University of Leicester

Graduate School

ANALYSIS OF THE FITNESS COST OF INTEGRONS IN CLINICAL ISOLATES OF *ACINETOBACTER BAUMANNII*

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by
Eva Horvath-Papp BSc (Hons)
Department of Infection, Immunity and Inflammation
University of Leicester

November 2014

Abstract

Analysis of the fitness cost of integrons in clinical isolates of *Acinetobacter baumannii*

Eva Horvath-Papp

Antibiotic resistance determinants carried on mobile genetic elements such as integrons are widespread in *Acinetobacter baumannii*. A total of 76 clinical isolates of *A. baumannii* were screened for class 1, 2 and 3 integron structures using PCR-based assays. Further chromosome walking and Southern blot-based analyses revealed a significant proportion of incomplete integrons, primarily due to IS element-mediated deletions or truncations at the 5' end of these integrons. Based on these multiple detection methods, a total of 72 integron structures distributed among 58 isolates were identified. Remarkably, 29 (40.3 %) were either incomplete, or had a truncated integrase gene. These data also revealed that using established PCR-based detection methods alone would yield high rates of false-negative and –positive results. The high frequencies of *intI1* and/or 5' end integron truncations suggest that IntI1 amelioration confers a significant selective advantage. This hypothesis was investigated in *E. coli* and *A. baumannii* backgrounds using an IPTG-inducible IntI1-expressing plasmid. Significant fitness costs were observed through growth curve analysis when IntI1 was overexpressed in *recA*-deficient *E. coli* lab strains, and also in an *A. baumannii* strain. The latter was predicted to have a ~1.5-fold higher number of non-canonical IntI1 recombination sites in a typical *E. coli* strain. The archetypal *intI1* repressor, LexA, is not present in the *A. baumannii* genome, so the role of the closest homologue, $UmuD_{Ab}$, was investigated through knockout studies. $\Delta umuD_{Ab}$ mutants were created through allelic exchange, and although the mutants showed a significant upregulation of *intI1* expression, when the mutants were complemented with a $umuD_{Ab}$-overexpressing plasmid, *intI1* expression was also increased, albeit to a lesser extent. This study demonstrates a complex DNA-damage response in *A. baumannii*, and will aid further investigation for a full understanding of the development and maintenance of antibiotic resistance in this important nosocomial pathogen.

Acknowledgements

I would like to thank my supervisor Dr Kumar Rajakumar for giving me the opportunity to undertake this PhD, and for introducing me into the many aspects of scientific world. With his assistance, I have achieved many of the objectives I set out to do at the beginning of my journey.

Furthermore, I'm very grateful to have been part of lab 212, and give thanks to all past and present members. First and foremost, many thanks to Hari Meenakshi Sundaramoorthy, who worked with me and provided a crucial sounding board and assistance for many aspects of this project. Without him, this project would not have developed in the same way. Many thanks go also especially to Dr Ewan Harrison, Dr Jon van Aartsen, Dr Barbara Rieck, Dr Mahmoud Tawfick and Dr Marialuisa Crosatti, to whom I am indebted for helping me master the basics of molecular biology in the early stages of my PhD. I especially thank Dr Barbara Rieck for also helping in the later stages of my PhD, with revisions of chapters and manuscripts. In addition, I want to thank the many others who have come and gone through 212 over the years – in particular Andrew Santiago-Frangos, Jainy Shah, Dr Natia Karumidze, Donna Mathews and Dona Reddiar - for cheering up dreary days in the lab through their companionship and wit.

I'm also very grateful to the wider members of the Infection, Immunity and Inflammation community, specifically Dr Jinyu Shan for assistance with the Southern blotting technique, and Dr Hany Kenawy for assistance with statistical analysis. I would also like to express my gratitude to Dr Galina Mukamolova and Professor Wilhelm Schwaeble for guidance and advice throughout my PhD. Many thanks also to Dr Primrose Freestone, who gave me the opportunity to learn teaching skills and work with some fantastic undergraduates, some of whom I now call friends. In addition, I'm grateful to all the students and staff who have been a source of useful discussions and friendly support in the computer room, including Dr Sadiyo Siad, Dr Nawal Helmi, Dr Nima Abbasian, Maryam Ghaderi, and many others.

A very special thanks to Dr Asha Akram, who I'm very grateful was able to accompany me throughout our respective PhD journeys, as we supported each other through the emotional ups and downs, right from the beginning of our studies. I'm also very happy that she was by my side to also assist as

bridesmaid at my wedding. Without Asha, my PhD journey would not have been the same.

I would also like to thank Prof Hong-Yu Ou for facilitating my very stimulating and exciting exchange visit to Shanghai Jiao Tong University, and all the members of his group who very kindly welcomed me and ensured that I was fully immersed in both the lab community and Chinese culture. Special thanks to Lu Liu who was always on hand to help with any issues and who welcomed me into her home, and also my fellow exchange colleagues Dexi Bi and Yingzhou Xie, who provided ample advice, much-needed computing assistance and never failed to be dependable in any other requests I had of them. 我永远不会忘记曾与在座各位在上海拥有的美好时光.

During my journey, I had the great fortune to meet with many interesting researchers. I'm very grateful to Dr Chris Holmes, who was very patient with me during my time as a research assistant at the UHL Microbiology department, and taught me the basics of several techniques, as well as being the source of plenty of PhD advice. I also give thanks to Dr Bruno S Lopes, for many discussions about *Acinetobacter* and the world. I'm also very grateful to have met Dr Pål Johnsen and Professor Kåre M. Nielsen, who provided invaluable discussion and exchange of ideas regarding our shared research interests. I give thanks to the other current and former group members at University of Tromsø, whose work has been vital for the understanding of my project. Many thanks also to Dr Thomas Jové, for technical assistance with sequence and integron analysis as well as being a constant source of jovial jokes and chats on integrons, despite having never met in person.

Thanks also to Dr Jorge Cham and Dr Nik Papageorgiou for helping to cheer up myself and many other PhD students worldwide, through their inspired and irreverent web comics "PhD Comics" and "The Upturned Microscope". Knowing that there are plenty of others in the same boat is a great motivator, and these cartoons remind us that during a PhD, you've got to laugh, or you'll cry.

This PhD would not have been possible without my parents, Dr Agnes Ayton, Dr Imre Horvath-Papp and Dr Andrew Ayton, who have supported me throughout my PhD, and continue to support all my education and betterment

as a person. They remain inspirational and encouraging in my life. I'm grateful for the support of my wider family as well, especially my sisters.

Most importantly, thanks to my husband Samuel, for showing me love and support throughout my PhD journey, and who taught me not to give up even when it seemed as though there was "no data", and to always remain positive in the face of adversity!

Lastly, I'd like to end with some quotes with significant meaning for me. The first is one by famous Hungarian Nobel Prize winner Albert Szent-Györgyi:

"*A tudományos módszer lényege, hogy a problémákat mint problémákat kezeli, így keresi a legjobb megoldást, előítéletek és sovinizmus nélkül. Nem azt kérdezzük, hogy kinek van igaza, hanem azt, hogy mi az igazság.*"

"*The scientific method is to treat the problems as problems; in this way, the best solution can be found without prejudice and chauvinism. We do not ask who is right, but what is right*"

The second is was one my friend Dr Maria Fraser shared with me at the beginning of my PhD, which I hold to be even more true after all the years spent on this journey:

"Οὔτοι ἀπ' ἀρχῆς πάντα θεοὶ θνητοῖς ὑπέδειξαν,

ἀλλὰ χρόνῳ ζητοῦντες ἐφευρίσκουσιν ἄμεινον."

"The gods did not reveal all things to mortals in the beginning;

But in long searching, man finds out that which is better"

~ Xenophanes, 6th c. B.C.

And lastly, I'd like to include a quote from my grandfather's PhD thesis, whose dedication and hard work in science has been an inspiration for me:

"*Only lunatics can be completely original*"

~ W. Durant

Abbreviations

λ	Lambda
µg	Microgram
µl	Microliters
µM	Micromolar
bp	Base pair
cDNA	Complementary DNA
CFU	Colony forming units
CS	Conserved segion
C_t	Cycle threshold
DCO	Double cross-over
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DR	Direct repeat
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraaceti c acid
EMSA	Electromobility shift assay
EtBr	Ethidium bromide
EUR	European acquired isolates
FRT	Flp recombinase target
g	Grams
gDNA	Genomic DNA
GC	Global clone
GI	Genomic island
h	Hours
HCN	High copy number
HGT	Horizontal Gene Transfer
HPI	High pathogenicity island
IC	International clone
ICE	Integrative conjugative elements
IMU	Integron mobilisation unit
IPTG	Isopropyl-β-D-thiogalactopyranoside
IS	Insertion Sequence
kb	Kilobase pair
kDa	Kilodalton
l	Litres
LA	Lysogeny broth agar
LB	Lysogeny broth
LCN	Low copy number
LF	Left homologous flank
M	Molar
Mb	Megabase pairs
MCS	Multiple cloning site
mg	Milligram
MGE	Mobile genetic element
min	Minutes
MITE	Miniature inverted repeat transposable elements
ml	Millilitres
MLST	Multilocus sequence typing
mM	Millimolar
ng	Nanograms
nH_2O	Nanopure, PCR-grade water
nM	Nanomolar
°C	Degrees Celsius
OD_{xxx}	Optical density at XXXnm
ORF	Open reading frame
PAI	Pathogenicity island
PCN	Plasmid copy number
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real time polymerase chain reaction
RF	Right homologous flank
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SCO	Single crossover
ssDNA	Single-stranded DNA

Table of Contents

List of Figures

List of Tables

Presentations and grants

Presentations

University of Leicester: Department of Infection, Immunity and Inflammation Annual Postgraduate Student Conference, April 2010

Oral presentation entitled: "*The genetics of aminoglycoside resistance in Acinetobacter baumannii*"

University of Leicester: Department of Infection, Immunity and Inflammation Annual Postgraduate Student Conference, April 2011

Oral presentation entitled: "Carriage of antibiotic resistance determinants on integrons in *Acinetobacter baumannii*"

University of Leicester: Department of Infection, Immunity and Inflammation Annual Postgraduate Student Conference, April 2013

Oral presentation entitled: "Understanding the contribution of Integrons to Multi-Drug Resistance in *Acinetobacter baumannii"*

British Society for Antimicrobial Chemotherapy, Antibiotic Resistance Mechanisms Workshop for Researchers, Holiday Inn, Birmingham, UK: November 2012

Poster presentation entitled: "Characterization of gene cassette arrays found in class 1 integrons in clinical isolates of *Acinetobacter baumannii*"

9th International Symposium on the Biology of Acinetobacter, Maternushaus, Cologne, Germany: June 2013

Poster presentation entitled: "Characterization of gene cassette arrays found in class 1 integrons in clinical isolates of *Acinetobacter baumannii*"

Grants

The Society for General Microbiology

Travel Grant for the attendance of the annual meeting, in Harrogate, UK, April 2010

The Society for General Microbiology

Travel Grant for the attendance of the Irish meeting, in Dublin, Ireland, September 2011

Federation of European Microbiological Societies

Travel Grant, for the attendance of "*Acinetobacter* 2013" - 9th International Symposium on the Biology of *Acinetobacter,* June 2013

Chinese Scholarship Council and British Council

Postgraduate Student Exchange grant, as part of the Sino-UK Higher Education Research Partnership, September - December 2013

Chapter 1. Introduction

The first documented description of an *Acinetobacter* species was in the early 1900s, when Beijerinck, a Dutch microbiologist identified an organism isolated from soil samples in enrichment in a calcium acetate-containing minimal medium. He referred to this organism as *Micrococcus calcoaceticus*. Since then, the taxonomy of the *Acinetobacter* genus has gone through a large number of changes as new organisms were discovered and reclassified under a range of different names. This has resulted in some papers being published in order to "dispel some of the confusion" (Henriksen 1973).

Although it has been identified as a human pathogen since the first descriptions, *Acinetobacter baumannii* in particular has become an increasing nosocomial problem in the last thirty years. This comes from a background in which as recently as the early 1970s, it was still possible to treat infections caused by this species with a range of antibiotics, compared to today, when multidrug resistance (MDR) is widespread, and in some cases there are no treatment options available (Pan-Drug Resistant or PDR). MDR strains are particularly a problem in the Middle East, which earned *A. baumannii* the colloquial term "Iraqibacter" after it affected significant numbers of military personnel in medical care during conflicts in Iraq and Kuwait (Howard et al. 2012).

The following sections will describe a brief introduction to the taxonomy of Acinetobacters and put into context its rapid development of multidrug resistance, which has propelled it into a global threat to the antibiotic era. This section will explore genetic features of the *Acinetobacter* genome which contribute to its MDR phenotype and virulence, focusing on the role of integrons and how they can cause rapid evolution by horizontal gene transfer of antibiotic resistance determinants. Lastly, the current model hypothesised for regulation

of integron activity and the differences in the regulation in *A. baumannii* will be outlined, in order to lay out the background for the aims of this project.

1.1. The *Acinetobacter* genus

1.1.1. Taxonomy

The name *Acinetobacter* was first put forward by Brisou and Prévot in the mid-1950s, as a way of differentiating from a closely related, but motile genus *Achromobacter* (Brisou, Prévot 1954). The name was chosen from the Greek for *ακινετοσ* (*akinetos*), meaning “non-motile”, and although there were still several years of confusion over the taxonomy, the name was used by Bauman et al. in a landmark study in 1968. In this paper, they demonstrated that a wide range of species previously separately identified under different names could be demonstrated using phenotypic characteristics to belong under one genus, *Acinetobacter*. This resulted in its recognition by several taxonomic groups throughout the mid-1970s, and hence established the genus name and paved the way for more focused research (Peleg, Seifert & Paterson 2008).

However, this study, which was mainly focused on nutritional phenotypes, yielded only two major groups within the genus, so a further, more thorough study was completed by Bouvet and Grimont (1986), in which they combined a range of other, more modern phenotypic tests, such as biochemical and auxotrophy tests as well as DNA hybridization. This thorough examination of a wider range of characteristics allowed them to refine the descriptions of *A. calcoaceticus* and *A. lwoffii* (the two main species noted in the study by (Baumann, Doudoroff & Stanier 1968), as well as identifying four new species in this genus, including *A. johnsonii*, *A. junii*, *A. haemolyticus* (previously *Achromobacter haemolyticus*) and also *A. baumannii*, the latter of which was the most common species isolated from humans, and was named in honour of

Paul and Linda Baumann, American microbiologists that made a significant contribution to the understanding of the *Acinetobacter* genus.

Table 1.1 Table showing a list of species within the *Acinetobacter* genus, registered on List of Prokaryotic names with Standing in Nomenclature database (accessed March 2015)

Species name	Reference
Acinetobacter apis	(Kim et al. 2014)
Acinetobacter baumannii	(Bouvet, Grimont 1986)
Acinetobacter baylyi	(Carr et al. 2003)
Acinetobacter beijerinckii	(Nemec et al. 2009)
Acinetobacter bereziniae	(Nemec et al. 2010)
Acinetobacter boissieri	(Álvarez-Pérez et al. 2013)
Acinetobacter bouvetii	(Carr et al. 2003)
Acinetobacter brisouii	(Anandham et al. 2010)
Acinetobacter calcoaceticus	(Bouvet, Grimont 1986)
Acinetobacter gandensis	(Smet et al. 2014)
Acinetobacter gerneri	(Carr et al. 2003)
Acinetobacter grimontii	(Carr et al. 2003)
Acinetobacter guangdongensis	(Feng et al. 2014)
Acinetobacter guillouiae	(Nemec et al. 2010)
Acinetobacter gyllenbergii	(Nemec et al. 2009)
Acinetobacter haemolyticus	(Bouvet, Grimont 1986)
Acinetobacter harbinensis	(Li et al. 2014a)
Acinetobacter indicus	(Malhotra et al. 2012)
Acinetobacter johnsonii	(Bouvet, Grimont 1986)
Acinetobacter junii	(Bouvet, Grimont 1986)
Acinetobacter kookii	(Choi et al. 2013)
Acinetobacter lwoffii	(Bouvet, Grimont 1986)
Acinetobacter nectaris	(Álvarez-Pérez et al. 2013)
Acinetobacter nosocomialis	(Nemec et al. 2011)
Acinetobacter oleivorans	(Kang et al. 2011)
Acinetobacter parvus	(Nemec et al. 2003)
Acinetobacter pittii	(Nemec et al. 2011)
Acinetobacter puyangensis	(Li et al. 2013)
Acinetobacter qingfengensis	(Li et al. 2014b)
Acinetobacter radioresistens	(Nishimura, Ino & Iizuka 1988)
Acinetobacter rudis	(Vaz-Moreira et al. 2011)
Acinetobacter schindleri	(Nemec et al. 2001)

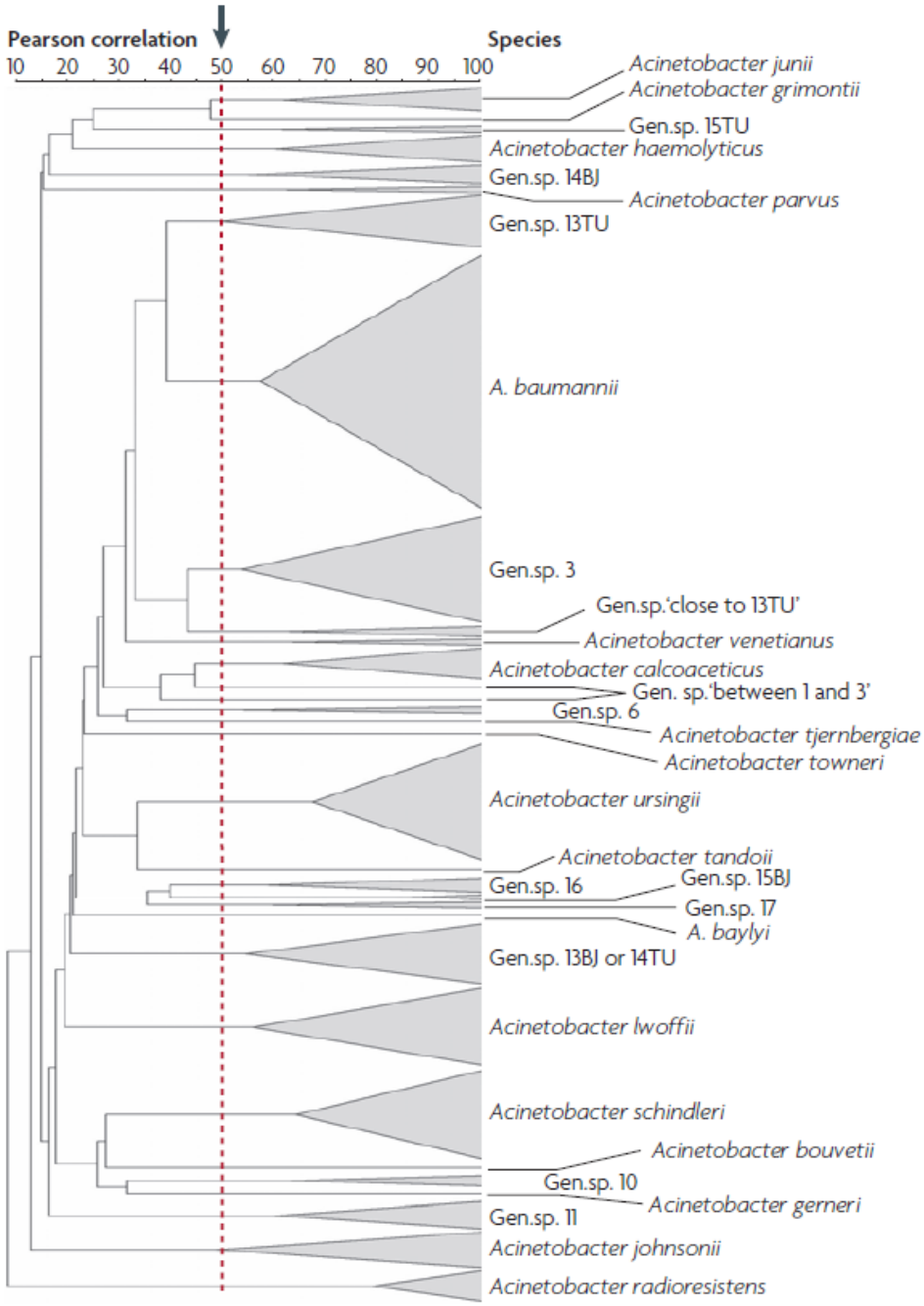
(Table continued)

Species name	Reference
Acinetobacter soli	(Kim et al. 2008)
Acinetobacter tandoii	(Carr et al. 2003)
Acinetobacter tjernbergiae	(Carr et al. 2003)
Acinetobacter towneri	(Carr et al. 2003)
Acinetobacter ursingii	(Nemec et al. 2001)
Acinetobacter venetianus	(Di Cello et al. 1997)

Differentiation methods between species have now progressed beyond the laborious methods such as DNA-DNA hybridisation to more refined typing methods which allow the detection of clonal groups within the same species, such as ribotyping (Gerner-Smidt 1992), pulsed-field gel electrophoresis (PFGE, Bou et al. 2000), amplified fragment length polymorphism (AFLP, Janssen et al. 1997), *rpoB* gene sequence (La Scola et al. 2006) and MLST (Bartual et al. 2005), the latter of which was used in this study.

