helsinki, Helsinki, Finland

ARTICLE INFO

Article history:
Received 5 September 2017
Accepted 8 January 2018
Available online xxx

Keywords:
Membrane transport
Gram-negative pathogen
Antimicrobial resistance
Efflux
PACE
Bacterial transmembrane pair domain

ABSTRACT

The proteobacterial antimicrobial compound efflux (PACE) family of transport proteins was only recently described. PACE family transport proteins can confer resistance to a range of biocides used as disinfectants and antiseptics, and are encoded by many important Gram-negative human pathogens. However, we are only just beginning to appreciate the range of functions and the mechanism(s) of transport operating in these proteins. Genes encoding PACE family proteins are typically conserved in the core genomes of bacterial species rather than on recently acquired mobile genetic elements, suggesting that they confer important core functions in addition to biocide resistance. Three-dimensional structural information is not yet available for PACE family proteins. However, PACE proteins have several very highly conserved amino acid sequence motifs that are likely to be important for substrate transport. PACE proteins also display strong amino acid sequence conservation between their N– and C-terminal halves, suggesting that they evolved by duplication of an ancestral protein comprised of two transmembrane helices. In light of their drug resistance functions in Gram-negative pathogens, PACE proteins should be the subject of detailed future investigation.

1. Introduction

In the broadest sense, drug resistance may arise in actively growing bacterial cells in two distinct ways: either the drug target site is protected from the toxic activities of the drug by modification or bypass, or the drug cannot reach the target site due to degradation, sequestration, reduced cellular entry or active efflux. Efflux is a major mechanism of drug resistance, and due to the high promiscuity in substrate recognition by the transport proteins involved, efflux-mediated resistance is found for a wide range of different antimicrobial compounds.

Drug efflux proteins from five distinct families of transport proteins were described between the 1970s and 2000, and have been studied extensively at both the functional and structural levels [1]. These families include the ATP-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the resistance-nodulation-cell division (RND) superfamily, the multidrug/oligosaccharidyl-lipid/polysaccharide (MOP) flippase superfamily and the drug/metabolite transporter (DMT) superfamily. In the last five years two new transporter families that include bacterial drug efflux systems have been identified; these are the proteobacterial antimicrobial compound efflux (PACE) family and the p-aminobenzoyl-glutamate transporter (AbgT) family [2–4]. Proteins from the PACE family transport biocides such as chlorhexidine and acriflavine, whereas AbgT family transporters transport sulphonamides.

2. The *Acinetobacter baumannii* AceI protein is a prototype for the novel PACE family of transport proteins

Drug efflux systems, and drug resistance factors in general, are frequently controlled by regulators that sense the transported drug

* Corresponding author. School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW, 2308, Australia.
E-mail address: Karl.Hassan@newcastle.edu.au (K.A. Hassan).

https://doi.org/10.1016/j.resmic.2018.01.001
0923-2508/

Please cite this article in press as: K.A. Hassan, et al., Pacing across the membrane: the novel PACE family of efflux pumps is widespread in Gram-negative pathogens, Research in Microbiology (2018), https://doi.org/10.1016/j.resmic.2018.01.001

substrates or their downstream effects in the cell. For example, TetR controls expression of the *tetB* tetracycline transporter gene in response to tetracyclines, and QacR controls expression of the multidrug efflux pump gene *qacA* in response to cationic antimicrobials [5]. For bacterial cells, this regulatory control means that efflux pump gene expression will proceed only when the pumps are required, saving cellular resources and preventing the potential toxic effects of constitutive high-level efflux pump expression [6]. From a research perspective, this tight regulatory control of drug efflux pump genes means that transcriptional changes may be used to identify either efflux pumps that might recognise substrates of interest, or novel factors that may be involved in drug resistance or tolerance.