As a greater understanding of this genus develops, new species are constantly being identified, refining and expanding the information available on this complex genus. A summary of the currently accepted species available on NCBI are given in Table 1.1, but new additions to the *Acinetobacter* genus are due to be made in the coming years. Sixteen strains of *A. baumannii* have now had their genome fully sequenced, assembled and annotated (Table 1.2).

Figure 1.1 Dendrogram showing phylogenetic relationship between *Acinetobacter* species



Dendrogram showing relationship between established *Acinetobacter* species based on AFLP analysis; figure from Dijkshoorn, Nemec & Seifert (2007). Note that gen. sp. 3 has now been renamed as *A. pittii*; 13TU as *A. nosocomialis* (Nemec et al. 2011); gen. sp. 10 as *A. bereziniae*; and gen sp. 11 as *A. guillouiae* (Nemec et al. 2010).

Table 1.2 Available sequenced, assembled and annotated genomes of *A. baumannii,* at the time of writing

***A. baumannii* strain**	**Chrs Size (Mb)**	**Plasmids (size in kb)**	**GC%**	**Reference**
ATCC 17978	4	pAB1 (13.4) pAB2 (11.3)	38.9	(Smith et al. 2007)
TCDC-AB0715	4.22	p1ABTCDC0715 (8.7) p2ABTCDC0715 (70.9)	38.9	(Chen et al. 2011a)
1656-2	4.02	ABKp1 (74.5) ABKp1 (8.0)	39.1	(Park et al. 2011)
AB0057	4.06	pAB0057 (8.7)	39.2	(Adams et al. 2008)
AB307-0294	3.76	-	39	(Adams et al. 2008)
ACICU	4	pACICU1 (28.3) pACICU2 (64.4)	38.9	(Iacono et al. 2008)
AYE	4.05	p1ABAYE (5.6) p2ABAYE (9.7) p3ABAYE (95.4) p4ABAYE (2.7)	39.3	(Fournier et al. 2006)
BJAB07104	4.04	p1BJAB07104 (70.2) p1BJAB07104 (20.1)	39	(Zhu et al. 2013)
BJAB0715	4.05	pBJAB0715 (52.3)	38.9	(Zhu et al. 2013)
BJAB0868	4.01	p1BJAB0868 (8.7) p2BJAB0868 (70.2) p3BJAB0868 (20.1)	38.9	(Zhu et al. 2013)
D1279779	3.71	pD1279779 (7.4)	39	(Farrugia et al. 2013)
MDR-TJ	4.15	pABTJ1 (77.5) pABTJ2 (111.0)	39.1	(Huang et al. 2012)
MDR-ZJ06	4.01	pMDR-ZJ06 (20.3)	39	(Zhou et al. 2011)
SDF	3.48	p1ABSDF (6.1) p2ABSDF (25.0) p3ABSDF (25.0)	39.1	(Fournier et al. 2006)
TYTH-1	3.96	-	39	(Liou et al. 2012)
ZW85-1	3.76	pAbNDM-1 (48.4) ZW85p2 (113.9)	39.1	(Wang et al. 2014)

The majority of the *A. baumannii* species were used in alignments for primer design for this study, but in particular, strains AYE, AB0057 and ACICU have been used as templates and in experiments in this thesis. A further 34 strains are available as scaffolds, and 750 more are in raw sequence data available on NCBI at the time of writing. As software developments aid faster genome gap-closure and annotation to complete these remaining strains, the

breadth of information available for *A. baumannii* research is set to expand exponentially.

1.1.2. *Acinetobacter* ecology and epidemiology

As a genus, *Acinetobacter* has a reputation for being ubiquitous throughout the environment. This feature has been documented from as early as the 1960s (Baumann 1968), but also, many of the recent species additions have been isolated from a range of soil and environmental-based water sources, such as wetlands, sewage treatment plant effluent, wastewater, forest soil and even the nectar of plants, as well as humans and animals (Álvarez-Pérez et al. 2013, Anandham et al. 2010, Carr et al. 2003, Kang et al. 2011, Kim et al. 2008, Vaz-Moreira et al. 2011). In addition to inhabiting moist environments, members of the *Acinetobacter* genus are also capable of withstanding long periods in dry environments, and studies have shown that *A. baummannii* can be recovered from hospital bed rails up to nine days after a patient is discharged (Catalano et al. 1999). Furthermore, they have been shown to be able to utilise alcohol from alcohol hand-rubs as a carbon source (Edwards, Patel & Wareham 2007), therefore, their propensity to survive in hospital environments is even higher.

During the conflicts in the Middle East in the years 2002 to 2004, there was a spike in the number of military personnel infected with *A. baumannii*, earning it the nickname “Iraqibacter” (Howard et al. 2012). Several reports found that many *A. baumannii* infections were associated with traumatic injuries and invasive infections such as deep wound infections and osteomyelitis. A study by Scott, et al. (2004) concluded that environmental contamination of the field hospitals was a major cause for this outbreak.

Nevertheless, not all species of this genus are found naturally in the environment. Many species of *Acinetobacter* can be found as a commensal on human skin, and one study found that up to 43 % of healthy human volunteers

in their study had skin colonisation by members of this genus (Berlau et al. 1999), and it was found to rise as high as 75% in hospitalised patients (Seifert et al. 1997), with *A. lwoffii* being the most dominant species found in both studies. It is thought that this could facilitate its ability to cause disease in injured or compromised individuals leading to its reputation as an opportunistic pathogen, targeting those with already weakened or stressed immune systems.

However, the most clinically relevant species of this genus, *A. baumannii*, was found to have much lower rates of carriage on human skin in these studies, at 3 % or below (Berlau et al. 1999, Seifert et al. 1997, Scott et al. 2004). It should be noted, however, that a study investigating *A. baumannii* carriage in human body louse found that up to 22 % of lice found on the homeless tested positive. Although the authors note that this could be due to undiagnosed transient bacteraemia in this population, cross contamination via an insect vector or host in this population should not be ignored.

This relatively low frequency of colonisation by *A. baumannii* may be contributing to the low rate of infections found in the community. The most common type of infection in this setting is community-acquired pneumonia; however the majority of these cases involved patients with other comorbidities, such as diabetes, long-term smoking or alcoholism (Dijkshoorn, Nemec & Seifert 2007). These types of infections in the community are also more common in tropical/subtropical zones and other countries that have warm and humid seasons.

A. baumannii infections in hospitals are increasing, and the significance of this species is highlighted by its inclusion within the ESKAPE group of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter sp.* which are a group of organisms known to be problem opportunistic pathogens in healthcare settings, with high rates of antibiotic

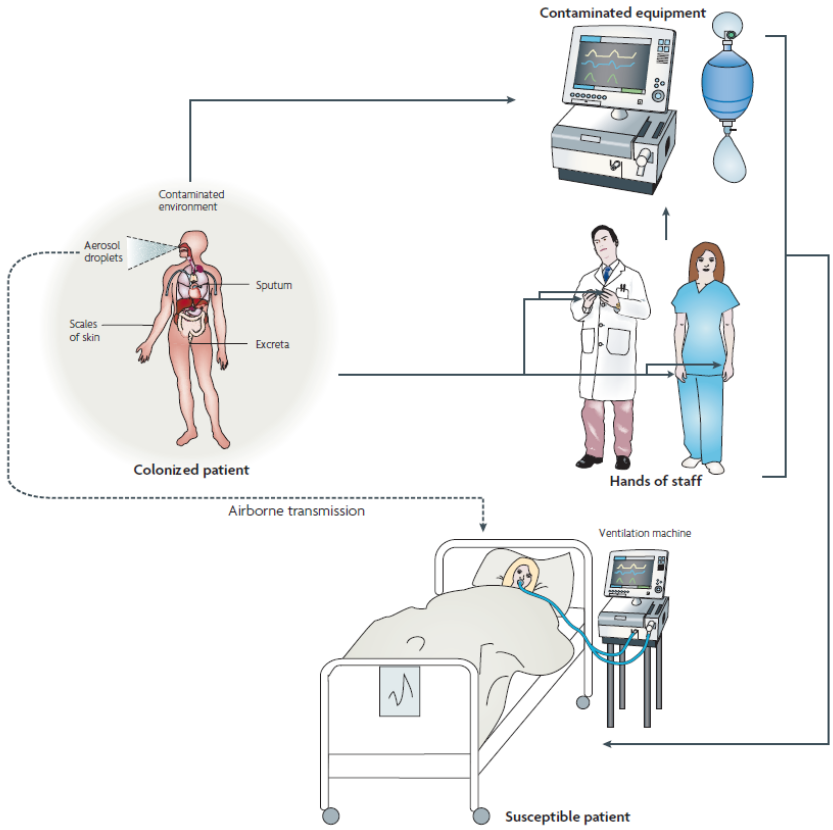
resistance. This group of organisms is responsible for up to 40 % of infections in intensive care units (Rice 2008).

This is certainly the case for *A. baumannii*, which most commonly affects critically ill patients, who have been through major surgery, trauma, burns, or premature birth. Infections can occur at a range of sites, such as skin and wound, urinary tract infections (UTI), ventilator-associated pneumonia (Dijkshoorn, Nemec & Seifert 2007), but the most serious tend to be bloodstream infections, which can have mortality as high as 45 % (Cisneros et al. 1996). Risk factors for bacteraemia include invasive procedures or serious injury, (as with the military personnel in the Middle East; (Cisneros, Rodriguez-Bano 2002). Nosocomial infections are predominantly caused by *A. baumannii,* but *A. pittii* and *A. nosocomialis*, which are very closely related, are also clinically relevant (Peleg, Seifert & Paterson 2008).

Treatment with broad-spectrum antibiotics is another significant risk factor for contracting these nosocomial species (Dijkshoorn, Nemec & Seifert 2007). Since many hospital strains of *A. baumannii* are already resistant to these first line drugs, numbers of commensal and competing bacteria are reduced, allowing the *Acinetobacter* strain to dominate a patient's microflora and become more successful in virulence.

However, there is continuing debate about the significance of *A. baumanni* in clinical mortality. Since *A. baumannii* seems to most commonly infect those that have severe injuries or have a significantly poor prognosis, on some occasions cause of death may be difficult to establish conclusively. A study by Blot, Vandewoude & Colardyn (2003) found that there was no significant correlation between *A. baumannii* infections and increase in mortality.

Figure 1.2 Routes of transmission of *A. baumannii* in healthcare settings



Simplified schematic of probable routes of transmission of *A. baumannii* in healthcare settings. Figure taken from Dijkshoorn, Nemec & Seifert (2007).

Conversely, a meta-analysis by Falagas, Bliziotis & Siempos (2006) found that there was a difference in mortality for *A. baumannii* infections in hospital patients compared with those in the intensive care unit with 7.8 to 23 % and 10 – 43 %, respectively.

1.1.3. Monitoring the spread and outbreaks of *A. baumannii*

In order to keep track of *A. baumannii* outbreaks, genetic testing can be completed, to help potentially identify the source. Strains of *A. baumannii* can have many similar physical characteristics, and so in order to differentiate them in such instances, various genetic methods have been used to categorise *A. baumannii* species into groups known as “clonal types”. In this case, a “clone” is described as:

> "*groups of isolates which, although similar, may not have any known epidemiological link between them, perhaps because they have been independently selected in different hospitals from a common ancestor*"
>
> (Turton et al. 2005)

The original establishment of the clonal system for *A. baumannii* was undertaken by Dijkshoorn, et al. (1996), based on various genotypic and phenotypic methods, such as AFLP and ribotyping. However, when a large number of isolates are sent to one reference lab for clonal identification, it becomes difficult to complete many laborious identification methods. As a simpler solution to this problem, researchers at the Public Health England institute in London established a simple multiplex PCR to identify the clonal types, which vastly increased the number of samples that could be processed and gave a faster turnaround time than previous diagnostic methods such as PFGE or AFLP (Turton et al. 2007). The PCR method was based on the identification of different allele combinations of three genes intrinsic to all *A. baumannii* strains, *ompA*, *csuE* and *bla*$_{\text{OXA-51}}$-like. Outer-membrane protein A (*ompA*) is a porin, whilst the *csuE* gene codes for a pilus segment involved in biofilm formation and the *bla*$_{\text{OXA-51}}$-like gene which codes for a carbapenemase enzyme. PCR results for different alleles of these genes broadly correlated with the European Clone classification system, so this method became widely adopted by labs worldwide. Consequently, the clones were renamed as "Global Clones" (GC) or "International Clones" (IC). There are now eight groups of GC (I to VIII), although groups I to III are the most prevalent in nosocomial infections. The GC groups are largely genetically stable, but there are some variations between the strains, which could be due to horizontal gene transfer.

Whilst this method yields quick and simple results that give a broad idea of the prevalence of clones, more detailed information can be gained by sequencing methods, such as MLST, which use the sequence of seven house-

keeping genes - *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD* – to identify sequence types (STs, (Bartual et al. 2005). This provides a more detailed method of analysing isolates and facilitates the construction of more specific phylogenetic trees. Currently, two MLST schemes are now available for the analysis of *A. baumannii* strains, the first published on the PubMLST website (Bartual et al. 2005) and also a second from the Institut Pasteur (Diancourt et al. 2010), although the former is used in this study. A full description of this technique is described in the Materials and Methods section (Chapter 2).

More recently, whole genome sequencing has also started to be used to monitor outbreaks of nosocomial infections, such as in *Staphylococcus aureus* (Harris et al. 2012), and may be used for other important outbreaks in future as genome sequencing costs continue to decrease, although assembly and quality may continue to be a bottleneck. All these typing methods can help to establish routes of transmission and reservoirs within a hospital setting, the most common routes of which are summarised in Figure 1.2.

1.1.4. Development of antibiotic resistance

Several contributing factors are thought to be involved in the development of antibiotic resistance. The overuse of antibiotics by doctors, in order to calm patients or to maintain their confidence is a major problem. Further afield, many third world countries suffer from a lack of regulation of antibiotic prescription or distribution, as well as sanitation and waste disposal issues, meaning that many bacterial strains are frequently exposed to sub-inhibitory levels of antibiotics. In addition, many patients fail to follow guidelines for the required length of time for treatment, and tend to stop taking antibiotics once their symptoms are alleviated. The overuse of disinfectants and antiseptics has also become a concern in recent years (Aiello, Larson & Levy 2007), as pharmaceutical companies strive to sell more products with

“antibacterial” taglines. All these factors contribute to low level antibiotic exposure that naturally selects for those strains that contain antibiotic resistance factors.

Furthermore, antibiotics are frequently used in veterinary agriculture, not just as a form of treatment, but also as prophylaxis, and as growth promoters. Although the mechanism by which certain antibiotics help animals to grow larger and more efficiently is not fully understood, the difference is significant enough for many farms to justify their use (McEwen, Fedorka-Cray 2002).This means that many healthy animals are being unnecessarily treated with antibiotics, and there is concern that these antibiotics are finding their way into humans through consuming such meat, and exposing our microbiota into subinhibitory concentrations of antibiotics. Such concerns resulted in an EU ban in using antibiotics for growth promotion in 2006, but they continue to be used for other purposes, such as prophylaxis or disease treatment. The cessation of antibiotics for prophylaxis has been investigated to establish whether it can reduce the spread of antibiotic resistance. Whilst the rates of antibiotic resistance in farm animals were reduced (Aarestrup et al. 2001), so far, studies into whether this has an impact on human diseases has been inconclusive. There has been an attempt to restrict antibiotics into different groups for humans and animals, to avoid encouraging resistance against the same antibiotics, but many of the antibiotics used for animals are simply analogues of human ones, and therefore promote the development of very similar antibiotic resistance mechanisms

The spread of MDR strains throughout hospitals and communities is a situation of great concern. It means that there is an increase in infection-associated mortality, prolonged hospital stays, and an increase of reliance on last-line, more expensive and potentially more toxic drugs in order to treat infections. The lack of any significant new drug discoveries in the last twenty years and few current trials to introduce new products have caused alarm in the

microbiology community and have led to the formation of the “Antibiotic Action” group in the UK, that has attempted to thrust this vitally important issue into the media and to the attention of the government.

One of the main problems is that major pharmaceutical companies have shown a deliberate shift away from drugs that are only used in the short term to long-term conditions, such as cancer chemotherapy, which yields higher investment returns, due to higher selling price and longer time period of use (Projan 2003). Yet at the same time, there has never been a more vital moment to have a full understanding of the development and spread of antibiotic resistance, and attempt to combat this situation.

1.1.5. Mechanisms of antibiotic resistance in *A. baumannii*

One of the reasons why *A. baumannii* thrives in hospital environments is its rapid ability to acquire antibiotic resistance determinants and survive in a harsh, sanitised environment. Many strains are able to acquire genes that confer resistance to several different antibiotics, sometimes to many different classes. The sequencing of the *A. baumannii* strains has revealed large “islands” of DNA that have been thought to have been acquired horizontally from other species. Analyses of the GC content for genes in these regions are often found to be different to the rest of the chromosome that is normal in *A. baumannii*, which suggests that they have been externally acquired. Many of the resistance genes found in *A. baumannii* have also been previously found in *Pseudomonas* spp, *Salmonella* spp. and *E. coli* (Fournier et al. 2006). In addition, these regions were surrounded by determinants known for genetic mobility, such as transposons, insertion sequences (IS) and integrons.

Not all antibiotic resistance is externally acquired however – many *Acinetobacter* species are intrinsically resistant to many β-lactamases, and this is one of the reasons why $bla_{\text{OXA-51-like}}$ genes are used for the detection of GC

type. AmpC cephalosporinases are also inherent to all *A. baumannii* strains (Bou, Martínez-Beltrán 2000), which has an association with an *Acinetobacter*-specific IS element, IS*Aba1*. IS elements are able to carry a promoter, which when inserted upstream of certain genes, can result in overexpression (Corvec et al. 2003).

Other enzymatic forms of resistance found in *A. baumannii* include extended-spectrum β-lactamases (ESBLs; (Peleg, Seifert & Paterson 2008). Resistance to carbapenems by class A or class D β-lactamases are now also a major concern in *A. baumannii* infections. The recent discovery of NDM metallo-β-lactamases (MBLs) made headlines in 2010 when they were discovered in *E. coli* and *K. pneumoniae*, and were reported very shortly after in *A. baumannii* strains across the world, from Germany to Egypt and China (Kaase et al. 2011, Pfeifer et al. 2011, Chen et al. 2011b).