The *Acinetobacter* chlorhexidine efflux protein (AceI) was identified by analysing the transcriptomic response of *A. baumannii* to the membrane active biocide chlorhexidine [2,7]. *A. baumannii* has recently emerged as major cause of Gram-negative infections in hospitals. Strains of *A. baumannii* are becoming increasingly resistant to antibiotics. Consequently, carbapenem-resistant strains of *A. baumannii* are listed as "Priority 1: CRITICAL" targets for development of new antibiotics World Health Organisation [8]. Chlorhexidine is listed as an essential medicine by the World Health Organisation, and is commonly used as an antiseptic in wound dressings, hand washes and mouthwashes. The transcriptome of *A. baumannii* ATCC 17978 cells exposed to a subinhibitory concentration of chlorhexidine, equivalent to half the minimum inhibitory concentration, was compared to control cells. The major gene expression changes were to genes encoding the AdeAB components of the AdeABC multidrug efflux pump and a gene annotated as encoding a hypothetical protein, A1S_2063 [2].

From its sequence, the A1S_2063 gene was predicted to encode an inner membrane protein with four transmembrane helices (Fig. 1A). The gene was cloned into an *Escherichia coli* expression vector and was shown to confer significant levels of resistance to chlorhexidine when overexpressed in *E. coli*. Deletion of the A1S_2063 gene in *A. baumannii* ATCC 17978 and its orthologue in *Acinetobacter baylyi* ADP1 halved the chlorhexidine resistance in the host strain, demonstrating that the genes had a resistance function in native hosts [2,9]. The Biolog Phenotype Microarray system was used to screen over 200 additional antimicrobials against A1S_2063 expressing *E. coli*. This analysis demonstrated no significant hits apart from chlorhexidine, suggesting that chlorhexidine had an apparent specificity as a substrate [2].

When overexpressed in *E. coli*, the A1S_2063 protein was identified in the inner membrane. The protein could be readily extracted from the membrane by detergent solubilisation and purified. The detergent-solubilised protein bound to chlorhexidine with high affinity (K_d in the low μM range) as determined by tryptophan fluorescence quenching and near-UV synchrotron radiation circular dichroism [2]. Transport experiments using [^{14}C]-chlorhexidine demonstrated that the A1S_2063 protein prevented the high-level accumulation of chlorhexidine when expressed in *E. coli* until the proton-motive-force across the membrane was collapsed using a protonophore. The protein could also mediate the efflux of chlorhexidine from *E. coli* cells preloaded with chlorhexidine [2]. Together, these results suggested that the A1S_2063 protein was a novel chlorhexidine efflux protein and that transport is likely to be proton-coupled. The protein was named AceI (*Acinetobacter* chlorhexidine efflux protein I).

3. PACE proteins are a family of multidrug efflux systems conserved across many Gram-negative pathogens

Genes encoding proteins homologous to AceI are found in the genomes of many bacterial species, including pathogens such as *Pseudomonas*, *Klebsiella*, *Enterobacter*, *Salmonella* and *Burkholderia* species. These genes are particularly common among Proteobacteria, but can be found in some Actinobacteria and in a limited number of other unrelated bacterial species. To determine whether, like AceI, these proteins can mediate chlorhexidine resistance, more than 20 phylogenetically diverse homologues were cloned into an *E. coli* expression system and examined by routine minimum inhibitory concentration analyses. Most of the cloned proteins were expressed at detectable levels, and about half could confer resistance to chlorhexidine [3]. Notably, at least two of the *aceI* homologues found to confer chlorhexidine resistance are also highly expressed in their native hosts, *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, in response to a chlorhexidine treatment [10,11].

Additional resistance tests were performed to determine whether the antimicrobial recognition profiles of these homologues might extend beyond chlorhexidine. Many of the proteins were able to confer resistance to several additional biocides, including acriflavine, proflavine, benzalkonium and dequalinium [3]. The substrate profile of one pump, VP1155 encoded by *Vibrio parahaemolyticus*, was investigated using the Biolog phenotype microarray system. In addition to chlorhexidine, benzalkonium, proflavine and acriflavine, VP1155 appeared to confer resistance to 9-aminoacridine, domiphen bromide, guanazole and plumbagin [3].