If the cell cannot sufficiently break down the antibiotic molecules, it may also be able to mutate recognition sites such as on outer membrane proteins (OMPs) or to actively export molecules that have already gained entry by expelling them through efflux pumps. The main efflux pumps present in *A. baumannii* are summarised in Table 1.3, with members of the pumps Major Facilitator Superfamily (MFS); the Resistance-Nodulation-Division (RND) family; and the Multidrug And Toxic compound Extrusion (MATE) family. It is thought that these pumps originally evolved to eliminate waste products of the cell as well as molecules produced by the host for defence or regulation, and later became co-opted to mediate resistance to a range of classes of antibiotics. Indeed, their wide substrate specificity poses a major problem for treatment (Piddock 2006).

Table 1.3 Efflux pumps of *A. baumannii*

Efflux pump	Family	Antibiotics
Tet(A)	MFS	tetracycline
Tet(B)	MFS	tetracycline, minocycline
CmlA	MFS	chloramphenicol
AdeABC	RND	aminoglycosides, b-lactams, chloramphenicol, erythromycin, tetracyclines and ethidium bromide; reduced susceptibility to fluoroquinolones
AbeM	MATE	norfloxacin, ofloxacin, ciprofloxacin, gentamicin, 4',6-diamino-2-phenylindole (DAPI), triclosan, acriflavine, Hoechst 33342, daunorubicin, doxorubicin, rhodamine 6G and ethidium bromide

Adapted from Vila, Mart and Sanchez-Cespedes (2007)

The most common method of resistance against aminoglycosides is through the action of Aminoglycoside Modification Enzymes (AMEs). AMEs facilitate the inactivation of aminoglycoside antibiotics by the modification of the molecule on its –OH or $–NH_2$ ligands. This can occur by one of three ways: 1) *N*-acetyltransferases (AAC), which use acetyl-coenzyme A as a donor, and act on the amino group; 2) *O*-nucleotidyltransferases (ANT); and 3) *O*-phosphotransferases (APH) which use ATP as a donor, and act on the hydroxyl groups. A myriad of modification enzymes exist, of which more than 130 genes have been described to date (Ramirez et al. 2008), although only a small number are clinically significant. There are two different naming systems available for these classes of enzymes. The initial system designed by Novick, et al. (1976) was based on labelling the enzyme with the type of AME followed by assigning a letter to the specific site of action, and then a number for each gene allele, according to the order in which it is found. A more recent form of classification involves incorporating the location of the ligand that is modified by the enzyme, according to the carbon number on the ring to which it is attached. Different alleles are given roman numerals (Shaw et al. 1993). In this study, the former scheme is used to refer to AMEs. The AME class of resistance enzymes are an important focus of this study, as they are frequently carried on class 1

integrons within the genome, but there are also other types of resistance against aminoglycosides possible, such as *armA* and *rmt* genes A to C, which code for methylases that modify the target site in the 16S rRNA (Doi, Arakawa 2007).

Given the large number of antibiotic resistance determinants of *A. baumannii*, there are relatively few last-line drugs available. Until recently, colistin and polymyxins were considered to be suitable drugs in cases of severe MDR *A. baumannii* infection, despite their initial lack of favour due to high toxicity and cost. However, cases of *A. baumannii* infections that are resistant to these drugs have also now been found (Ko et al. 2007).

1.2. *A. baumannii* genome structure

One of the most notable features of *A. baumannii* is its rapid rise from an easily-treatable occasional cause of infection in the early 1970s, to becoming a major MDR pathogen within ten years. This rapid change has been attributed to the organism's ability to easily acquire external genetic elements into its genome. Indeed, close relatives such as *A. baylyi* are known for their natural competence and ability to take up external DNA (Vaneechoutte et al. 2006). This theory is matched by the ever expanding list of sequenced *A. baumannii* genomes, which frequently reveal resistance islands that are composite of several mobile genetic elements which can be traced to other Gram-negative species by comparative BLAST and GC content analysis (Fournier et al. 2006). These findings continue to emphasize the fluid nature of horizontal gene transfer between bacterial strains and species, and as the cost of genome sequencing continues to diminish, we are likely to see more such information and gather a greater understanding of the dynamic structure of microbial evolution.

1.2.1. Spread of antimicrobial resistance

1.2.1.1. Clonal spread

There are several pathways by which drug resistance may spread in a community of pathogenic strains, and the most basic of which can occur frequently in healthcare is simple clonal spread. This happens in environments when insufficient decontamination procedures are present and people and equipment move around frequently and come into contact with others (see Figure 1.2). When combined with circumstances where there is a high level of broad-spectrum antibiotics in use, it can create a situation where multi-drug resistant pathogens are at an advantage, and they will have a tendency to thrive and spread. Due to increased air travel, the spread of pathogens is now a global problem. For example, in a recent case, a patient at the Leicester Royal Infirmary was found to be infected with a highly virulent and pathogenic *A. baumannii* strain which resulted in the closure of the hospital's intensive care unit (data unpublished, strain included in this project). Antibiotic resistance genes first identified in countries as far away as India, such as the New Dehli metallo-β-lactamase NDM-1, have been documented in Europe only a matter of months later (Kumarasamy et al. 2010). So despite stringent rules enforced in some parts of the world on antibiotic use, the spread of antibiotic resistance will continue to perpetuate, unless a concerted, global effort can be implemented.

1.2.1.2. Horizontal Gene Transfer

A major contributor for the rapid spread of antibiotic resistance and emergence of MDR strains is horizontal gene transfer (HGT). In order for HGT to be successful, two main events must occur 1) DNA must be mobilised, and 2) incorporated into the genome of the recipient (Stokes, Gillings 2011).

The mobile genetic elements (MGEs) can be transferred between cells by a range of diverse mechanisms. The main modes of transfer are via plasmids, bacteriophages, IS elements, Integrative and Conjugative Elements

(ICEs), transposons (Tns) and the more recently described miniature inverted repeat transposable elements (MITEs). This exchange of genetic material vastly increases the genes available to bacterial strains, giving rise to an estimate of nearly 18,000 genes for *E. coli* alone, in its "pan genome" (Touchon et al. 2009).

However, the DNA which is taken up has to be compatible with the cell in order to be maintained, and some bacteria have developed resistance mechanisms against the uptake of foreign invasive DNA, for example, through restriction-modification systems such as CRISPR. In addition, the cell has to have the appropriate machinery to process the genes so that they can be expressed correctly.

1.2.1.3. Plasmids

Plasmids are one of the main mediums of MGE transfer. This is because they do not code for genes which are essential for bacterial growth, so they are separate entities from the main chromosome which does carry these housekeeping genes. Plasmids can be freely exchanged between cells, providing that the cell has the required machinery to recognise the plasmids replication and maintenance sites, the plasmid is not in the same incompatibility group as resident plasmids, and that the plasmid is not toxic to the cell. In addition, there has to be a compatible mechanism of transfer, as the cell surface is the main barriers to conjugation.

Since plasmids are frequently present in more than one copy, it is important that the genes they carry do not code for proteins that are detrimental or cause a heavy burden to the cell. Even in these situations, certain plasmids may be maintained due to toxin-antitoxin (TA) systems, where a plasmid carries genes coding for a long-acting toxin, and a short-acting antitoxin, so that in the event that the plasmid is lost, the cell is killed. This is an example of a so-called "selfish gene" situation, where the plasmid carries genes that ensure its maintenance within a cell. In most cases though, mega-resistance plasmids

contain genes that code for proteins that confer a selective advantage to the cell, often proteins that confer resistance to antimicrobial compounds.

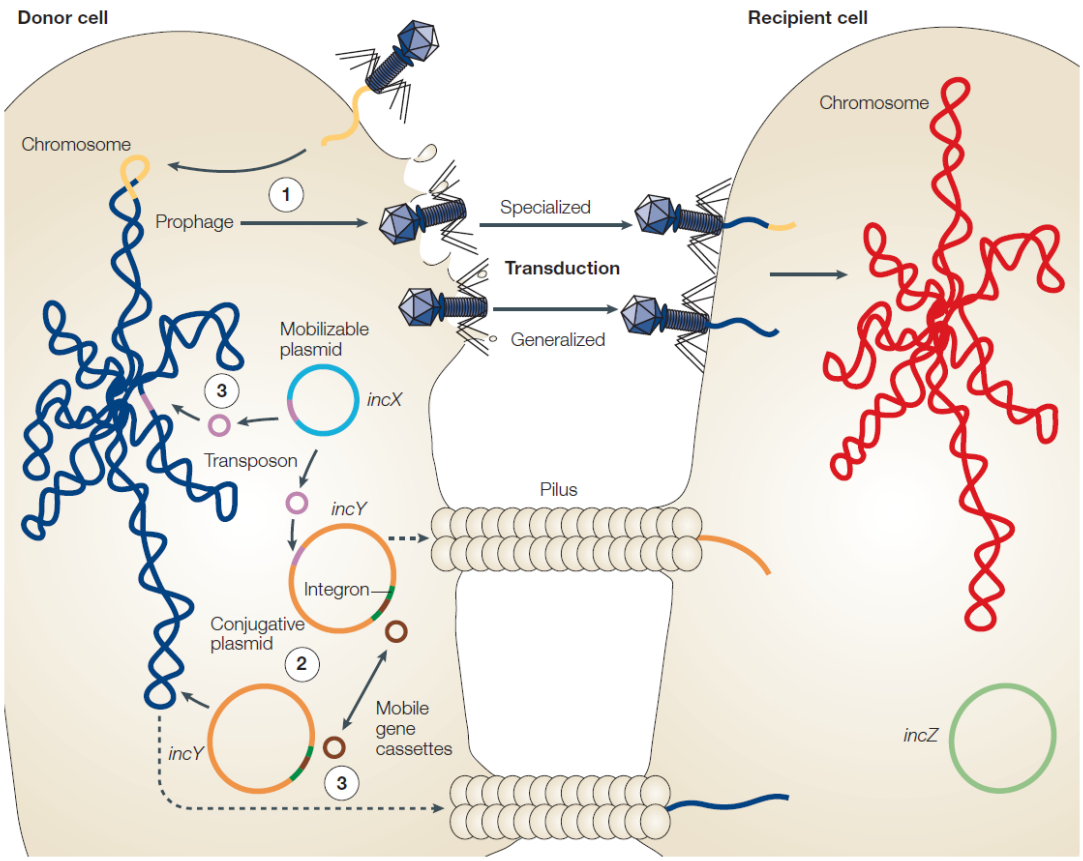
Most sequenced *A. baumannii* strains contain at least one plasmid, and Table 1.2 shows the plasmids contained in the currently available completed genomes. Plasmids have previously been used in studies to type *A. baumannii* strains (Seifert et al. 1994). Many carry β-lactamases such as bla_{OXA-23} or bla_{OXA-58}, which can have a very high level of expression due to the ubiquitous presence of IS elements (Héritier et al. 2005). Surprisingly, in the case of AYE, it was found that no antimicrobial markers were found on any of the four plasmids (Fournier et al. 2006), but the genes contained on the plasmids most likely gave another advantage that resulted in their maintenance in this strain.

Plasmids can also contain functions that facilitate their own horizontal gene transfer, and these are known as conjugative plasmids. Plasmids can be transferred whole by conjugation, or may also contain smaller MGEs described above, which can be transferred individually. Conjugation is one of the most important methods of DNA transfer between cells, and can also occur across species. For conjugation to successfully occur, the element has to contain a mobility (MOB) module that codes for a relaxase, and coupling proteins. A mating-pair formation (MPF) module is also required, which provides a type 4 secretion system (T4SS) type channel that allows the DNA to be transferred (Smillie et al. 2010). In addition, an origin of transfer (*oriT*) must be present in the DNA to be transferred, since this is the area that the relaxase recognises and binds to in order to initiate conjugation. The single stranded DNA can then be transferred through the mating channel, and the complementary strand is synthesised once the DNA has been received in the recipient cell. Non-conjugable plasmids can also be transferred if there are other conjugation elements present in the donor strain or if the plasmid is present in the environment and the recipient strain is naturally competent. This is one currently accepted model of plasmid transfer, but there are others.

1.2.1.4. Natural transformation

Natural transformation is the ability of cells to take up DNA from their environment and incorporate them into their own genome. It is now known to be an important part of acquiring foreign DNA and in some cases is thought to be a defence mechanism (Charpentier, Polard & Claverys 2012). The DNA taken up can originate from living or dead cells, and since DNA does not degrade immediately, it can be taken up after a later date from inanimate surfaces.

Figure 1.3 Schematic of major methods of DNA transfer in bacteria



The major mechanisms of DNA transfer between bacterial cells: 1) transduction showing a segment of the host's chromosomal DNA becoming incorporated into the phage head and delivered into the recipient's cell. 2) Movement of large, conjugative plasmids being transferred through a pilus. ICEs also use this kind of structure to transfer into a recipient cell. 3) Transposition occurring within the cell, where transposons (in pink) move to a different location, or gene cassettes (brown) move within a more localised region of an integron (green). Taken from Frost, et al. (2005).

Natural transformation is a particularly significant mechanism for DNA transfer in *Acinetobacter*, a genus which is known for its naturally high levels of competence, seen for example in strains like *A. baylyi* ADP1 (Vaneechoutte et al. 2006, Young, Parke & Ornston 2005). It is thought that this feature accelerated the development of the MDR phenotype in *A. baumannii*, and studies have shown that certain *A. baumannii* strains are capable of natural transformation (Ramirez et al. 2010). The transformation in *A. baylyi* as well as *Streptococcus pneumoniae* is reliant on being in a particular phase of exponential growth, whereas some other species such as *Neisseria gonorrhoea* are known to be constitutively in a transformable state (Solomon, Grossman 1996).

1.2.1.5. Transduction

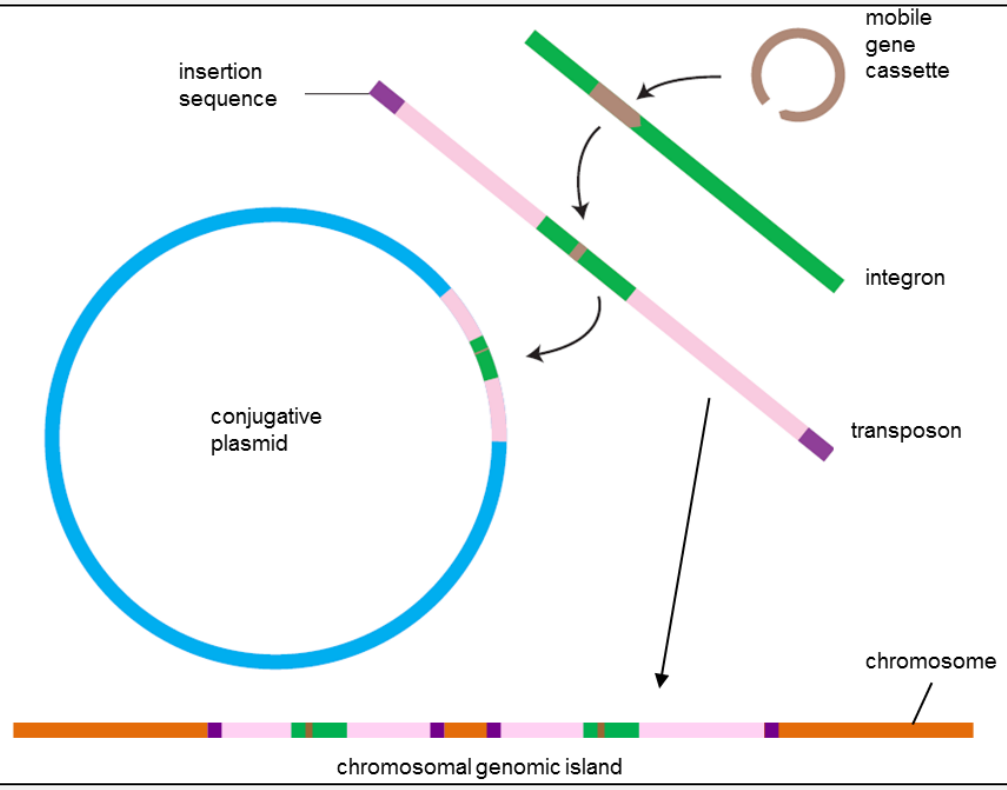
Transduction is bacteriophage-mediated transfer of DNA that occurs when DNA from the host can be incorporated into the phage head during replication, and is transferred to a recipient cell upon infection. This tends to mainly occur between closely related strains, because it is dependent upon successful recognition and attachment from the phage particle. Therefore, their range is smaller than that of conjugative plasmids, so their impact on the spread of antibiotic resistance is much lower. A summary of how the different horizontal gene transfer mechanisms function is illustrated in Figure 1.3.

1.2.2. *A. baumannii* genome plasticity and mobile genetic elements (MGEs)

In addition to these inter-cellular genetic elements, there are a number of smaller genetic structures which can be mobilised and move to another site within the same cell (intra-cellular mobility), or can be transferred to other cells (intercellular mobility). The most important ones involved in *A. baumannii* virulence are integrons, IS elements and transposons, which can become

interrelated to form composite structures. These can then be transferred on plasmids, or incorporated into hotspots on the chromosome where a high density of pathogenicity determinants may become a Genomic or Pathogenicity Island (GI or PI, see Figure 1.4 for illustration of how this can occur). Such MGEs and their structures have contributed significantly to the rapid development of MDR phenotypes in *A. baumannii*, so an understanding of their functioning is vital in establishing effective treatment methods for infections whilst preventing the development of resistance.

Figure 1.4 Schematic demonstrating possible relationship between different mobile genetic elements in bacterial genomes

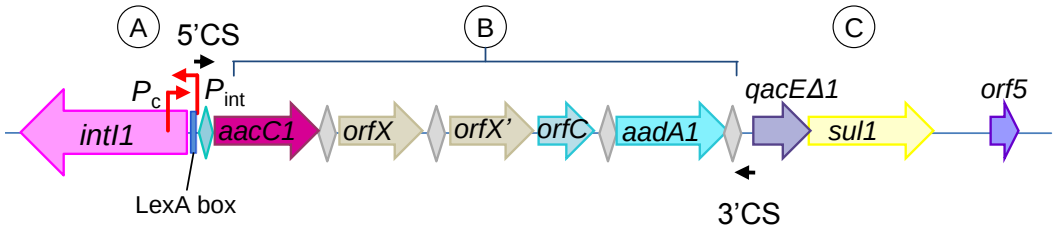


Schematic showing an example of the hierarchical relationship that various MGEs can be interacting with each other. Individual gene cassettes can be incorporated within integrons, which can be mobilised as a whole by transposons. These transposons can be mobilised through the function of IS transposases in the case of composite transposons. These in turn may be incorporated into a conjugative plasmid that can be transferred to other cells. These mechanisms are also the ways by which these MGEs can become embedded and accumulated into chromosomal "hotspots", giving rise to genomic pathogenicity islands (GI or PAIs). Image adapted from Norman, Hansen & Sørensen (2009).

1.2.2.1. Integrons

Integrons are semi-mobile genetic structures that were discovered during the late 1980s, first described by Stokes and Hall (1989), with later elucidation of their structure and mechanism by Collis and Hall (1992a). They are agents for bacterial evolution, through their ability to construct different arrays of gene cassettes, in what has been described as an "assembly platform" (Mazel 2006). Integrons are able to do this through the action of a tyrosine recombination enzyme (the integrase), which can excise or incorporate special *attC* flanked gene cassettes into site-specific "attachment" sites, *attC* or *attI*, on the host genome. This assembly structure inserts the gene cassettes in the same orientation so that they may be expressed by the P_c promoter at the 5' end of the structure, with an inverse relationship between the strength of the promoter and distance of the gene cassette.