The demonstration that many AceI homologues are able to confer resistance to compounds such as proflavine and acriflavine presented the possibility of assaying transport by measuring their fluorescence in real time [12]. These compounds intercalate into nucleic acids, which leads to a quenching of their fluorescence. This property facilitates a convenient assay for their transport in cells expressing an efflux pump [13]. Cells expressing the protein of interest can be loaded with proflavine or acriflavine in the presence of a protonophore, such as carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), then washed and re-energised by the addition of an energy source such as D-glucose. Fluorescence can be monitored before and after energisation to examine transport [12]. These transport experiments have been performed for a number of AceI homologues and identified proteins that mediate transport of these compounds. For example, the *B. cenocepacia* HI2424 homologue Bcen2424_2356 is able to transport acriflavine, whereas at least one other homologue encoded by this strain, Bcen2424_5347, does not (Fig. 2). Bcen2424_2356 has been previously shown to confer resistance to chlorhexidine, benzalkonium, proflavine and acriflavine. The Biolog phenotype microarray antimicrobial resistance tests confirmed several of these phenotypes and suggested that Bcen2424_2356 also confers resistance to benzethonium, 9-aminoacridine, methyl viologen, guanazole and plumbagin (Supplemental Fig. S2).

The observation that several AceI homologues can confer resistance to multiple biocides and can mediate transport of the fluorescent substrates proflavine and acriflavine led to their designation as a new family of efflux pumps. This family was called the Proteobacterial Antimicrobial Compound Efflux (PACE) family, due to their abundance in Proteobacteria [3]. To date at least ten putative substrates have been identified for PACE family proteins (Fig. S3). Proteins from this family have been incorporated into the Transporter Classification Database [14] under the original family title, the proteobacterial chlorhexidine efflux (CHX) family (TCDB number: 2.A.117), and are captured in the TransportDB 2.0 database [15], which catalogues all putative transport proteins from sequenced genomes in the NCBI RefSeq database.

4. Predicted topology and sequence conservation in PACE pumps

All PACE family proteins analysed to date are predicted to contain four transmembrane α-helices, organised into two tandem

Please cite this article in press as: K.A. Hassan, et al., Pacing across the membrane: the novel PACE family of efflux pumps is widespread in Gram-negative pathogens, Research in Microbiology (2018), https://doi.org/10.1016/j.resmic.2018.01.001

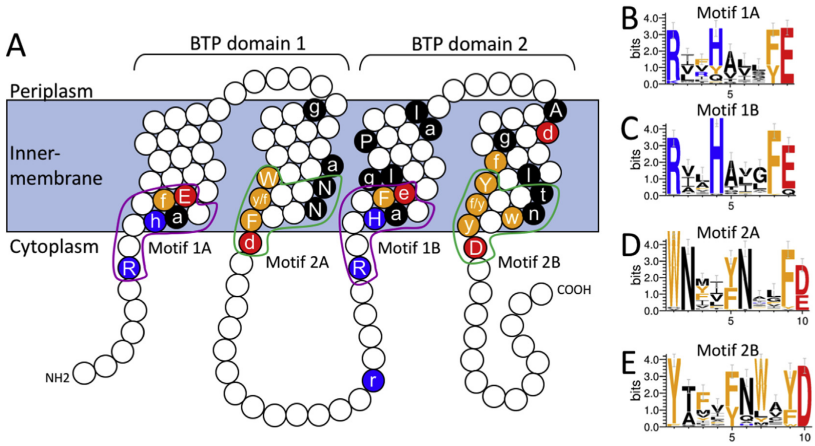


Fig. 1. Predicted transmembrane topology and conserved amino acid sequence motifs present in PACE family proteins. An amino acid sequence alignment of 47 diverse PACE family proteins (Supplemental Fig. S1), encoded by a broad range of hosts, was used to identify amino acid sequence motifs that are conserved across the family (A) Predicted topology of PACE family proteins. Upper case characters are conserved in greater than 90% of protein sequences and characters in lower case are conserved in greater than 65% but fewer than 90% of the aligned protein sequences. Conserved residues are coloured (red: negatively charged, blue: positively charged, orange: aromatic, black: others). The similar amino acid sequence motifs in helices 1 and 3 (1A and 1B, respectively) are surrounded by purple lines, and the similar sequence motifs in helices 2 and 4 (2A and 2B, respectively) are surrounded by green lines (B–E) Sequence logos, made using WebLogo 3 [24], showing conservation of amino acid residues in the four sequence motifs identified in PACE proteins; error bars show an approximate Bayesian 95% confidence interval [24]. (For interpretation of the references to color/colour in this figure legend, the reader is referred to the Web version of this article.)

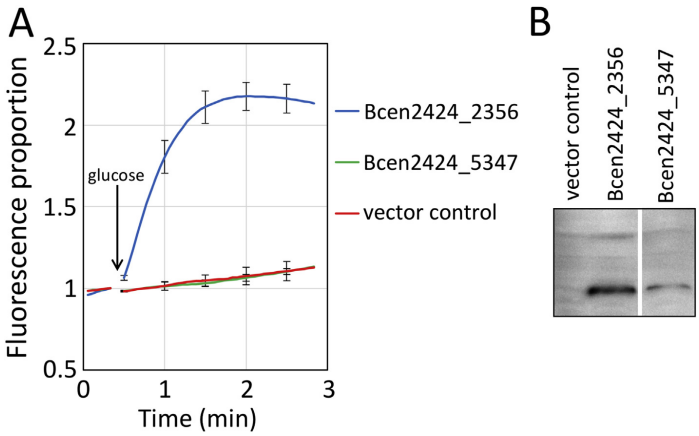


Fig. 2. Acriflavine transport mediated by PACE family proteins encoded by the human pathogen *Burkholderia cenocepacia* HI2424. A) Transport experiments performed using *E. coli* OmniMax cells expressing the proteins of interest, essentially as described previously [3]. The cells were preloaded with 5 μM acriflavine in the presence of 10 μM CCCP. The cells were washed and re-energised using glucose at the point marked with an arrow and transport monitored fluorimetrically. Acriflavine fluorescence is quenched when it is intercalated into nucleic acids. Therefore, its transport out of the cell is associated with an increase in fluorescence. B) Western blot performed on the cells used in the assay, probed using His-Probe-HRP. The *B. cenocepacia* HI2424 PACE family proteins Bcen2424_2356 and Bcen2424_5347 were expressed at easily detectable levels after the 1-h induction used for this assay. Bcen2424_2356 promoted the rapid efflux of acriflavine, whereas Bcen2424_5347 did not promote acriflavine transport above background. The third PACE family protein encoded by *B. cenocepacia* HI2424, Bcen2424_5167 was not expressed and was excluded from the figure. Bcen2424_5167 and Bcen2424_5347 are phylogenetically distinct from Bcen2424_2356. Error bars show the standard deviation of three replicate experiments.

bacterial transmembrane pair (BTP) domains (Fig. 1; pfam: PF05232) [16]. Given their small size, it seems very likely that PACE proteins function as oligomers. However, the oligomeric state of PACE family proteins remains unresolved. Several PACE family proteins have been experimentally characterised by overexpression and purification (Henderson et al., unpublished). When expressed in *E. coli*, these proteins localise to the inner membrane and can be readily purified by extraction with a mild detergent such as n-dodecyl-β-D-maltoside [2], or using a styrene maleic acid co-polymer (Supplemental Fig. S4) [17]. Analysis of the purified

Please cite this article in press as: K.A. Hassan, et al., Pacing across the membrane: the novel PACE family of efflux pumps is widespread in Gram-negative pathogens, Research in Microbiology (2018), https://doi.org/10.1016/j.resmic.2018.01.001

detergent-solubilised proteins by far-UV circular dichroism has confirmed their high α-helical content and demonstrated that they typically show structural stability to around 50–60 °C [2].