Figure 1.5 Schematic of basic class 1 integron structure



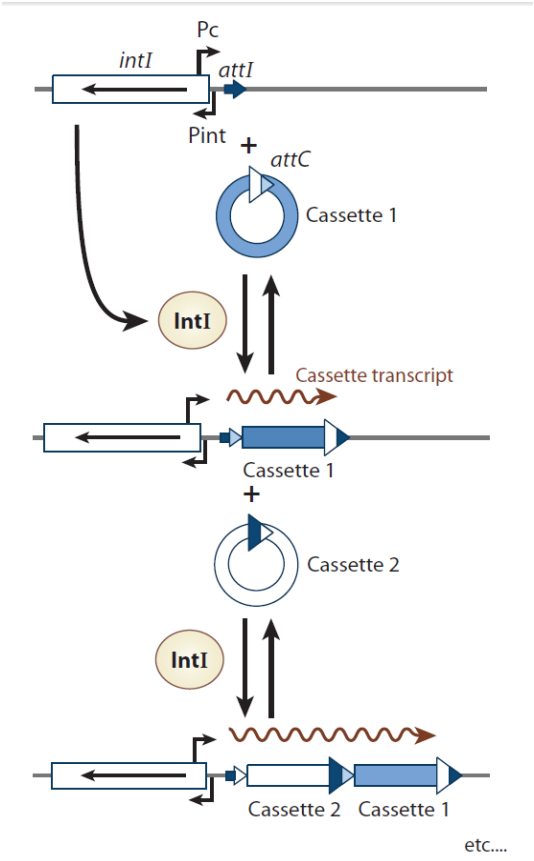
A) The 5' conserved region contains the integrase in a reverse orientation to the gene cassettes. The 5' region also contains the P_c and P_{int} promoters in opposite orientations (red bent arrows). Note that the P_c is coded within the 5' end of the integrase gene. The *attI* site (blue diamond) is the first site at which a cassette can be inserted. The LexA binding box (navy blue rectangle) is the location where the LexA repressor protein binds to prevent expression of *intI1*.

B) The region between the 5' and 3' end can contain variable number and types of gene cassettes. The array included in this diagram shows an example of a common array type, Type II in this study, In616. Gene cassettes are flanked by *attC* sites (grey diamonds).

C) The 3' conserved region contains the *qacEΔ1-sul1* gene fusion structure, and usually, but not always, a further gene of unknown function, *orf5*. Primers 5'CS and 3'CS are present at conserved regions (Levesque et al. 1995) and are indicated by small black arrows.

Gene cassettes can be in any number or arrangement, and currently more than 200 gene cassettes linked to integrons have been identified (Partridge et al. 2009). Once acquired, these cassettes can be moved around to different orders, thus modulating their expression, in the most favourable arrangement for the current environment. In this way, a "bank" of antibiotic resistance genes may be maintained at a low or zero fitness cost, which can be up regulated when required (Cambray, Guerout & Mazel 2010).

Figure 1.6 Mechanism of gene cassette movement by integrase



Schematic of mechanism showing the insertion of a circularised gene cassette into the attI site. Figure taken from Cambray, et al. (2010).

There are five classes of integrons that are based upon differences in the amino acid sequence of the integrase, but only 1 – 3 have been implicated as being important in nosocomial isolates, and only 1 and 2 have been documented in *A. baumannii*. Class 1 integrons are the most common in all nosocomial isolates; therefore they are the focus of this study. Class 2 integrons have been documented in *A. baumannii*, but they are relatively uncommon (Ploy et al. 2000, Ramirez, Quiroga & Centron 2005).Although Figure 1.5 gives a guide to the general structure of an integron, there can be many differences. The P_c promoter actually has different variants, which have different strengths. The most common types are the P_cS (strong), P_cW (weak) and PcH1 (hybrid 1), the latter of which is a mix of the -35 of P_cW and the -10 signal of P_cS. These

promoters differ by only one or two base pairs from each other, but have differing strengths in the expression of downstream genes, with P_cS being 25- and 4.5-fold stronger than P_cW and P_cH1 respectively (Jové et al. 2010). In addition, a second promoter closer to the gene cassettes may be formed when there is a GGG insertion in the LexA binding box in the 5' region of the integron. The LexA protein has been established as binding to this region in the integron, and acts as a repressor of the integrase (Guerin et al. 2009). Although this insertion disrupts the LexA binding, it results in the separation of two pre-existing -10 and -35 promoter signals to the optimal 17 bp gap (Collis, Hall 1995). This new promoter of the gene cassettes is known as the *P*2 promoter, and has a strength that is intermediate between P_cW and P_cS variants.

Regardless of the P_c type, the P_c is always present in the 5' region of the *intI1* ORF. Due to this incorporation to the coding region, means that variations in P_c type can affect the integrase efficiency. It has been shown by Jové, et al. (2010) that this is an inverse relationship.

Each gene cassette is flanked by recombination sites known as *attC* sites (previously 59-base pair elements; represented by grey diamonds in Figure 1.5). These allow individual genes to be excised as transient circular entities, which can be moved around and reinserted at other *attI* or *attC* sites in the cell, in a "cut and paste" mechanism. This plays an important role in the development of antimicrobial resistance because it means that a bank of genes coding for the resistance of a range of antimicrobials can be moved closer to the P_c and hence upregulated very easily, should the bacterium find itself under new antibiotic selective pressures.

Since the gene cassette array can be variable and many different configurations are possible, Moura, et al. (2009) established an online database, INTEGRALL, which keeps a record of different integron types, and assigns reference numbers to each array. Although this INTEGRALL scheme

does not always document the structure of the 5' and 3' conserved regions, it allows easy reference between studies for integrons, and facilitates the monitoring of integron epidemiology. For this reason, the INTEGRALL system is applied to the integrons found in this study.

The 3' conserved region of the integron usually starts with a gene fusion of *qacEΔ1-sul1*, which code for quaternary ammonium compound efflux pump and sulphonamide resistance, respectively. Due to the fusion, only the first 390 bp of the *qacE* gene is present, however, experimental evidence has confirmed that the *sul1* gene is able to confer resistance to sulphonamide antibiotics. The overlap suggests that at one point, the two genes may have been separate cassettes (Stokes, Hall 1989). An open reading frame of unknown function (*orf5*) is commonly present downstream of *sul1*, and there can also be a further *orf6* (not shown in Figure 1.5), and these are considered part of the 3' conserved region, if present.

1.2.2.2. Insertion Sequences

IS elements are mobile DNA structures, that code for their own transposase, and are flanked by inverted repeats (IRs) or direct repeats (DRs), which is the edge of the transposition site. IS elements are able to move to many locations in the chromosome as simple "cut and paste" mechanisms (Schneider, Lenski 2004).

One important feature of IS elements lies in their ability to carry strong, outwardly directed promoters that increase the expression of genes when the elements are inserted at close proximity upstream of a gene (Charlier, Piette & Glansdorff 1982, Safi et al. 2004, Lewis et al. 2004). In *A. baumannii*, IS elements such as IS*Aba1* and IS*26* are widespread and have been associated with a range of β-lactamases, including OXA-23-like, OXA-58-like and OXA-51-like (Peleg, Seifert & Paterson 2008).

Many IS elements are promiscuous, that is to say, they don't have a specific site of insertion, and as a consequence, there can be many copies of an IS element at different sites within one strain's genome. These elements can be recognised by the cells recombinational machinery, which means that when multiple copies are present, crossover events can be possible. IS element-mediated deletion events have been documented as early as the mid-1970s (Reif, Saedler 1975). However, not all of these mutations may be deleterious, and they can be advantageous if the element deletes or truncates a region that is no longer useful to the cell (Dawes et al. 2010).

Figure 1.7 Structure of IS elements

Basic structure of an IS element, with IS*26* used as an example. A typical IS element consists of a transposase gene, flanked by two inverted repeats (IRL and IRR, represented by grey boxes). In some cases, more than one transposase may be present. The two flanking boxes containing "XYZ" are representative of the short direct repeats which are created as a consequence of the insertion process. The rest of the host's genome is represented by "NNNN". The transposase promoter which is partly located in the IRL is represented by a red bent arrow. Diagram adapted from Mahillon and Chandler (1998).

IS elements have a significant role in *A. baumannii*, and many of these elements are ubiquitous throughout the genomes. There are even IS elements unique to the species, for example IS*Aba1* (Segal, Garny & Elisha 2005). Through the carriage of promoters, insertion-deletion and recombinational events, IS elements are able to both upregulate and decrease gene expression simply through their insertion at the appropriate place. In this way, they can be seen as important modules whereby the cell can increase its rate of evolution by increasing the amount of new configurations and hence variability in the gene pool.

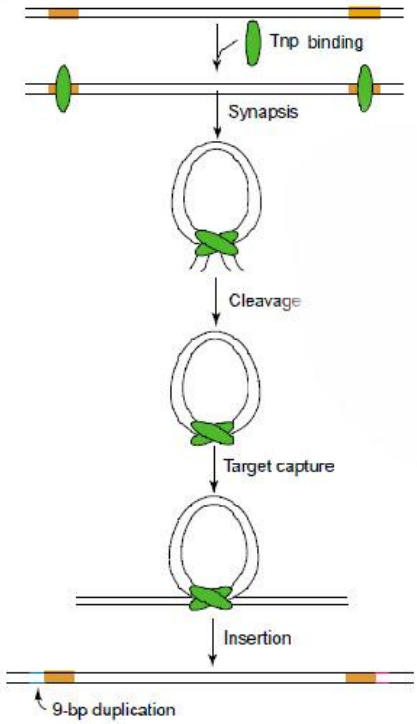
1.2.2.3. Transposons

Transposons are mobile genetic elements that can facilitate their own movement and also require integration into the host genome. They tend to be much larger than IS elements and can carry several genes. Some transposons can only travel within the same cell (composite transposons) and others are able to move between different bacterial cells (conjugative transposons or ICEs).

Composite transposons are able to make use of IS elements for their mobility, as they are flanked by IS elements on each side. The intervening region carries advantageous genes that contribute to virulence or pathogenicity, but it does not have its own mobility genes, and the IS elements are still capable of acting independently (Bennett 2004). Complex or unit transposons contain their own transposase, resolvase and other accessory genes. These function as one mobile unit, without the involvement of any IS elements.

Conjugative transposons or Integrative Conjugative Elements (ICEs) are known as a hybrid

Figure 1.8 Mechanism of composite transposon mobilisation



The mechanism of the Tn*5* transposon, with IS elements shown in orange, and the transposase in green. From Steiniger-White, Rayment & Reznikoff (2004)

structure in between transposons and plasmids. This is because they carry their own determinants for excision and conjugation, and are transferred in a circular form intermediate. However, they cannot be maintained as an independent circular element, and must be integrated into the recipient's chromosome. These structures tend to be much larger, and can carry a number of resistance genes (Bennett 2004, Roberts et al. 2008).

1.2.2.4. Miniature Inverted-repeat Transposable Elements

Miniature Inverted-repeat Transposable Elements (MITEs) are non-autonomous mobile elements that have recently been investigated in *A. baumannii*. They consist of small (<200 bp) inverted repeat sequences at terminal ends of their structure, and are found randomly inserted in the genome of several diverse bacteria (Delihas 2008). Although they don't code for any of their own specific genes, they have been known to carry any genes that are present in between the repeats. MITEs are mobilised through transposases of other transposons that may be present within the same cell. MITEs are thought to be quite an ancient genetic element, since they are also present in *Archaea* and eukaryotes, and some activity between cross-species transposases on MITE repeats has also been demonstrated (Feschotte et al. 2005).

Recently, it has been shown that MITE-like repeats are able to mobilise whole integron structures. This is highly important because this means that integrons are not only able to swap gene cassettes around within the cell, but that favourable structures created in other strains may be transferred intact to new cells of even other species. This structure was first identified in *Enterobacter cloaceae* by Poirel, et al. (2009), and named the "integron mobilisation unit" (IMU).

Since the demonstration of this unit as mobilisable by a transposase (supplied in trans in the original study), other similar MITE-like structures mobilising integrons have been discovered. An IMU-like structure was subsequently described in *A. baumannii* (Domingues, Nielsen & da Silva 2011), and it is possible that through more thorough analysis of the genetic context of mobile elements, more IMUs may be discovered. Furthermore, it has now been demonstrated that it is possible for some strains to uptake whole integrons and

transposons through natural competence in *Pseudomonas stutzeri* and *Acinetobacter baylyi* (Gestal, Liew & Coleman 2011, Domingues et al. 2012), which means that MITE-facilitated mobilisation of integrons may be a significant contributor to horizontal gene transfer of integrons, in addition to carriage on composite transposons and conjugative plasmids.

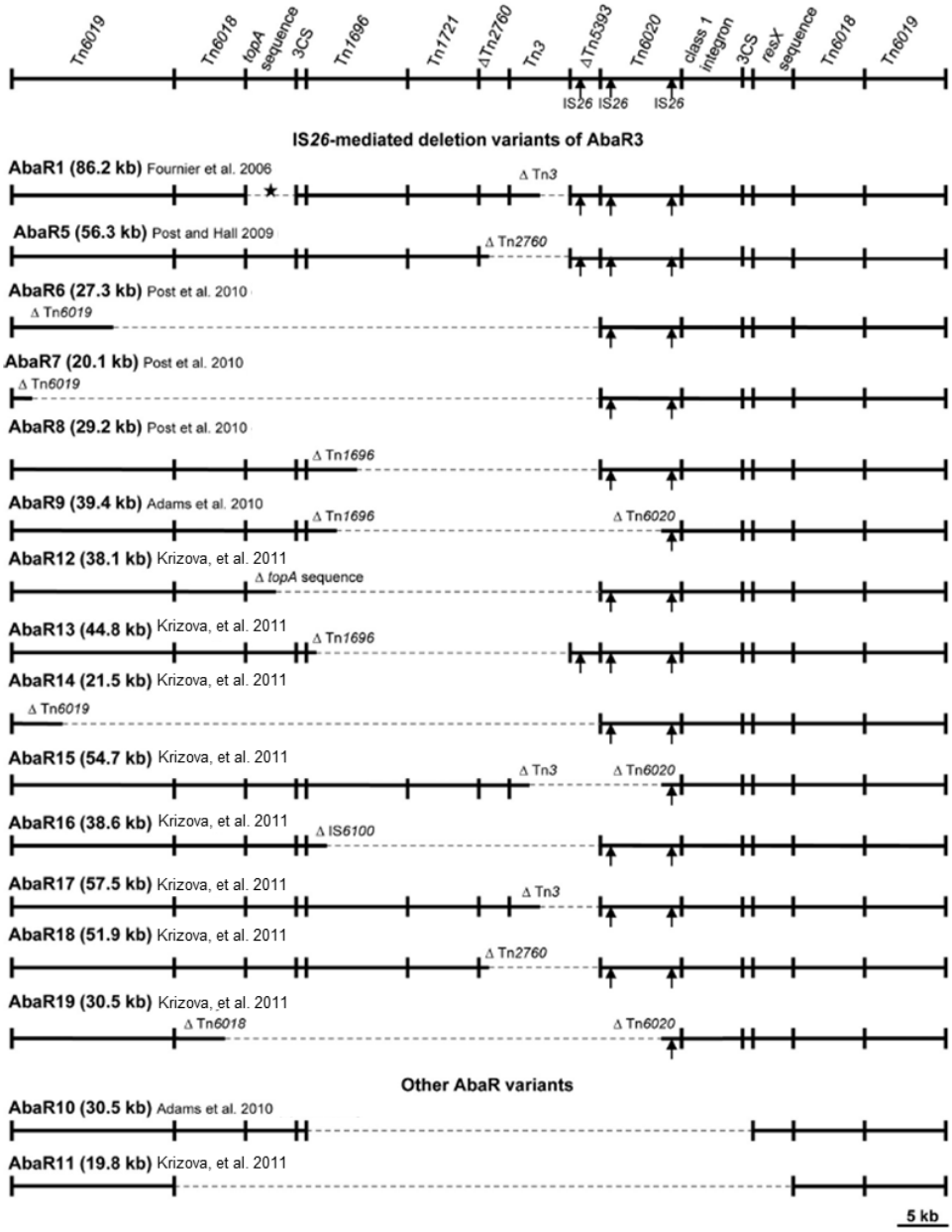
1.2.3. AbaR genomic islands

Many of the mobile genetic elements mentioned above can be found clustered in recombinational hotspots that acquire a range of external, horizontally transferred genetic elements. As mentined earlier, these structures are referred to as “genomic islands” (GIs), since they are separate entity from the indigenous housekeeping genome of the host strain. The advent of whole genome sequencing has facilitated the discovery of these regions, as it is possible to deduce the origins of some of these elements by analysis of codon usage and GC content compared to the levels considered normal for certain species. One of the first GIs identified in *A. baumannii* was also one of the largest, discovered during the sequencing project of strain AYE, an epidemic strain in France which contains an 86 kb island, known as AbaR1 (Fournier et al. 2006). After this discovery, further GIs of this type were discovered in *A. baumannii*, and there are now in excess of 23 different variations, containing a mixture of different IS elements, transposons and integrons, carrying different antibiotic resistance or other pathogenicity determinants. The GI structures of *A. baumannii* are thought to be related, since they all have a Tn*6019* backbone of 16.3 kb in length, which is interrupted by a large compound transposon flanked by two copies of Tn*6018,* except for AbaR6 and AbaR7*,* which only have one copy of the Tn*6018* transposon, due to large deletions (Post, White & Hall 2010). Indeed, recent evidence shows that resistance islands may be able to excise as one entity, and whilst this may be a rare event, bioinformatic analysis of island termini suggested that these islands may share a common ancestor with Tn*7* (Rose 2010, Kochar et al. 2012). In light of these findings these genomic islands will be referred to using the Tn*AbaR* terminology.

Currently, the evidence seems to suggest that Tn*AbaR3* was the ancestral form of these islands in Global Clone I (GCI) isolates. This is thought

to be so because most of the reported Tn*AbaR* types are truncated versions of the Tn*AbaR3* structure, and only Tn*AbaR1* so far seems to have additional DNA incorporated in comparison to Tn*AbaR3* (Krizova, Dijkshoorn & Nemec 2011).

Figure 1.9 Overview of IS*26*-mediated deletion variants of Tn*AbaR3*



Schematic of Tn*AbaR* (formerly AbaR) variants in Global clone I adapted from Krizova, Dijkshoorn & Nemec (2011). A diagram of Tn*AbaR3* is shown for reference at the top. The other Tn*AbaR*s are aligned below this, according to the presence of the regions homologous to those of Tn*AbaR3*. Unbroken and dashed horizontal lines indicate the Tn*AbaR3* regions present or absent in each of the Tn*AbaR*s, respectively. The positions of three copies of IS*26* sequences present in Tn*AbaR3* are indicated by black, vertical arrows. The sizes of individual Tn*AbaR*s are proportional to the scale.

Since the discovery of these large genomic structures, there have been studies on strains isolated as far back as the 1970s, which also showed the presence of a Tn*AbaR3*-like structure, indicating that this GI composition has been present in *A. baumannii* for over thirty years (Krizova, Nemec 2010).

The role of IS elements in shaping the development of these Tn*AbaR* islands, is demonstrated by the study completed by Krizova, Dijkshoorn and Nemec (2011). They showed the high frequency of IS*26* elements in the genomic islands, and the variation in different islands which had regions in between IS*26* elements deleted, possibly due to recombination. This thesis illustrates how IS elements can be involved in shaping the dynamic nature of an organism's genome. The high frequency of IS*26* elements discovered also shows the importance of IS*6*-type insertion sequences in *A. baumannii* genomic islands, and their shaping of other MGEs.

1.2.4. The role of the SOS-response in regulation of MGEs such as integrons

Although all the MGEs working together in genomic islands and resistance plasmids make the biggest impact on the pathogenicity of nosocomial isolates, integrons are a particularly important factor in *A. baumannii* due to their high rates of carriage. In many studies, the majority of *A. baumannii* strains contain integrons (Turton et al. 2005, Liu et al. 2014, Gallego, Towner 2001). Of the currently available sequenced reference genomes, ten out of sixteen clinical isolates of *A. baumannii* contain at least one integron or partial integron structures.

Yet conversely, it is known that integrons, like many antibiotic resistance mechanisms, carry a fitness cost. The expression of large antibiotic modification enzymes are a heavy burden to a cell (Andersson 2006). However, they are maintained in strains because their benefits outweigh their costs, or there are regulatory mechanisms in place that can repress or reduce the expression of the genes.