A high level of amino acid sequence conservation is apparent between members of the PACE family. Based on an alignment of 47 diverse PACE family proteins from a variety of bacterial genera (Supplemental Fig. S1), four amino acid residues appear to be universally conserved across these proteins: a glutamic acid residue within transmembrane helix 1, an asparagine residue in transmembrane helix 2, an alanine residue at the periplasmic membrane boundary of transmembrane helix 4 and an aspartic acid residue at the cytoplasmic membrane boundary of transmembrane helix 4 (Fig. 1A). The functional importance of the conserved asparagine, alanine and aspartic acid residues has not yet been investigated, but neutralisation of the glutamic acid residue in the prototypical PACE family member AceI by substitution with a glutamine abolished chlorhexidine resistance and transport [2]. This mutant (E15Q) was still able to bind chlorhexidine with only slightly reduced affinity compared to the parental protein. Furthermore, the mutant protein was less thermostable than the parental protein in the absence of chlorhexidine, but was significantly more stable than the parental protein in the presence of a molar excess of chlorhexidine, supporting a binding interaction. These results suggest that the glutamic acid residue is not required for substrate binding. The high conservation of this residue in PACE proteins that have different substrate specificities suggests that it may be involved in an aspect of transport common to these proteins, such as an energy coupling reaction. The position of this residue within the transmembrane region is consistent with this possibility. In other efflux pumps from the MFS, SMR and MATE families, membrane embedded carboxyl residues participate in coupling reactions [18–20].

PACE family proteins contain several highly conserved amino acid residues in addition to the four universally conserved residues. The amino acid sequence conservation is particularly strong close to the predicted cytoplasmic boundaries of the transmembrane helices, where four amino acid sequence motifs have been identified (Fig. 1). In line with the PACE proteins containing tandem BTP domains, the amino acid sequence motif in transmembrane helix 1 (motif 1A; RxxhaxxfE, where upper case residues are conserved in more than 90% of proteins and lower case residues in at least 65% of proteins) is very similar to that in transmembrane helix 3 (motif 1B; RxxHaxxFe) (Fig. 1B and C), and the motif in transmembrane helix 2 (motif 2A, WNxxy/fNxxFd) is very similar to that in transmembrane helix 4 (motif 2B; Ytxxf/ynwxyD) (Fig. 1D and E). The notable features of the sequence motifs in helices 1 and 3 are the membrane-embedded glutamate residue (universally conserved in helix 1), and histidine and arginine residues at the membrane boundary. The motifs found in helices 2 and 4 notably contain several aromatic residues along one helical face adjacent to polar asparagine residues, and an aspartate residue at the membrane boundary (universally conserved in helix 4) (Fig. 1A).

Based on the distribution of charged residues within the loop regions, the N– and C-termini of most PACE family proteins are predicted to lie within the cytoplasm, but this is yet to be experimentally tested (Fig. 1). Some PACE family homologues, primarily from *Acetobacter*, contain predicted N-terminal signal sequences, suggesting that the N-terminus is moved across the cytoplasmic membrane and that they may exist in an alternative topology, e.g. APA01_04520 and APO_1949 from *Acinetobacter pasteurianus* IFO 3283-01 and *Acinetobacter pomorum* DM001, respectively. Representatives of these proteins have been expressed in *E. coli*, but as yet, no resistance or transport functions have been identified (Hassan et al., unpublished). These proteins may be part of a separate protein subfamily from those that mediate drug resistance.

5. Conservation of PACE family genes

PACE family proteins are typically highly conserved in the genome of the bacterial species in which they are found. For example, genes encoding three different PACE proteins have been identified in the *A. baumannii* pan-genome (based on the genomes of 623 strains) [7]. Of these, two were conserved in 100% or close to 100% of the strains and can be considered to be part of the core genome. The third gene was found in only two strains and is part of the accessory genome. Similar to *A. baumannii*, *P. aeruginosa* isolates have two PACE proteins encoded in the core genome and one in the accessory genome, which is found in only a few strains, and *B. cenocepacia* strains encode three PACE pumps in their core genome [7]. This high level of conservation suggests that PACE pumps are acquired vertically and have been maintained in their host species since their divergence from related organisms. They are thus likely to have an important core function that may be unrelated to drug resistance. Indeed, the biocides that are recognised by PACE family pumps have only been present in the environment for 50–100 years, and are therefore very unlikely to be the physiological substrates of these proteins.

In contrast to the species described above, *E. coli* do not encode PACE pumps in their core genomes; four different genes encoding PACE homologues were found among the genomes of 1986 sequenced *E. coli* strains, but these were each found in 0.2% of strains or less [7]. These accessory genes are likely to move between related species on mobile genetic elements. However, there is as yet no strong evidence for how these genes are mobilised.

6. Evolution of the PACE family

The conservation of sequence motifs between the N– and C-terminal halves of PACE proteins suggests that these proteins may have evolved by a duplication event of an ancestral single BTP domain protein. To investigate this further, the N– and C-terminal BTP domains were compared between 47 diverse PACE family proteins (Supplemental Fig. S5). The level of amino acid identity between the N– and C-terminal BTP domains in these proteins ranged from 6.6% to 33.3% (mean 23.3%). The presence of such high levels of sequence identity between the N– and C-terminal BTP domains across diverse PACE family proteins suggests that these proteins may not have diverged significantly since the occurrence of the duplication event(s). Along with the distribution of these proteins almost exclusively within the Proteobacteria, and their likely vertical acquisition, due to their presence on the core genome, this may suggest that this protein family is relatively young compared to other families of transport proteins, which show lower levels of sequence conservation between domains that are thought to have arisen via duplication [21].

To further examine the evolution of PACE family proteins, the levels of sequence identity between the N-terminal and C-terminal BTP domains of different PACE proteins were determined. It was found that the N-terminal BTP domains of PACE family proteins almost always have higher identity to the N-terminal BTP domains of other PACE proteins than they do to their own C-terminal BTP domain, or the C-terminal BTP domain of other PACE family pumps (Supplemental Fig. S5). This suggests that a BTP domain duplication event occurred only once in an ancestral gene, and that there is little or no recombination between the N– and C-terminal BTP domains in individual strains. The C-terminal BTP domains of different PACE pumps typically show even higher levels of sequence identity than the N-terminal domains (Supplemental Fig. S5). The high conservation of sequence within the C-terminal domain of different proteins may reflect the involvement of the C-terminal domain in a core part of the

Please cite this article in press as: K.A. Hassan, et al., Pacing across the membrane: the novel PACE family of efflux pumps is widespread in Gram-negative pathogens, Research in Microbiology (2018), https://doi.org/10.1016/j.resmic.2018.01.001

functional mechanism, whereas the N-terminal domain may play a bigger role in substrate recognition.

7. Concluding remarks

The PACE family of transport proteins is one of two transporter families discovered only recently to mediate drug efflux. From currently available analyses, PACE family proteins display somewhat restricted drug substrate recognition profiles, which include primarily synthetic biocides such as chlorhexidine and acriflavine, rather than the multitudes of diverse antibiotics and biocides recognised by transport proteins from families such as the RND superfamily. This may be a primary reason for the family being only recently identified, 15 years after the first descriptions of MATE family pumps [22,23]. However, PACE proteins are highly conserved in a range of opportunistic Gram-negative pathogens, including *A. baumannii*, *P. aeruginosa*, *B. cenocepacia* and *Klebsiella pneumoniae*, and in serious human pathogens such as *Yersinia pestis*, *Francisella tularensis* and *Burkholderia pseudomallei*. Therefore, the role of these proteins in drug resistance warrants future investigation.