Integrons also carry another kind of fitness cost. Although integrases are site-specific enzymes, it is possible that they may erroneously recognise similar

sites in a genome, and cause illegitimate recombination, potentially causing lethal damage to a cell. A study by Starikova, et al. (2012) found that the presence of an intact integrase placed a 7 – 11 % fitness cost on *A. baylyi*, even when inserted in a single copy on the chromosome. In addition, many non-functional integrases have now been recognised in genomes, suggesting that there may be negative selection in action, and that their ability to incorporate new cassettes is not a strong enough benefit for their maintenance (Nemergut et al. 2008).

It has been discovered during the last decade that integrons can be repressed by LexA, as mentioned in section 1.2.2.1. LexA is a global repressor within the cell, which binds to many sites. During the SOS response, RecA coats single stranded DNA which builds up at stalled replication forks and this filmament (known as RecA*) in turn mediates the proteolytic self-cleavage of LexA (Rao, Radding 1995). Once LexA has broken down, the genes downstream of the binding box can be expressed. In this way, cells can minimise expression of potentially damaging proteins such as error-prone polymerases or recombination enzymes, but enable their activation when the cell is under stress from changes in the environment or exposure to harmful substances, when these enzymes may be useful. This response is specifically activated during and aimed at combating DNA damage. A classical SOS-response was first identified in *E. coli*, as a way of upregulating error-prone enzymes to help repair DNA damage under stressful conditions for the cell, and subsequently extensively studied (Gudas, Pardee 1975, Radman 1975). Activation of this response could be induced by a range of DNA-damaging conditions, such as exposure to UV irradiation, dessication or exposure to antibiotics that result in DNA damage. The resulting effects included prophage induction, cell filamentation and mutation. When it was discovered that not only was the SOS response also involved in expressing the enzymes of MGEs, such as integrases of integrons and ICE elements (Guerin et al. 2009), it was realised that understanding of this mechanism would be vital in combating the global problem of the spread of multidrug resistance. This ground-breaking finding facilitated the realisation that antimicrobial chemotherapy was actually promoting the development of antibiotic resistance.

These original studies in *E. coli* and *V. cholerae* became the textbook examples of how bacteria cope with stress, and through thorough studies into this mechanism, at least 40 genes have been established as regulated by the SOS network (Fernández de Henestrosa et al. 2000). With the advent of microarray analysis, it has been shown that the expression of thousands of genes can be changed during the SOS-response (Quillardet, Rouffaud & Bouige 2003).

1.2.5. Atypical DNA-damage response in *A. baumannii*

It was originally assumed that this SOS-response mechanism was universally present across all bacterial species. However, as studies increased to a wider net of organisms, it was soon discovered that this was not the case. There are many notable exceptions in human pathogens that do not have a LexA homologue, such as *Neisseria gonorrhoeae* (Black, Fyfe & Davies 1998), *Legionella pneumophila* (Charpentier et al. 2011), *Streptococcus pneumoniae* (Gasc et al. 1980), *Helicobacter pylori* (Dorer, Fero & Salama 2010) as well as *A. baumannii* (Aranda et al. 2011). In fact, the whole of the Moraxellaceae family does not have a LexA, with the exception of one species Perlucidiaca piscinae, which has a putative homologue with 49 % identity. Since this discovery, a range of studies have been conducted into what alternative pathways these species use to cope with stress.

Since the discovery that *A. baumannii* does not have a LexA homologue, several studies have attempted to investigate the full extent of the *A. baumannii* SOS response and its differences from the well-established pathways in other Gram-negative pathogens (Aranda et al. 2011, Aranda et al. 2013, Hare, Perkins & Gregg-Jolly 2006). It's clear that RecA is a vital feature of both the classical SOS responses and that of *A. baumannii*. In both systems, RecA is involved in facilitating DNA repair after damage from UV light, molecules such as mitomycin C and ethidium bromide, heat shock and dessication (Aranda et al. 2011). It is thought that RecA helps a cell to cope with such genetic insults by facilitating the repair of DNA homologous recombination. Studies in *Acinetobacter* strains show that expression of *recA* is upregulated during these DNA-damaging conditions, and that *recA* mutants have reduced pathogenicity

in *in vivo* models (Aranda et al. 2011, Hare, Perkins & Gregg-Jolly 2006, Rauch et al. 1996).

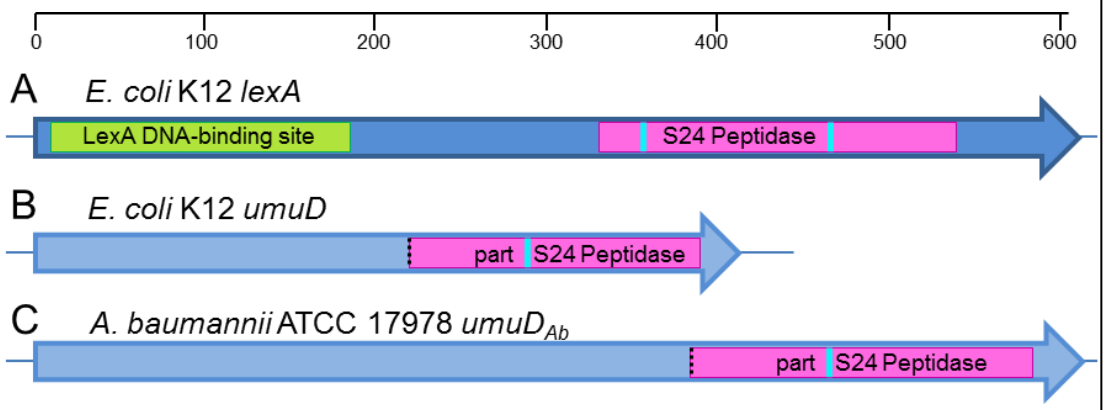
However, the similarities in DNA-damage response between *Acinetobacter* and the other Gram-negative pathogens appear to end there. The precise downstream actions of RecA are not well understood at the present time, in contrast to the clear cascades known in *E. coli*. In addition, members of the *Acinetobacter* genus appear to lack several important genes which are upregulated in *E. coli* during DNA-damaging conditions, such as polB (error-proner polymerase), stress response factor σ^{38}, cell division inhibitor *sulA* and *dinI*, a gene coding for a protein that inhibits LexA cleavage (Robinson et al. 2010). In addition, whilst both system contain genes expressing UmuC and UmuD proteins, it is clear that there are significant structural differences between the two sets of proteins, and in fact, in *A. baumannii* $UmuD_{Ab}$ is the closest homologue to LexA (Figure 1.10),

In *A. baumannii*, $UmuD_{Ab,}$ was identified as having some similar motifs to LexA, for example the conserved serine-lysine catalytic dyad (S24 peptidase) in the carboxy-terminal, which is required by both for self-cleavage. Moreover, it was established that this self-cleavage was RecA dependent, much like LexA. However, only this part of $UmuD_{Ab}$ is shared with LexA, and the amino-terminal DNA-binding domains of LexA are absent, even though the two proteins are of a similar size, as can be seen in Figure 1.10 (Hare et al. 2012a). This places doubt onto whether $UmuD_{Ab}$ may be able to bind to promoter sites and function in a similar repressor role as LexA. Furthermore, several studies have shown that $umuD_{Ab}$ mutants do not have affected growth or survival rates when exposed to UV irradiation, as might be expected from a *lexA* mutant (Hare, Perkins & Gregg-Jolly 2006, Aranda et al. 2014). However, unpublished data from A. MacGuire and V. G. Godoy, suggest that $umuD_{Ab}$ is not able to complement a ΔlexA mutant of *E. coli*.

However, it is thought that whilst $UmuD_{Ab}$ is likely involved in the SOS response mechanism, since it has been established that UmuD in other strains such as *E. coli* act with UmuC upon cleavage to form DNA polymerase V, which is an error-prone, trans-lesion polymerase (Reuven et al. 1999). $UmuD_{Ab}$ in fact

has a higher homology to these UmuD proteins in other species, at 42 – 46 % amino acid identity, when compared to 37 % similarity to LexA (Hare et al. 2012a). It is clear that $UmuD_{Ab}$ is an unusual protein that differs significantly to both of its nearest relatives, and warrants further study.

Figure 1.10 Schematic for comparison of conserved domains in *E. coli* and *A. baumannii* LexA, UmuD and *A. baumannii* $UmuD_{Ab}$



Comparison of conserved domains between *E. coli* K12 *lexA* (A), *umuD* (B) and *A. baumannii* ATCC 17978 $umuD_{Ab}$ (C). The region coding for the LexA DNA-binding site is denoted by a green box, and is only present in *lexA*. The S24 peptidase required for self-cleavage is shown by a pink box. Regions coding for important catalytic sites of the protein are represented by turquoise vertical lines. Truncation of domain region is shown by vertical dashed black lines. The scale bar at the top represent nucleotide numbers

Furthermore, while studies on $UmuD_{Ab}$ have been conducted regarding ability to cope with UV damage and its mechanism of self-cleaving has been elucidated, to our knowledge, there have not been any studies analysing whether $UmuD_{Ab}$ has a role in integron repression in the same way as LexA. Investigation of what kind of interaction, if any, $UmuD_{Ab}$ has in integrons, would give a greater understanding of this unusual protein, shed greater light on the DNA damage mechanism in *A. baumannii*, and would contribute to understanding how MGEs such as integrons may function differently in different species.

1.3. Aims and objectives of this study

This project was focussed on understanding how integrons are carried and regulated in *A. baumannii*. Based on existing knowledge of largely chromosomal integron carriage and the absence of a confirmed LexA repressor homologue, we hypothesized that integron regulation differs significantly in *A. baumannii* compared to other Gram-negative pathogens. Since it has been recently discovered that antibiotic exposure can encourage the activation of these MGEs, it was deemed important to establish how these mechanisms may operate in a significant nosocomial pathogen, as *A. baumannii*. Through the elucidation of this regulation, this study could help to ascertain how these MDR strains maintain and evolve antibiotic resistance, and may possibly influence clinical guidelines in the treatment of such pathogens.

1. To survey a large group of *A. baumannii* clinical isolates for the presence of integron structures

A diverse range of *A. baumannii* clinical isolates will be selected for screening of their integrons. These strains will primarily consist of the laboratory strain collection, which were isolated from the local hospital University Hospital Leicester NH Trust, and also from the private collection of Dr Kevin Towner. The selection of these strains may have some bias towards particularly pathogenic, virulent or interesting strains, however, they are sourced from different geographical locations and times. Their diversity will be confirmed through the analysis of their clonal lineage through MLST and other typing methods. In addition, the main aim of the project will be to analyse the pathogenicity of problematic nosocomial isolates that are difficult to treat, therefore, this selection will provide a useful dataset from which to infer clinically relevant conclusions.

Integron analysis will start with PCR screening for integron features, such as the integrase gene and gene cassette arrays. Subsequently, their integron structures and their relative frequencies will be determined. To expand the number of strains analysed, these analyses will also be

completed on *A. baumannii* strains that have available genome sequences on NCBI.

2. To conclude from the patterns observed trends in integron gene cassette arrangements, carriage and maintenance in *A. baumannii*

Integron carriage in *A. baumannii* has been shown to be significantly different to other Gram-negative nosocomial pathogens. This study will attempt to draw conclusions for why this may be the case. The differences between integrons of *A. baumannii* when compared with other species may give some clue about different evolutionary pressures and fitness costs of integrons in this species.

3. To quantify integrase activity within *A. baumannii* and any fitness costs

Analysis of integrase activity and fitness cost will be analysed through the design and use of molecular tools that allow the artificial induction of integrase activity under experimental conditions. The activity will be compared in both *A. baumannii* and *E. coli* backgrounds, and the efficiency of integrase expression and activity will be compared through qRT-PCR. Fitness costs will be approximated via growth curves.

4. To analyse the role of $UmuD_{Ab}$ as a possible LexA homologue *in A. baumannii*

LexA has been shown to have a repressive role for a number of genes, including *intI1*. This repression can be removed through activation of the SOS response. To analyse whether the role of $UmuD_{Ab}$ in *A. baumannii* is homologous to LexA, gene expression studies will be completed on sequenced reference strains that are established model MDR strains of *A. baumannii* (AYE and AB0057). Analysis of integron-related gene expression in wild-type and *$umuD_{Ab}$* mutants will be analysed under conditions known to promote integrase activation. Tools for creating *A. baumannii*-compatible plasmids will also explored.

Chapter 2. Materials and methods

2.1. Bacterial strains and plasmids

A. baumannii isolates were obtained from two main sources. 50 strains were collected from clinical samples at the Queen's Medical Centre (University of Nottingham), from the private collection of Dr Kevin Towner. These strains were collected over a number of years from patients in the QMC and collaborators of Dr Towner. Ten strains were obtained from clinical samples obtained from University Hospital of Leicester, NHS Trust between the years of 2007 and 2009. In each case, strains were stored at -20°C / -80°C in brain heart infusion broth with 30 % glycerol. Strains were routinely grown at 37°C using lysogeny broth (LB) or agar (LA), unless otherwise specified. When required, *E. coli* growth media was supplemented with 100 µg/ml ampicillin, 50 µg/ml kanamycin, 30 µg/ml chloramphenicol, 15 µg/ml gentamicin, 10 µg/ml tetracycline or 140 µg/ml hygromycin. Conversely, *A. baumannii* strains were routinely grown in media supplemented with 200 µg/ml carbenicillin, 30 µg/ml chloramphenicol, 200 µg/ml gentamicin, 10 µg/ml tetracycline, or 140 µg/ml hygromycin unless otherwise specified.

Bacterial strains used and constructed in this work are summarised in Appendix 3 and Appendix 4, respectively. Plasmids used and constructed in this work are summarised in Appendix 5.

2.2. DNA-related techniques and methods

2.2.1. Polymerase chain reaction (PCR)

The majority of PCR products in this work were generated using GoTaq DNA polymerase (Promega). Long-range PCR products were amplified using Phire Hot Start DNA polymerase (Finnzymes). KOD Hot Start II DNA polymerase (Novagen) was used when high fidelity amplification of short- or long-range PCR products was required. All enzymes were used according to

manufacturer's instructions. Representative cycling conditions for each enzyme type is shown in Table 2-1. Cycling conditions were adjusted according to primer melting temperature (T_m) and predicted amplicon size.

Touchdown PCR was used in instances when non-specific bands needed to be eliminated (Don et al. 1991).

Regular GoTaq DNA PCR		
Step	**Temperature**	**Time**
Denaturation	95°C	30 s
Annealing	(Lowest $T_{m)}$°C	30 s
Extension	72°C	1 min / kb

Regular KOD Hot Start DNA PCR		
Step	**Temperature**	**Time**
Denaturation	95°C	30 s
Annealing	(Lowest $T_{m)}$°C	30 s
Extension	70°C	20 s / kb

2.2.2. Oligonucleotide design and synthesis

The sequences of oligonucleotides (primers) used in this project are listed in Appendix 6. Primers were designed using Primer3 (Rozen, Skaletsky 1999) and synthesised by Sigma-Aldrich. All primers were dissolved in nH_2O at 10 pmol/µl and stored at -20°C.

2.2.3. Colony PCR

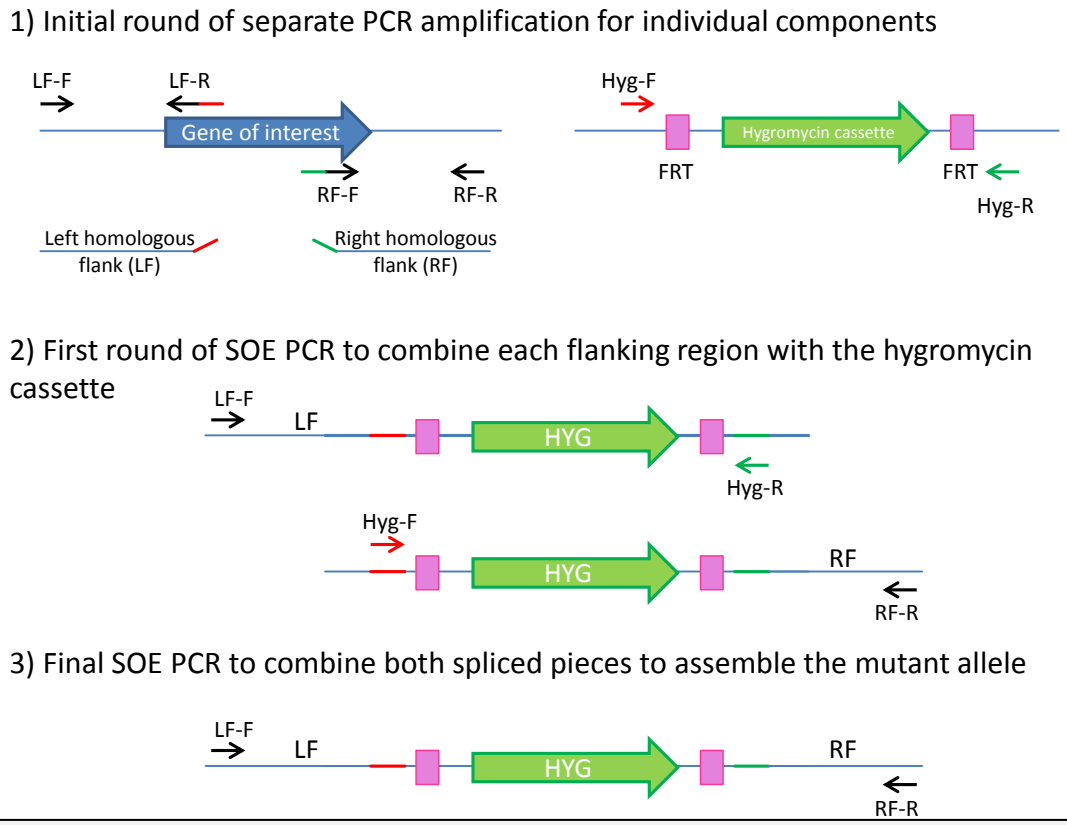
A single bacterial colony was resuspended in 30 µl of nH_2O and heated at 100°C for 10 min. Cells were then pelleted at 16000 × g for 1 min and 1 µl of the supernatant was then used as template for PCR.

2.2.4. Splice overlap extension-PCR: mutant allele construction

Splice overlap extension-PCR (SOE-PCR) was used to create hybrid PCR products to facilitate allelic exchange by recombination (Heckman, Pease

2007). This technique allows the construction of mutant alleles in a simple way by providing a method whereby DNA molecules could be recombined at specific junctions, independent of the nucleotide sequence or restriction sites present. Mutant alleles consisted of a left homologous flank (LF), an antibiotic resistance cassette (HYG) and a right homologous flank (RF), spliced together in the manner depicted in Figure 2.1. The flanking region size was ~ 400 bp in keeping with known sizes required for successful recombination, and they were amplified from the target strain to be mutated, in order to be homologous to sequences upstream and downstream of the region intended for deletion.

Figure 2.1 Schematic of SOE PCR used to create mutant alleles



1) The left hand and right hand flanks around a gene of interest are amplified by PCR, using primers with overhangs containing regions that are homologous to the antibiotic gene used (homologous regions in primers shown in red and green for the left and right hand, respectively).
2) First round of PCR involves annealing and amplification of the left flank with the hygromycin cassette, and also the cassette with the right flank
3) The final stage of the SOE PCR involves annealing of the two amplicons from stage 2, to produce the full mutant allele, which is then amplified by the LF-F and RF-F primers

Initially, primer pairs LF-F/LF-R, RF-F/RF-R, and Hyg-F/Hyg-R were used to separately amplify PCR fragments corresponding to the LF, HYG and RF, respectively (Figure 2.1). All fragments were then gel purified, as described in section 2.2.6. Primers LF-R and RF-F were designed so that their ends contained ~20 bp of sequence complementary to the HYG fragment primers Hyg-F and Hyg-R, respectively. This added an overhang to PCR products amplified which, when mixed, denatured and annealed in SOE-PCR, the complementary fragment ends annealed, producing a spliced product where two fragments have been merged.

The extracted PCR products from the first individual rounds of PCR were assessed for concentration, and then the LF and RF fragments were mixed with the HYG fragment individually, at a molar ratio of 1:1. The resultant PCR produced the LF-HYG cassette. PCR cycling conditions were as mentioned in section 2.1, but primers were omitted from the initial reaction mixture and only added during the extension phase of the 5th cycle. In a similar manner, the RF-HYG fusion product was produced using primer pair Hyg-F/RF-R and fragments RF and HYG. The LF-HYG and HYG-RF fragments were then purified from a gel and mixed in a 1:1 molar ratio, to be used as template for step 3, amplification by using primer pairs LF-F and RF-R, producing an SOE-PCR assembled mutant allele.

Using this protocol, one mutant allele was produced. Mutant allele PCR products were cloned into lambda *pir*-based suicide vectors for plasmid-based allelic exchange (section 2.6.3).

2.2.5. Genomic and plasmid DNA extraction

2.2.5.1. Kit-based DNA extraction

Genomic DNA (gDNA) was routinely extracted from 500 µl of overnight culture using the 5Prime ArchivePure DNA Purification Kit (VWR). Plasmid DNA was isolated from 5 ml overnight cultures using either the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). Mega-resistance plasmids from clinical isolates

were purified from 60 ml overnight culture in LB using the Qiafilter Midiprep Kit (Qiagen). All kits were used according to manufacturer's protocol.

2.2.5.2. Phenol-chloroform extraction

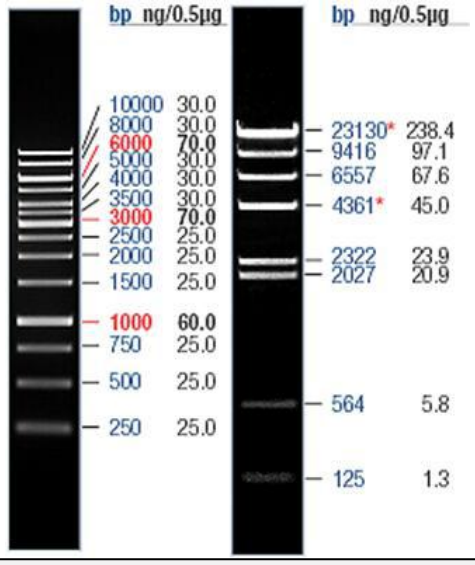
Phenol-chloroform extraction was performed using an adapted method based on the outline in Cold Spring Harbor Protocols (Sambrook, Russell 2006). Briefly, an equal volume of Phenol:Chloroform:Isoamyl Alcohol 25:24:1 (Fisher Scientific) was added to the solution from which DNA was extracted, and after vigorous vortexing for 20 s, tubes were centrifuged for 10 minutes at 4 °C at 16000 x *g*. The aqueous phase was then removed into a new eppendorf tube without disturbing the phenol-chloroform layer. For impure cultures with a high protein content, this process was repeated until no protein was visible at the interface between the two layers.

After the separation of the aqueous layer, 300 µl of isopropanol was added and mixed by inverting the solutions several times. The mixture was centrifuged again for 10 minutes at 4 °C at 16000 × *g*. The supernatant was poured away and the tubes allowed to drain upside down on a paper towel, before washing with 300 µl 70 % ethanol. The tubes were again centrifuged for 10 minutes at 4 °C at 16000 × *g*, before discarding the supernatant, and allowing the pellets to air dry. The DNA was then resuspended in the appropriate volume of TE or nanopure H_2O.

2.2.6. Gel electrophoresis, DNA purification and DNA sequencing

Gel electrophoresis for the visualization of DNA fragments was routinely performed in agarose gels made from 1 x TAE buffer containing 0.5 µg/ml ethidium bromide. Agarose was routinely used at 0.8 % concentration, unless stated otherwise, DNA markers λ-HindIII or GeneRuler™ (Fermentas) were used for relative size estimation (Figure 2.2). When required, DNA fragments were excised from agarose gels without ultraviolet light exposure using an IMS-cleaned scalpel and extracted using the PCR DNA purification kit (Geneflow).

Figure 2.2 DNA markers used in this study



The left hand side shows the 1 kb GeneRuler and the right hand picture shows the lambda HindIII ladder. The left hand columns of numbers denote the band sizes in bp of the markers. The right hand columns of numbers represent the equivalent ng per 0.5 µg marker for approximate quantification of DNA

2.2.7. Restriction enzyme digestion of DNA

Restriction enzymes were obtained from Promega, NEB and Roche, and were used for digestions carried out according to manufacturer's protocols. The reaction volume was routinely between 20 – 50 µl and was adjusted according to the enzyme chosen, total mass of DNA and length of time for digestion. When reactions were required for downstream applications, heat-inactivation was completed as per manufacturer's instructions.

S1 nuclease digestion was completed by suspending bacterial colonies in 8 µl of PBS and boiling at 95 °C for five minutes before being cooled on ice. The appropriate dilution of buffer and 1µl of enzyme (Promega) was added to the suspension. The reaction was incubated at 37 °C for one hour and then separated by gel electrophoresis.

2.2.8. Dephosphorylation of DNA

Plasmid DNA linearised by one restriction enzyme is prone to self-ligation in subsequent ligation reactions. This can be prevented by adding alkaline phosphatase which removes the 5' phosphate groups from DNA. Thermosensitive alkaline phosphatase (Promega) was added to plasmid restriction digests at 1 U/µg DNA. When the reaction was complete, TSAP was inactivated at 74°C for 15 min.

2.2.9. Ligation of DNA fragments

Ligation reactions were performed in a final volume of 10 – 20 µl containing 3 U of T4 DNA ligase (Promega) and 1 – 2 µl of 10 × ligation buffer. The reaction was incubated first at room temperature for 60 min and then overnight at 4°C, and subsequently transformed into *E. coli*.

2.2.10. Chromosome walking

2.2.10.1. Inverse PCR-mediated chromosome walking method

This technique was adapted from the method outlined by Khairatkar-Joshi and Ahmad (1998). Briefly, 3 µg of genomic DNA was digested with HincII in the case of strains containing incomplete integrons with the *intI1* gene region missing, and AleI in the strain which contained an incomplete integron with the *qacEΔ1-sul1* region missing. These enzymes were selected after analysis showed that no restriction sites were found in the centre of the gene regions, and that they cut with sufficient frequency in the remainder of the *A. baumannii* genome to facilitate the production of digestion product sizes to be ligated into sizes which could be amplified by PCR.

Following digestion, the DNA was purified by phenol-chloroform extraction and dissolved in 80 µl TE buffer. Ligation was performed in a total volume of 120 µl at 16 °C for 15 hours, followed by 24 hours at 4 °C. Ligations were subsequently extracted using phenol-chloroform extraction again, and the dried DNA was reconstituted in 10 µl TE buffer. Of this solution, 1 µl was used as a template for each inverse PCR, where primers facing outwards from the

known region were used to amplify different PCR products. The PCR products were separated by gel electrophoresis and the appropriately sized band was extracted using the gel extraction methods outlined above, and then the DNA sent for sequencing.

2.2.10.2. Two-step gene walking method

The method outlined by Pilhofer et al. (2007). An outline of the technique is given in **Error! Reference source not found.**. For the incomplete integrons, the alking primer used was PR1751, and the sequencing primer used was PR2523 (see **Error! Reference source not found.** A). For the method of testing for multiple integron types, primer PR2523 was used for walking, and PR2525 was used for sequencing. Both procedures were carried out using Phusion Hi-Fidelity DNA polymerase.

Figure 2.3 Two-step chromosome walking

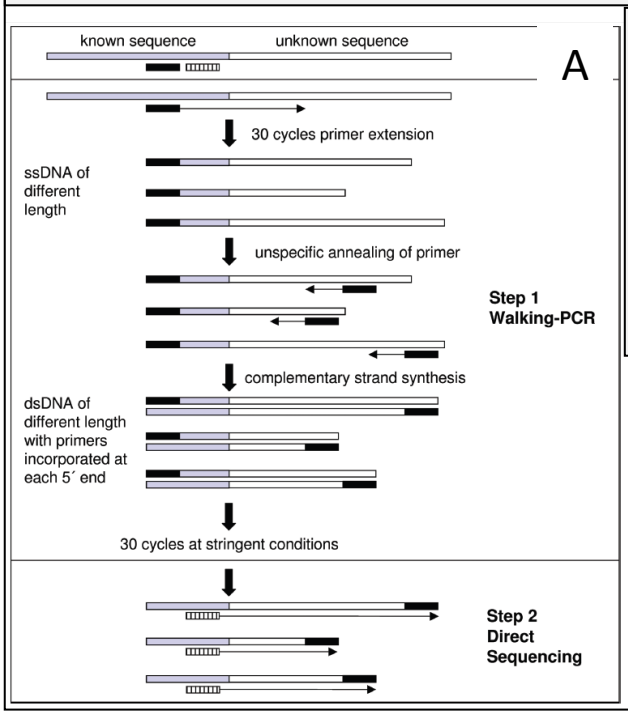


Figure showing the basic principle behind the two-step chromosome waking method outlined by Pilhofer *et al.* (2007). A) Specific walking primer is shown in black, whilst the nested sequencing primer is striped. B) Cycling conditions of one round of stringent conditions, one round of non-specific conditions, and finally another PCR under stringent conditions (taken from Pilhofer, *et al*. 2007).

B

Table 1. Walking-PCR cycling program

Round	Cycles	Temperature	Time	Action
		94°C	4 min	Primary denaturation
1	30	94°C 56°C 72°C	30 s 30 s 3 min	Specific primer extension; ssDNA synthesis
2	1	94°C 40°C 72°C	30 s 30 s 3 min	Unspecific binding of primer; dsDNA synthesis
3	30	94°C 56°C 72°C	30 s 30 s 3 min	Specific exponential amplification
		72°C	10 min	Final elongation

2.3. Typing methods: MLST, DNA sequencing and Global Clone identification

For the purposes of MLST, the normal Go*Taq* DNA polymerase conditions were completed with primers at 500 nM and dNTPs at 100 µM for 35

cycles, in order to maximise product concentration and lower the risk of contamination of the sequencing reaction by residual primers and dNTPs. The PCR reaction was then diluted 1 in 5 by taking 10 µl of the reaction and diluting it with 40 µl nH_2O, and Sanger sequencing was completed by GATC Biotech or Source Bioscience.

The primers used for MLST were taken from the scheme developed by Bartual, et al. (2005), except for those of alleles *gdhB* and *gpi*, which were redesigned in this study due to inconsistent amplification across the clinical isolates. Primers were redesigned after analysis of aligned sequences from currently available reference genomes of *A. baumannii* on NCBI.

Briefly, BLAST analysis of the region 150 bp upstream and downstream from each allele was used to identify and compile matching sequences from the regions around both alleles. These sequences were then aligned using Clustal X (Larkin et al. 2007). Conserved regions were targeted for primer design by Primer 3, and in the case of *gpi*, it was necessary to include one and two wobbles in the forward and reverse primer respectively, in order to amplify all the analysed strains in the relevant region. The new primers were tested and successfully amplified in all *A. baumannii* strains in our collection, and when tested for sequencing, facilitated the assignment of allele numbers to all the strains.

The sequences were checked for their allele numbers and ST types using the PubMLST website for *A. baumannii* (http://pubmlst.org/abaumannii/), and new alleles or sequence types were also submitted onto this database when required.

Phylogenetic trees drawn on the basis of the allele sequences were completed using the Sequence Type Analysis and Recombinational Tests (START 2) software, using the neighbour-joining method (Jolley et al. 2001).

Global clone number was assigned to isolates on the basis of bands obtained by multiplex PCR as outlined in the scheme by (Turton et al. 2007) targeted at different alleles of *ompA*, *csuE* and $bla_{\text{OXA-51-like}}$ genes, where possible.

2.4. RNA-related techniques and methods

2.4.1. RNA extraction

Following antibiotic induction, 5ml of cells were harvested by centrifugation for 5 min at 4000 rpm, and resuspended in 1 ml RNALater (Invitrogen) and stored at 4°c. The total RNA was extracted the next day using an amended version of the RNeasy mini kit protocol (Qiagen) as described below. The pellet was resuspended in 200 µl TE buffer containing 1 mg/ml lysozme and incubated at room temperature for 10 min with vortexing every two minutes. 700 µl buffer RLT (Qiagen; 1/10 volume of ß-mercaptoethanol was added to buffer RLT before use) and the solutions were mixed well. 500 µl 100 % ethanol was added and the RNA fixed to the membrane in an RNeasy mini spin column (Qiagen) by centrifugation for 15 s at 9000 × *g* in a benchtop microfuge and the flow-through discarded. The sample was washed by centrifugation of 350 µl buffer RW1 through the column for 15 s at 9000 × *g*. DNA in the sample was digested by pipetting 80 µl buffer RDD containing 10 µl DNaseI stock solution (Qiagen) onto the column and incubating it at room temperature for 15 min The DNA was washed off the membrane by adding 350 µl buffer RW1 and centrifuging the column for another 15 s at 9000 × *g*. The sample was washed by addition of 500 µl buffer RPE (Qiagen) onto the column and centrifugation for 15 s at 9000 × *g*. Another 500 µl buffer RPE was pipetted onto the column and it was centrifuged for 2 min at 9000 *g*, the flow-through was discarded and it was centrifuged for a further 1 min at 9000 × *g*. The RNA was eluted from the column into a 1.5 ml microcentrifuge tube by addition of 30 µl RNase-free water and centrifugation for 1 min at 9000 × *g*. RNA solutions were stored at -20°C until needed.

2.4.2. Reverse Transcription

Total RNA was by quantified spectrophotometry at A_{260} (Nanodrop 2000; Fisher ThermoScientific), and cDNA was created by taking 1 µg of RNA was for each reverse transcription, and the procedure was completed according to the Qiagen Reverse Transcription kit (Qiagen), including a ‘genomic DNA wipe’

step. In each case, a simultaneous reaction was set up without the reverse transcriptase enzyme to provide a negative control for gDNA contamination

2.4.3. gPCR in detection of integron gene cassettes circularization as an indicator of integrase activity

Integrase functioning was analysed by the detection of circular gene cassette structures within crude cell extract. For both plasmid and chromosomal integrons in both wild type and constructed integron structures, samples were prepared according to the method outlined by (Yang et al. 2009). Briefly, strains containing these structures were cultured overnight in LB, and subcultured the next day. After reaching OD_{600} 0.2, cultures were induced using either IPTG or an SOS-response inducing antibiotic (either mitomycin C, ciprofloxacin or trimethoprim). After the desired induction time, 500 µl of the culture was removed and washed twice with 1 ml molecular grade water, before being reconstituted in 500 µl molecular water, and boiled for 10 minutes at 100 °C on a variable temperature heat block. The cell suspension was centrifuged at 16000 × *g* for 3 min, and the supernatant stored at – 20°C.

The circular form of the control single gene cassette integron (bla_{IMP-5}) was detected by the use of primers that are orientated outwards with respect to the bla_{IMP-5} gene (PR1679/PR1680).

2.4.4. SOS induction

5 ml overnight culture of *A. baumannii* or *E. coli* was subcultured 1:100 into fresh LB and then allowed to grow until OD_{600} = 0.2. At this point, for those cultures with SOS induction, antibiotics were added to a final concentration of 6 µg ml^{-1}/0.2 µg ml^{-1} for mitomycin C (MTC) for *A. baumannii*/*E. coli* respectively, and 0.4 µg ml^{-1} for ciprofloxacin (CIP) and 10 µg ml^{-1} for trimethoprim (TMP) for *A. baumannii* only. A non-induced control was allowed to grow along side these cultures on each occasion, under identical conditions, but without the addition of any antibiotic. For gene expression analysis, the entire 5 ml culture was centrifuged and the RNA extracted from the pellet as outlined in section 2.3.

For the purposes of PCR to detect circular forms of gene cassettes, 500 µl of the culture was taken and prepared for PCR as described above (Yang et al. 2009). Briefly, cells were pelleted from 500 µl of the cultures by centrifugation at top speed for 2 minutes, and then washed twice with 1ml ultrapure water. The cells were then reconstituted in 500 µl ultrapure water and then boiled by heating to 100°C for 10 min. The suspension was centrifuged at 10000 × *g* for 3 minutes to pellet the cells, and the supernatant was separated for use as template in PCR reactions, and stored at -20°C for further analysis.

The excision of the gene cassettes was tested in *A. baumannii* strains AYE (for the genes *aac3*, *aadA1*, *orfX*, *dfrA1 and* bla_{VEB-1}, using primer pairs PR1953/PR1954, PR1955/PR1956, PR2044/PR2045 and PR1957/PR1958 respectively), AB0057 (for genes *aac3*, *aadA1*, *orfX* as before, and for *orfC* with primer pairs PR2023/PR2024) and ACICU (for genes *aac6* and *aadA1*, using primer pairs PR2233/PR2234 and PR1955/PR1956, respectively). qRT-PCR analysis was completed as outlined in section 2.4.6.

2.4.5. IPTG induction of pQE-based plasmids

5 ml overnight culture of *A. baumannii* or *E. coli* was subcultured 1:100 into fresh LB and then allowed to grow at 37°C with shaking, until it reached OD_{600} = 0.2. Then IPTG was added to induce the strains to a concentration of 1.0 mM, and the cultures were then incubated, with 500 µl of the culture removed at time points 4, 8 and 24 hours for crude DNA preparation as described above (Yang et al. 2009). The supernatant was used as template for qPCR analysis.

2.4.6. Quantitative PCR

Reactions were performed in duplicate 20 µl reactions using 2 µl of a 1 in 4 dilution of the cDNA, using the SensiMixPlus SYBR Green mix (Bioline) on the Corbett Rotorgene 6000 platform (Qiagen). Cycling conditions consisted of 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 20 s. The cycle threshold was always set at 0.1, and the slope correct function applied when required to give a reading at the exponential phase of the amplification.

Analysis for expression levels was completed using the REST software (Relative Expression Software Tool), from Qiagen (Pfaffl, Horgan & Dempfle 2002), or manually using the equation below (also used in the REST software), using 16S rRNA or *gyrB* as a reference gene for the expression analysis, and ColE1 *oriR* for the experiments analysing copy number of genetic entities in comparison to plasmid copy number.

$$\text{Gene expression ratio} = \frac{(E_{target})^{\Delta Cq \text{ target (control – sample)}}}{(E_{ref})^{\Delta Cq \text{ ref (control – sample)}}}$$

Efficiencies for each primer pair were calculated using the creation of standard curves and the efficiency calculated using the equation:

$$E = 10^{(-1/slope)} - 1$$

The specificity of the qPCR assay was checked by analysis of the melting curve for each reaction to ensure that the samples all gave a single sharp peak at a specific melting temperature. Any samples that gave a different peak were excluded from the subsequent data analysis. Template-free control reactions (NTC) were used to control for false positives, and to check the possibility of primer dimer formation.

2.5. Absolute quantification of plasmid copy number by qPCR

Quantification of plasmid copy number (PCN) was calculated against standard curves of known concentrations of purified genomic or plasmid DNA as appropriate. DNA concentration was measured by spectrophotometry at A_{260} (Nanodrop 2000; Fisher ThermoScientific), and then DNA copy number was calculated according to the following equation:

$$\text{DNA (copy)} = \frac{6.02 \times 1023 \text{ (copy/mol)} \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660 \text{ (g/mol/dp)}}$$

A graph of DNA concentration against C_t value was plotted using GraphPad Prism, and the absolute copy number of the relevant DNA was interpolated using the software, from the C_t readings of an 8 h sub-culture for each strain. The template for the timed cultures was a crude DNA preparation directly from

the culture, as outlined in section 2.4.4 (Yang et al. 2009). This was to avoid any specific extraction type that may have a bias for more efficient extraction of either plasmid or chromosomal DNA. For both *E. coli* and *A. baumannii* strains, their respective *gyrB* copy numbers were used for normalisation to the copy number of chromosomes (primers PR3509/PR3510 and PR2439/PR2440).