As mentioned above, the drug recognition profile of PACE pumps primarily includes synthetic biocides, most of which have only been in the environment for 50–100 years. However, genes encoding homologous PACE family proteins are found in the core genomes of bacterial genera that diverged much earlier than this, hundreds of millions of years ago. Therefore, these proteins are likely to mediate an important core function and may have common physiological substrates that are yet to be described. The importance of PACE family proteins is likely to extend beyond an apparently fortuitous role in drug resistance.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

Parts of this work were supported by Project Grants from the National Health and Medical Research Council of Australia to ITP, KAH and PJFH (GNT1060895 and GNT1120298), a grant from Macquarie University to KAH and LDHE (9201401563) and a grant from the BBSRC to AG (104399). KAH thanks the EU for the award of a Marie Skłodowska-Curie Research Fellowship (706499). PJFH thanks the Leverhulme Trust for the award of an Emeritus Fellowship (EM-2014-045). CLC thanks the BBSRC for support through a White Rose Research Experience Placement award.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.resmic.2018.01.001.

References

[1] Du D, van Veen HW, Murakami S, Pos KM, Luisi BF. Structure, mechanism and cooperation of bacterial multidrug transporters. Curr Opin Struct Biol 2015;33:76–91.
[2] Hassan KA, Jackson SM, Penesyan A, Patching SG, Tetu SG, Eijkelkamp BA, et al. Transcriptomic and biochemical analyses identify a family of chlorhexidine efflux proteins. Proc Natl Acad Sci USA 2013;110:20254–9.
[3] Hassan KA, Li Q, Henderson PJF, Paulsen IT. Homologs of the *Acinetobacter baumannii* AceI transporter represent a new family of bacterial multidrug efflux systems. mBio 2015;6. e01982–14.
[4] Su CC, Bolla JR, Kumar N, Radhakrishnan A, Long F, Delmar JA, et al. Structure and function of Neisseria gonorrhoeae MtrF illuminates a class of antimetabolite efflux pumps. Cell Rep 2015;11:61–70.
[5] Grkovic S, Brown MH, Skurray RA. Regulation of bacterial drug export systems. Microbiol Mol Biol Rev 2002;66:671–701.
[6] Andersson DI, Levin BR. The biological cost of antibiotic resistance. Curr Opin Microbiol 1999;2:489–93.
[7] Hassan KA, Elbourne LD, Li L, Gamage HK, Liu Q, Jackson SM, et al. 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 2015;6:333.
[8] World Health Organization. Global Priority List of Antibiotic-resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics. Geneva. 2017.
[9] Tucker AT, Nowicki EM, Boll JM, Knauf GA, Burdis NC, Trent MS, et al. Defining gene-phenotype relationships in Acinetobacter baumannii through one-step chromosomal gene inactivation. mBio 2014;5:e01313–4.
[10] Nde CW, Jang HJ, Toghrol F, Bentley WE. Global transcriptomic response of Pseudomonas aeruginosa to chlorhexidine diacetate. Environ Sci Technol 2009;43:8406–15.
[11] Coenye T, Van Acker H, Peeters E, Sass A, Buroni S, Riccardi G, et al. Molecular mechanisms of chlorhexidine tolerance in Burkholderia cenocepacia biofilms. Antimicrob Agents Chemother 2011;55:1912–9.
[12] Turner RJ, Taylor DE, Weiner JH. Expression of Escherichia coli TehA gives resistance to antiseptics and disinfectants similar to that conferred by multidrug resistance efflux pumps. Antimicrob Agents Chemother 1997;41:440–4.
[13] Blair JM, Piddock LJ. How to measure export via bacterial multidrug resistance efflux pumps. mBio 2016;7. e00840–16.
[14] Saier Jr MH, Reddy VS, Tsu BV, Ahmed MS, Li C, Moreno-Hagelsieb G. The transporter classification database (TCDB): recent advances. Nucleic Acids Res 2016;44:D372–9.
[15] Elbourne LD, Tetu SG, Hassan KA, Paulsen IT. TransportDB 2.0: a database for exploring membrane transporters in sequenced genomes from all domains of life. Nucleic Acids Res 2017;45:D320–4.
[16] Finn RD, Coggill P, Eberhardt RY, Eddy SR, Mistry J, Mitchell AL, et al. The Pfam protein families database: towards a more sustainable future. Nucleic Acids Res 2016;44:D279–85.
[17] Lee SC, Knowles TJ, Postis VL, Jamshad M, Parslow RA, Lin YP, et al. A method for detergent-free isolation of membrane proteins in their local lipid environment. Nat Protoc 2016;11:1149–62.
[18] Fluman N, Ryan CM, Whitelegge JP, Bibi E. Dissection of mechanistic principles of a secondary multidrug efflux protein. Mol Cell 2012;47:777–87.
[19] Soskine M, Adam Y, Schuldiner S. Direct evidence for substrate-induced proton release in detergent-solubilized EmrE, a multidrug transporter. J Biol Chem 2004;279:9951–5.
[20] Radchenko M, Symersky J, Nie R, Lu M. Structural basis for the blockade of MATE multidrug efflux pumps. Nat Commun 2015;6:7995.
[21] Saier Jr MH. Tracing pathways of transport protein evolution. Mol Microbiol 2003;48:1145–56.
[22] Morita Y, Kodama K, Shiota S, Mine T, Kataoka A, Mizushima T, et al. NorM, a putative multidrug efflux protein, of *Vibrio parahaemolyticus* and its homolog in *Escherichia coli*. Antimicrob Agents Chemother 1998;42:1778–82.
[23] Brown MH, Paulsen IT, Skurray RA. The multidrug efflux protein NorM is a prototype of a new family of transporters. Mol Microbiol 1999;31:394–6.
[24] Crooks GE, Hon G, Chandonia JM, Brenner SE. WebLogo: a sequence logo generator. Genome Res 2004;14:1188–90.
[25] Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and clustal X version 2.0. Bioinformatics 2007;23:2947–8.
[26] Okonechnikov K, Golosova O, Fursov M. Team U. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics 2012;28:1166–7.
[27] Ward A, Sanderson NM, O'Reilly J, Rutherford NG, Poolman B, Henderson PJF. The amplified expression, identification, purification, assay, and properties of hexahistidine-tagged bacterial membrane transport proteins. In: Baldwin SA, editor. Membrane transport: a practical approach. Oxford: Oxford University Press; 2000. p. 141–66.
[28] Stark MJ. Multicopy expression vectors carrying the *lac* repressor gene for regulated high-level expression of genes in *Escherichia coli*. Gene 1987;51:255–67.
[29] Campanella JJ, Bitincka L, Smalley J. MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinf 2003;4:29.