2.6. Genetic manipulation

2.6.1. Construction of plasmids for the analysis of integron function

2.6.1.1. pQE-80L-based IPTG-inducible plasmids

In order to study the functioning of class 1 integrons in *A. baumannii*, an *intI1* gene was cloned into an IPTG-inducible vector pQE-80L (Qiagen) in order to confirm its ability to excise and insert gene cassettes. The ABAYE3575 gene, coding for a class 1 integrase, in strain AYE was amplified using primers PR1719 and PR1569, which have restriction sites EcoRI and HindIII, respectively. pQE-int was constructed by digesting pQE-80L with EcoRI and HindIII to remove the protein-expression related His-tag-coding sequence. After double digestion of the PCR product with the corresponding enzymes, this amplicon was ligated into the digested vector. The PCR amplicon does not contain the native P_{int} promoter of the integrase gene but the primers were designed in such a way that when the resulting amplicon was ligated into the specified restriction enzyme sites, the open reading frame of the integrase gene would be in line with the LacZ expression apparatus, and hence *intI1* would then be directly controlled by the P_{lac} promoter.

To further study the activity of the cloned integrase, a region from a single cassette-bearing integron containing a bla_{IMP-5} gene cassette and surrounding *attI* and *attC* sites, was cloned into the XbaI site on the pQE-80L backbone using primers 5'CS (PR121) and 3'CS (PR122) with XbaI sites added. This created a plasmid in which both the *intI1* gene and the bla_{IMP-5} cassette were in equal molar ratio. The excision of the cassette was measured by the detection of the closed circular junction, which was confirmed by sequencing.

2.6.2. Construction of plasmids compatible with *A. baumannii* and other Gram-negative pathogens

In order to make the plasmid compatible with *A. baumannii* strains, the origin of replication was amplified by PCR from the pWH1277 (a cryptic plasmid from an *A. lwoffii* strain) section of pWH1266 (an *Acinetobacter – E. coli* shuttle plasmid, (Hunger et al. 1990), using the primer pair PR3136 and PR3137, which contain BamHI sites at the 5' end. pQE-WH-bla was created by the ligation of this section into the BamHI section of pQE-bla, the addition of which allowed the maintenance of the plasmid within *A. baumannii* A457. In each case where only one restriction enzyme site was used, the vector was subjected to alkaline dephosphatase prior to ligation using thermosensitive shrimp alkaline phosphatase (TSAP), to prevent relegation.

The pQE-BHR2 was constructed in a similar way, except the pRO1614 origin of replication was amplified by PCR from pFLP2 using primers PR2546/PR2614 to produce a 1341 bp product containing both the *oriR* sequence, and a gene coding for a replication protein. This was ligated into the BamHI site of pQE-bla as outlined above.

2.6.3. JTOOL-3 based Lambda pir suicide vectors

Gene knockouts were constructed by cloning SOE mutant alleles into a pDS132-based vector pJTOOL-3, constructed by van Aartsen (Philippe et al. 2004, van Aartsen, Rajakumar 2011). pDS132 is known to be a suicide vector in *A. baumannii* (Rieck et al. 2012), which facilitates the selection of recombinant strains only. The construct to knock out $umuD_{Ab}$ was created using the SOE method outlined in section 2.2.4. Briefly, regions flanking $umuD_{Ab}$ were amplified and then spliced to an FRT-flanked hygromycin gene cassette. The resultant SOE product was amplified with primers PR3002 and PR3005, which had PstI restriction sites incorporated at the 5' ends. The amplicon and pJTOOL-3 were both digested with PstI, and when ligated together, formed pJ-$umuD_{Ab}$-hyg.

2.6.4. Preparation and transformation of electro-competent bacteria

A single colony was used to inoculate 5 ml of LB, which was then grown overnight at 37ºC, 200 rpm. This culture was used to inoculate fresh LB at a ratio of 1:100 (e.g. 1 ml to 100 ml) which was grown to an OD_{600} of 0.4 – 0.5 after which the cells were cooled to 4°C by incubating on ice for 20 minutes. Following this, the cells were harvested by centrifugation (3000 × g, 15 min, 4ºC) and gently washed three times with successively smaller volumes of ice cold 10 % (v/v) glycerol. After the final wash, the cells were resuspended in a volume of ice cold 10 % (v/v) glycerol equal to $1/100^{th}$ of the initial culture volume and stored as 50 µl aliquots at -80ºC. Electroporation was completed using 0.2 cm cuvettes in the Bio-Rad Gene Pulser system (Bio-Rad) at the following settings: 25 kV/cm, 25 µF and 200 Ω. Immediately after transformation 950 µl of SOC (Appendix 1) was added and cells were incubated at 37ºC for 1 h. Transformed cells were then plated onto LA containing appropriate antibiotics and incubated at 37ºC, unless otherwise specified. This method was employed both for the preparation of electrocompetent *E. coli* and *A. baumannii*.

2.6.5. Suicide vector-based allelic exchange

The donor strain routinely used in conjugation for the mobilization of suicide vectors into *A. baumannii* was *E. coli* S17-1λ*pir.* This latter strain encodes the broad-host-range *tra* (transfer) genes of the RP4 conjugation machinery which enables mobilisation of plasmids that possess the RP4 plasmid mobilisation (*mob RP4*), such as that present in pDS132-based plasmids.

Briefly, recipient *A. baumannii* and donor *E. coli* S17-1λ*pir*/plasmid (in this case, pJ-umuD_{Ab}-hyg) strains were grown overnight in LB (plus required antibiotics) at 37ºC, 200 rpm. Next, overnight cultures were subcultured 1:100 into fresh LB without antibiotics and grown to an OD_{600} of 0.6 – 0.8. Donor and recipient strains were mixed at a 1:1 ratio (1 ml : 1 ml), pelleted at 7000 × g for 5 min, resuspended in 100 µl 10% glycerol and spotted onto pre-warmed LA plates, and incubated at 37ºC overnight, or in some cases for 36 hours. When

necessary, control conjugations were performed using a 1:1 ratio of donor:donor and recipient:recipient. Next, matings were scraped from the plate in 1 ml of 10% glycerol and appropriate dilutions were plated onto Simmon's Citrate Agar (SCA - Appendix 1) supplemented with hygromycin and incubated overnight at 37ºC. Single colonies were then parallel patched onto LB only, LB plus chloramphenicol and LB supplemented with hygromycin to screen for integration of suicide plasmids. The merodiploid (single-crossover) genotype was confirmed by colony PCR to detect the suicide vector backbone (primers to detect *sacB* - PR1656 and PR807) and the FRT-flanked *hph* gene cassette (primers PR351 and PR2773).

Next, a single merodiploid colony was grown overnight in LB and appropriate dilutions were plated on LA media containing 10 % sucrose (Appendix 1), which were incubated overnight at 37ºC. Colonies were then screened by patching for the double-crossover resistance phenotype (hygromycin-resistant and chloramphenicol sensitive). Additionally, suicide vector loss was confirmed by colony PCR testing the loss of the plasmid backbone and mapping to the targeted area by primer pairs that bound within the hygromycin gene cassette and both upstream and downstream of the $umuD_{Ab}$ region (primer pairs PR3075/PR3313 and PR3314/PR3076).

2.7. Other methods

2.7.1. Southern blotting

2.7.1.1. DNA and probe preparation

Southern blotting was completed using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche), according to the manufacturer's instructions. Probes were produced by PCR using the PCR DIG Probe Synthesis Kit (Roche) to create a 569 bp probe of a conserved region internal to *intI1* genes, and a 902 bp section that covers the *qacEΔ1-sul1* fusion junction, to be specific to known conserved regions of class 1 integrons. This 902 bp fusion-junction region, encompassing both fused genes, was chosen in

order to ensure that only class 1 integron-related regions would be detected, rather than alternative, but closely related, *qacE* or *sul* genes.

The primer pairs PR1241 and PR1242, and PR978 and PR1213 were used for the amplification of the *intI1* and the *qacEΔ1-sul1* probes respectively. Briefly, 100 pg of plasmids pQE-bla and pG-AYEi, each bearing a region of an integron separately, were used as template in a 50 µl reaction which contained DIG-labelled dUTP (used at the standard concentration of 70 µM). This becomes incorporated into the PCR products and facilitates chemoluminescent detection of fragments to which the probe is bound. The synthesis of a correctly labelled probe was confirmed by comparison with an identical PCR containing unlabelled dUTP visualised by gel electrophoresis, with the correctly labelled probe running at a higher point in comparison to the non-labelled PCR product.

All genomic and plasmid DNA preparations were prepared for gel electrophoresis before Southern blotting in the following way. For each strain, 500 ng and 50 ng of genomic and plasmid DNA respectively were digested using AleI, and run on a 4 mm thick 0.8 % agarose gel containing 0.5 × working concentration of Gel Red (Biotium), with genomic and plasmid DNA for one strain alongside each other, at 75 V for 1.5 hours. The use of ethidium bromide (EtBr) was avoided for these gels, since it is known that EtBr can cause uneven background problems in Southern blotting. Following electrophoresis, the gel was depurinated in 0.25 M HCl for 30 min with slow agitation. The gel was then rinsed in ddH_2O and soaked in transfer buffer (0.4 M NaOH).

2.7.1.2. Blotting and hybridisation

The blotting steps were completed using capillary action with filter paper and 0.4 M NaOH as the transfer buffer in order to transfer the DNA onto an appropriately sized piece of positively charged nylon membrane (Roche). After correct assembly of the capillary transfer, the gel and membrane was routinely left for 3 hours to overnight. Following blotting, the successful transfer of the DNA was confirmed by visualising the gel again using UV illumination, to check for significant or total reduction of DNA signal compared to the image prior to blotting.

After neutralisation of the membrane in 5 × SSC for 10 min, it was placed on a piece of Whatman paper dampened with 10 × SSC, and the DNA was fixed by crosslinking using 254 nm UV light, at 120 J/cm^2 (Stratalinker; Stratagene).

Following this, the membrane was pre-hybridised by incubation at the optimum hybridisation temperature T_{opt} temperature for 30 min in 10 mL hybridisation solution using a rolling hybridisation bottle. The pre-hybridisation solution was then discarded, and the PCR probe added to 10 mL hybridisation solution at a concentration of 2 µl/mL, and the membrane incubated at T_{opt} overnight. T_{opt} was calculated using the equation in the DIG High Prime DNA Labelling and Detection Starter Kit II manual.

$T_m = 49.82 + 0.41$ (% G + C) - (600/l) [l = length of hybrid in base pairs]
$T_{opt} = T_m$ – 20 to 25°C

This temperature was 52 °C and 50.6 °C for the *intI1* and *qacEΔ1-sul1* probes respectively.

For immunological detection, the membrane was washed twice in ample 2 × SSC 0.1% SDS solution for 5 min under constant agitation, then twice in pre-warmed 0.5 × SSC, 0.1% SDS at 65 °C for 15 min. This was followed by 5 min wash in 100 ml Washing buffer, 30 min wash in 100 ml Blocking solution, 30 min wash in 20 ml Antibody solution, twice in 100 ml Washing buffer for 15 min and then for 5 min in 20 ml Detection buffer. All washes were completed with gentle agitation and at room temperature (20 – 25 °C) unless otherwise stated.

The membrane was placed in a development bag, and 1 ml CSPD was applied before incubation at room temperature for 5 minutes, and then at 37°C for 10 minutes to enhance the luminescent reaction. Exposure of the membrane to photographic film was routinely allowed for 5, 10 and 20 min, in order to get a range of signal strengths and detect both high and low signal results.

2.7.1.3. Stripping and re-probing of blots

After the appropriate images were taken, then membrane was stripped of the first *intI1* probe according to the method outlined in the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche). Briefly, the membrane was rinsed thoroughly in double distilled water, before washing two times for 15 minutes 37°C in 0.2 M NaOH containing 0.1% SDS to remove the DIG-labeled probe. Then, the membrane was rinsed thoroughly 5 minutes in 2 × SSC. Following these steps, the membrane was then treated to the same prehybridization and hybridization steps as outlined in 2.7.1.2, but with a second probe, binding to a region across the fusion junction of the *qacEΔ1-sul1* part of the 3' end of class 1 integrons

2.7.1.4. Growth curves

Analysis of fitness of plasmid carriage was undertaken by analysis of growth curves of bacterial strains with and without the plasmids, under and in the absence of IPTG induction. Briefly, strains were grown overnight in LB (~1 × 10^9 CFU/ml), then diluted 1/100 in LB, with appropriate concentrations of antibiotic added for the maintenance of plasmids, and IPTG, where required. Cultures were pipetted into a 96-well flat-bottomed plate, with 150 µl in each well. All strains and conditions were repeated eight times. The absorbance at OD_{600} was measured every ten minutes for 24 hours using the Varioskan Flash Reader (Thermo Fisher Scientific), and then normalised to an LB-only control. Readings were converted into a graph growth curve using Graphpad Prism.

2.7.2. Data analysis and statistics

Statistical analysis of gene expression was completed using Student's unpaired *t*-test, comparing baselines from either wild-type strains or uninduced strains grown under normal conditions.

Growth curves were plotted using GraphPad Prism, and differences between the wild-type strain and presence of plasmids and IPTG induction were analysed by a two-way ANOVA of the total area under each curve.

P values less than 0.05 were considered statistically significant.

2.8. Bioinformatics

Table 2.1 Table showing the computing software used in this project

Name	Functions	Reference
BLAST		
BLASTn	Searches DNA database for similar DNA sequences	(Altschul et al. 1990)
BLASTx	Translates DNA in six frames and searches a protein database for similar sequences	
tBLASTx	Translates DNA in six frames and searches a database of nucleotide sequences also translated in six frames	
Sequence analysis		
A Plasmid Editor	Sequence editor of GenBank format files for assembly of sequencing traces, virtual digests, sequence annotation, and sequence schematic representation	(Davis 2013)
Finch TV	Sequence trace file viewer	(Geospiza 2006)
Other		
ClustalX	Visual multiple sequence (nucleotide and protein) alignment tool	(Larkin et al. 2007)
In silico PCR	Online program to perform *in silico* PCR	(Bikandi et al. 2004)
Oligo Calc	Oligonucleotide analysis	(Kibbe 2007)
Primer3	Primer design	(Rozen, Skaletsky 1999)
START2	Software tool for analysis of MLST data, including lineage assignment and data summary	(Jolley et al. 2001)
REST	Relative Expression Software Tool for analysis of RNA expression data	(Pfaffl, Horgan & Dempfle 2002)
Graphpad Prism 6	Analysis of data and graph construction	www.graphpad.com
ACID	Annotation of Cassette and Integron Data	(Gillings et al. 2009)

Chapter 3. Survey of integrons in clinical isolates of *A. baumannii* from Leicester and other European sources

3.1. Introduction

A high prevalence of integrons in clinical isolates of *A. baumannii* have meant that there have been several recent studies documenting integrons gene cassette arrays and epidemiology in this important nosocomial species (Turton et al. 2005, Liu et al. 2014). Indeed, Turton et al. (2005) have highlighted that all epidemic strains of *A. baumannii* found in their study contained class 1 integrons, suggesting that there is a significant advantage conferred to strains containing these genetic elements.

Although there have been more than six classes of integrons discovered, the most common class present in clinical isolates of Gram-negative pathogens, in particular in *A. baumannii,* is class 1. Class 2 integrons have also been discovered in *A. baumannii*, but strains bearing these structures are relatively rare (Ploy et al. 2000, Ramirez, Quiroga & Centron 2005).

These studies have also attempted to establish a system linking integron types to strain types, but tend to be mainly successful in groups of isolates collected in a short space of time or localised geographical area (Koeleman et al. 2001, Turton et al. 2005). In order to gain full epidemiological value, the integron types must be analysed alongside genotype grouping systems. There are also a significant number of descriptive studies of gene cassette prevalence. These studies are valuable in documenting the type and prevalence of integrons, as well as providing an insight into horizontal gene transfer or clonal expansion events between outbreak strains. However, the majority of these studies employ a simple PCR assay of using primers that bind in the 5’ and 3’ conserved regions. This produces a bias to only detecting complete integrons and so does not give information about the changes in integrons due to deletions and insertions. Alternative methods detecting the

intI1 gene alone only give a simple indication of the presence or absence of a small internal region of this gene, not yielding any information regarding functionality. Very little focus is placed on atypical integron structures which may have truncations or inversions, and aren't amenable to detection by these conventional methods, and are subsequently largely ignored.

In addition, whilst in some cases the genetic context of integrons are noted (for example, if it is part of a composite transposon), many of these studies do not investigate whether the integrons are located on the plasmid or chromosome. Since there are potentially many more copies of plasmids than chromosomes carried within each cell, genes expressed on plasmids may carry a heavier fitness cost. There are a multitude of cases in which integrons have been reported on plasmids of common Gram-negative pathogens such as *E. coli*, *Salmonella enterica* spp. and *Klebsiella pneumoniae* (Villa, Carattoli 2005, Chen et al. 2006, Wang et al. 2003, Winokur et al. 2001). In fact, simple literature and BLAST searches for class 1 integron features in these species produces mainly plasmid-based hits. Conversely, only three separate plasmids in *A. baumannii* have been identified as carrying integrons (Zhu et al. 2013, Opazo et al. 2012, Huang et al. 2012) to date, despite 70 complete plasmids available for analysis (4.29 %). In comparison, integron structures are contained within 42/303 (13.8 %) in *E. coli,* 33/121 (27.27%) in *K. pneumoniae,* and 4/10 (40 %) in *P. aeruginosa* plasmids.

A reason why this might be the case could be due to the regulation of integrase expression. A regulation mechanism was first discovered in *Vibrio cholerae,* linking integrase activity to the SOS response system via a LexA-mediated repression, a system also found in other Gram-negatives, such as *E. coli* (Guerin et al. 2009). It has since been shown that a similar integrase repression mechanism was present in other species, for example, *Pseudomonas aeruginosa* (Hocquet et al. 2012).

Whilst it seemed clear that LexA had a vital role in integrase expression regulation in these Gram-negative species, there has been limited investigation as to how integrase expression may be regulated in a LexA-negative species. In the absence of a repression system, integron carriage and epidemiology may

be affected (Cambray et al. 2011). There are many genera of bacteria that do not have a LexA homologue and have a different SOS-response defence mechanism, for example *Streptococcus*, *Neisseria* as well as *Acinetobacter*. This issue was discussed by Cambray, et al. (2011), who concluded that SOS-type regulation is the natural, ancestral state of integrons, based on phylogenetic evidence, and that unregulated integrons are likely to be under negative selection. This led to the hypothesis that integrons in *A. baumannii* may also be under negative selection, and hence may come to have deleted regions or pseudogenes, and would be most often present in one copy on the chromosome. At the same time, it has been shown that integrons are widespread in *A. baumannii*, suggesting a strong positive selective pressure for the maintenance of these genetic elements. This project attempts to put forward a hypothesis that resolves these two cost-benefit issues, through analysis of integron frequency, structures and locations in clinical isolates of *A. baumannii*.

To investigate how integrons function and are carried, the first part of this project was to screen two groups of *A. baumannii* clinical isolates, one from the local University Hospitals of Leicester (UHL; n = 10), collected between 2007 and 2008, and compare these with a collection of isolates obtained from European-wide sources (EUR; n = 50), mainly from Kevin Towner (Queen's Medical Centre, Nottingham). A total of 60 isolates were screened for the presence of class 1 integrons, and the structures of associated cassette arrays were determined. The results of this screening was analysed against a background of all fully sequenced and annotated reference genomes available on NCBI. These isolates are therefore representative of past and recent strains which were unusually difficult to treat or were saved in archives for other reasons. This selection puts the focus of this project on looking at the problem nosocomial isolates, and thus makes this work more clinically relevant. In all cases, every effort was made to ensure that no two strains were taken from the same patient, to avoid duplication and thus biasing of results. The development and findings of different biomolecular techniques for detecting multiple and incomplete integrons within a strain are outlined in this chapter.

3.2. Results

3.2.1. Integron survey and gene cassette array mapping

3.2.1.1. Gene cassette identification in the A. baumannii isolate collection

In order to study integrons in *A. baumannii*, a collection of clinical isolates were surveyed using a PCR screening method. Initial screening involved using the 5'CS and 3'CS primers to yield one or multiple amplicons. Those isolates which did not yield a product were screened using separate primers for the integrase genes associated with integron classes 1, 2 and 3, using primers intI1L/intI1R, intI2L/intI2R and intI3L/intI3R (Ploy et al. 2000).