Please cite this article in press as: K.A. Hassan, et al., Pacing across the membrane: the novel PACE family of efflux pumps is widespread in Gram-negative pathogens, Research in Microbiology (2018), https://doi.org/10.1016/j.resmic.2018.01.001

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

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

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

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.

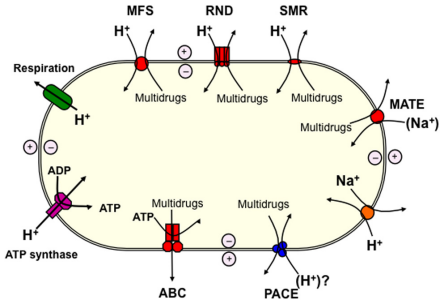


FIGURE 1 | Schematic diagram showing the basis for the energisation of multidrug efflux pumps operating in bacteria. The large oval represents the bacterial cell. An electrochemical H^+ gradient (proton-motive-force) across the cytoplasmic membrane is generated as a result of respiration (green). Energy 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

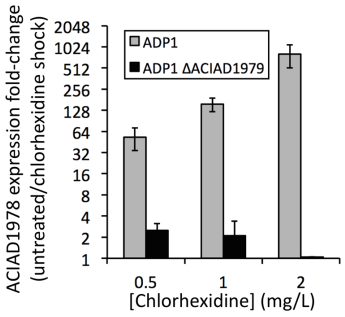


FIGURE 2 | Expression of the PACE family gene, ACIAD1978, in wild-type and mutant (ΔACIAD1979) *A. baylyi* ADP1 in response to chlorhexidine shock treatments. Cells were grown in LB broth to OD_{600} = 0.6, then split, with one half of each culture treated with chlorhexidine and the other half receiving 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.