All 55 clinical isolates were screened for class 1 integrons using 5'CS and 3'CS primers. Isolates that gave products had their gene cassette array mapped by PCR and restriction digestion mapping. Digestion of 5'CS and 3'CS amplicons were first completed alongside sequenced strains in order to establish which strains harboured identical integrons. PCR mapping was used to confirm the presence of most common gene cassettes, *aac3*, *orfX*, *orfC*, *aadA1a*, *aacA4*, *aadB*, *dfrIA*, bla_{VEB-1}, and *catB8* (using primers PR1953/PR1954, PR1957/PR1958, PR2023/PR2024, PR1955/PR1956, PR2233/PR2234, PR2257/PR2320, PR2044/PR2045, PR2020/PR2021 and PR2436/PR2437 respectively). Wherever possible, strains known to contain those cassettes, or certain arrangements were included in the PCR as a positive control, but in some cases, novel arrangements were found. The sequences of any remaining unknown regions were established by DNA sequencing of PCR products and chromosome walking (described in section 2.2.10).

Isolates found to be PCR-negative for class 1 integrons using the 5'CS and 3'CS primers were also screened for the presence of the *intI1* gene (using primers PR1241 and PR1242), present at the 5'CS region, and also the *qacEΔ1-sul1* gene fusion at the 3'CS region (using primers PR978 and PR1213).

In two instances, more than one integron was detected by 5'CS (PR121) and 3'CS (PR122), primers in the conserved region. In these strains, individual mapping on the integron PCR products to determine the cassette array was hampered by the carryover of each band during separation. For this reason, the array of these integrons was determined by restriction digestion of the integron amplicon with HinfI and SacI, depending upon the predicted array, and the digestion was pattern compared with known integron gene cassette arrays. In particular, this method was used in the two strains A401 and A418 in which two integrons were detected, as PCR mapping alone difficult to interpret.

Chromosome walking was used in instances when incomplete integrons were identified (see section 2.2.10). This included two strains where only the *qacEΔ1-sul1* region could be detected (no integrase present), 16 strains where one complete integron was present alongside one incomplete integron, one strain in which only the *intI1* region was detected (no *qacEΔ1-sul1* region), and lastly two cases where two complete integrons were detected by Southern blotting (see later section), but only one amplicon was produced by PCR. Two main methods of chromosome walking were employed – the classical chromosome walking method and a two-step PCR method (Pilhofer et al. 2007). The classical method involved digestion of the genomic DNA, self-ligation of the resultant fragments in a large ligation reaction, followed by inverse PCR across the ligated region. Conversely, the two-step method only involves a round of PCR with one primer under non-stringent, then stringent conditions, in order to produce PCR products which can be sequenced

Two isolates were found to be positive for the 3' conserved region, but absent for the 5' region by PCR screening. Using the two-step chromosome walking method, it was discovered that the whole of the 5' end and the corresponding gene cassette arrays had undergone a deletion, most likely due to the insertion of an IS*Aba1* element, interrupting this region. A further strain (A424) in our collection was found to contain an incomplete integron also interrupted by an IS element, following genome sequencing of the Tn*AbaR23* (Kochar et al. 2012). The integron from this strain was classified in this study as Type XVII. A summary and schematics of all the different integron types found in this study are shown in Figure 3.1.

Figure 3.1 Class 1 integrons detected in *A. baumannii* clinical isolates included in this study, showing high diversity of gene arrays

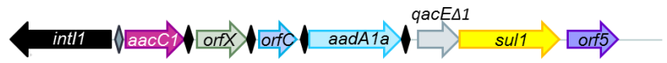
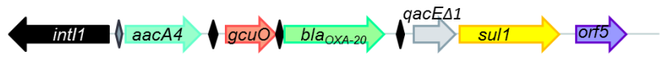
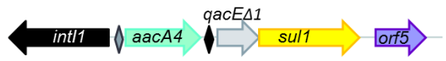
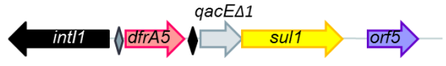
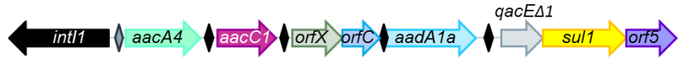
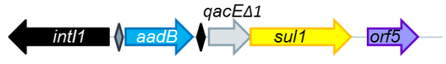
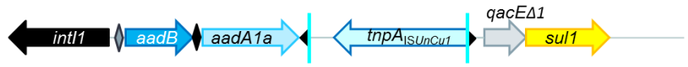
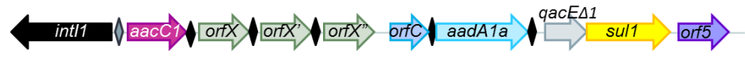
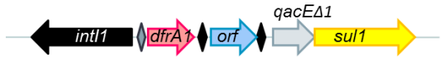
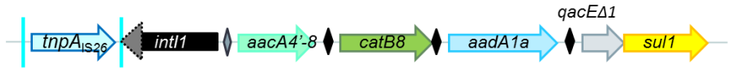
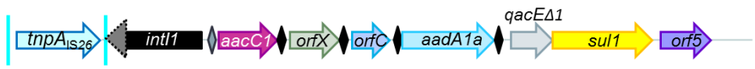
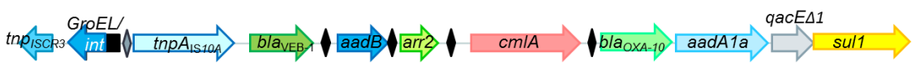
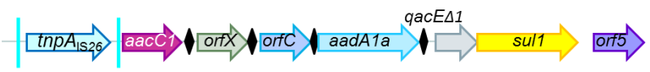
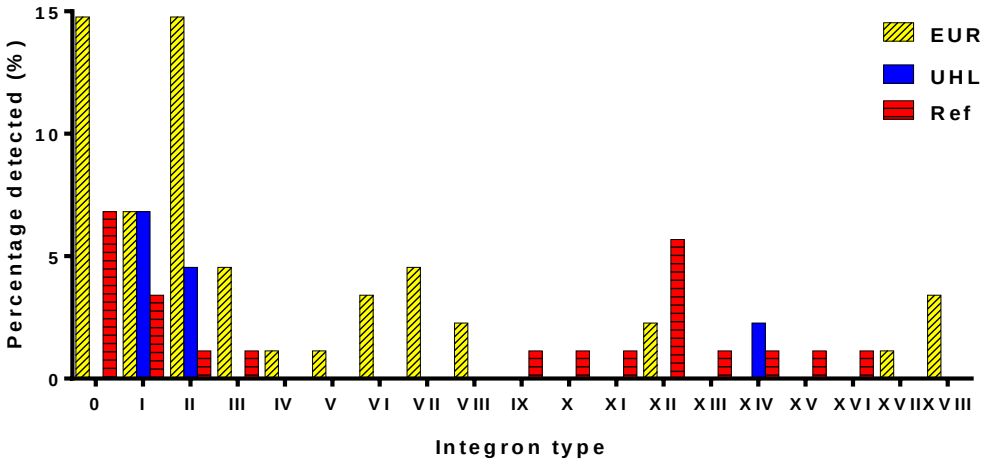
Integrall	Type	Index strain	Cassette array	Accession no.
In561	I	AB0057		KM972598
In616	II	AYE		KM972595
In426	III	ACICU		KM972600
In46	IV	A63		KM972589
In13	V	A297		KM972590
In611	VI	A387		KM972594
In7	VII	A329		KM972591
In290	VIII	A473		KM972597
In906	IX	MDR-TJ		NC_017847
In711	X	TCDC-AB0715		NC_017387
In363	XI	AYE		NC_010410
In439	XII	A380		KM972593
In561	XIII	MDR-ZJ		NC_017172
In266	XIV	1656-2		KM972599
In1056	XV	1656-2		NC_017162
In622	XVI	AYE		NC_010410
In561	XVII	A424		N676148
Unassigned	XVIII	A343		KM972592

Diagram showing schematic of whole and partial integron array types identified in this study, with named type strains from sequenced genomes where possible. Gene cassette arrays are labelled with the identification numbers of the online integron database Integrall. However, the database does not cover integrons without integrases (labelled as "unassigned"). Gene cassettes confer resistance to antibiotics as follows: *aacA4* – amikacin; *aacC1* – gentamicin; *aadA1a* – streptomycin/spectinomycin; *aadB* – kanamycin/gentamicin; arr2 - rifampicin; bla_{OXA-10} – oxacillin; bla_{OXA-20} – carbapenems; bla_{VEB-1} - cephalosporins; *catB8* – chloramphenicol; *cmlA* – chloramphenicol; *dfrA5* - trimethoprim; *orfX*/*orfC*/*orf5*/*gcuO* – unknown function; ***qacEΔ1*** – quaternary ammonium compounds (truncated); *sul1* – sulphonamide resistance. Inverted repeats of IS elements are shown as light blue vertical bars flanking their transposase gene.

Figure 3.2 Frequency of detection for integron types



Bar graph showing the frequency of each integron type in the two sets of clinical isolates (European set = diagonal striped yellow bars; UHL set = plain blue bars; reference sequenced genomes = horizontal striped red bars), based on the typing system outlined in Figure 3.1.

A total of 68 class 1 integrons were detected in 57 strains, of which 52 were full integrons with complete *intI1* genes, but no class 2 or 3 integrons were discovered in this isolate collection. All integron were assigned Roman numeral Type numbers for reference within this study (Figure 3.1). The In numbers corresponding to the gene cassette arrays documented in the INTEGRALL database for ease of cross-reference between studies. The most common integron gene cassette array types detected were In616, In561 and In439. All the integron types identified had previously been reported in *A. baumannii*, with the exception of the incomplete integron Type XVIII, which resulted from an IS*Aba1*-mediated deletion event (Figure 1).

3.2.2. Phylogeny and integron type designation

After establishment of different integron types, it was analysed whether integrons could be linked to different typing systems, in order to see whether it would be possible to glean some information regarding integron development or movement. Two main typing systems in *A. baumannii* are multi-locus sequence typing (MLST) and global clone (GC) assignment (Bartual et al. 2005, Turton et

al. 2007). MLST functions by analysing the sequence of internal regions of seven house-keeping genes – *gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD* – and then assigning different numbers for each new allele, allowing the allocation of a sequence type (ST). The method by Turton, et al. (2007) employs a simpler method where a multiplex PCR assay using two sets of three primer pairs can assign an *A. baumannii* strain according to a positive or negative result from each primer pair. Since both systems are widely used in *A. baumannii* studies, both procedures were undertaken on the strain collection, to establish whether a link could be made between phylogeny and integron carriage. However, there were some problems found with both methods, since the primers did not amplify a PCR product for all the *A. baumannii* clinical strains.

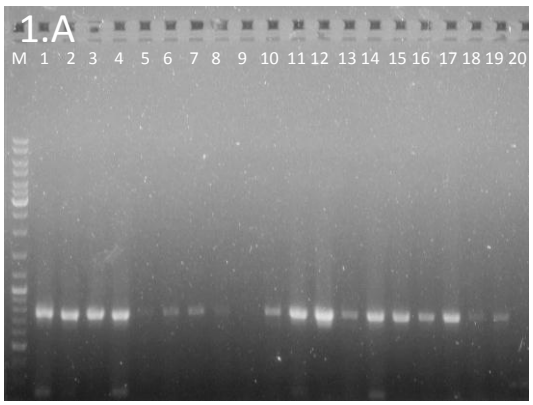
3.2.2.1. Optimisation of MLST by redesigning of gdhB *and* gpi *primers*

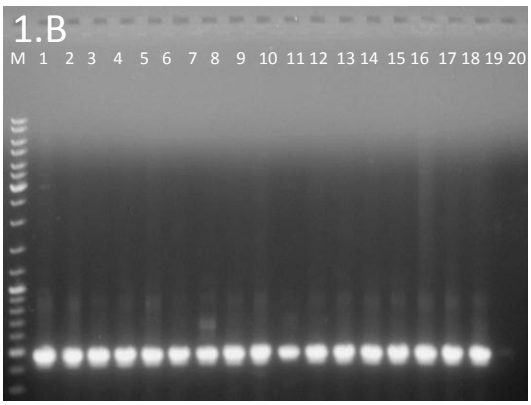
In particular, there were significant problems with primers used to amplify the genes *gdhB* and *gpi*, the most diverse genes in the MLST system designed by Bartual, et al. (2005), and have 144 and 199 different alleles respectively, at the time of writing. When using the updated primers published on the PubMLST website for *A. baumannii* (http://pubmlst.org/abaumannii/), only some strains yielded an amplicon for *gdhB*, and almost none for the *gpi* gene when tested on an initial selection of 18 from the isolate collection. This prompted a redesign of the primers used for the amplification of these genes (see Figure 3.3).

In order to maximise the number of *A. baumannii* strains to which the new primers would bind, the relevant region from sequenced genomes were aligned using Clustal X (Larkin et al. 2007), and conserved regions suitable for primer design were identified to be processed by Primer3 (Rozen, Skaletsky 1999).

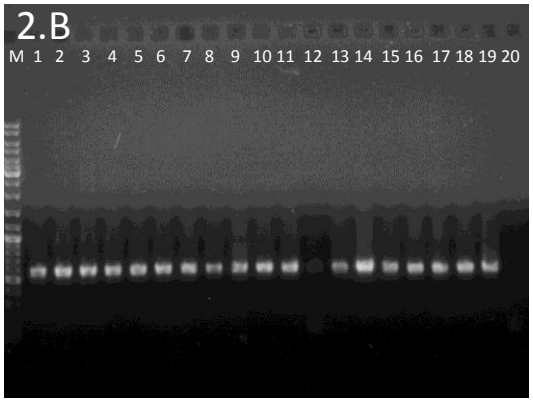
In the case of the *gpi* gene, the levels of variation were too high for simple primer design, and nucleotide wobbles had to be incorporated in order to ensure sufficient binding across all *A. baumannii* strains (see Figure 3.3, 2B).

Figure 3.3 PCR results for *gdhB* and *gpi* genes using established and new optimised primers redesigned in this study





Primers by Bartual, *et al.* (2005)		Redesigned primers in this study	
GDHB 1F	GCTACTTTTATGCAACAGAGCC	**gdhB M13 F**	CAGGAAACAGCTATGACCTAT GGGGGCCAGATAATCAA
GDHB 775R	GTTGAGTTGGCGTATGTTGTGC	**gdhB M13 R**	TGTAAAACGACGGCCAGTTGA GTCGGAGTATGCTGTGC



Primers by Bartual, *et al.* (2005)		Redesigned primers in this study	
gpi_F	GAAATTTCCGGAGCTCACAA	**gpi M13 F**	CAGGAAACAGCTATGACCGG RAAACCAATACAAGACCAAA
gpi_R	TCAGGAGCAATACCCCACTC	**gpi M13 R**	TGTAAAACGACGGCCAGTTTR CGTTTACCMTCGGAATA

Gel images to show inadequate binding of the current *A. baumannii* MLST primers to *gdhB* (1.A) and *gpi* (2.A) genes, compared to redesigned primers (1.B and 2.B). Underlined regions in primers denote M13 F and R sequences. Nucleotide wobbles in primers are highlighted in red letters, where R = A or G, and M = A or C.

PCR templates in lanes are M = λ HindIII; 1 = AYE; 2 = AB0057; 3 = ACICU; 4 = A457; 5 = A14; 6 = A230; 7 = A297; 8 = A442; 9 = A92; 10 = A473; 11 = A401; 12 = 1375; 13 = 1380; 14 = 1402; 15 = Crete; 16 = 1381; 17 = A387; 18 = 1404; 19 = A332; 20 = H_2O. Strain 1375 was subsequently identified to be *A. calcoaceticus*.

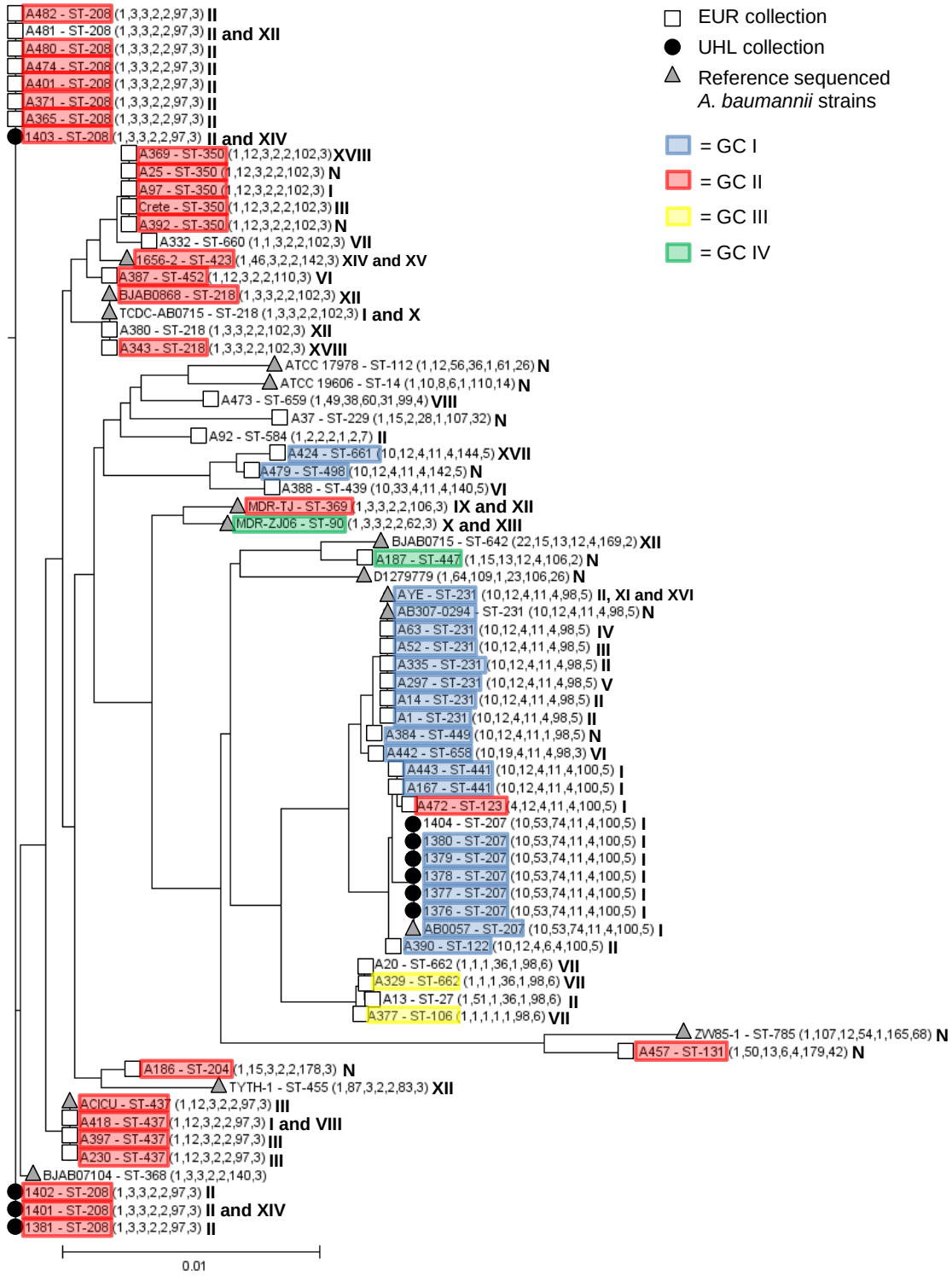
3.2.2.2. MLST typing suggests evidence of horizontal gene transfer

After the optimisation of the MLST primers, it was possible to complete sequence typing for all the 60 strains included in our study. Since the resulting MLST data yielded more detailed information across all the strains, it was used to create a phylogenetic tree that gives an estimate for the relatedness. In addition, there are several software programmes available which are able to process the complex data results produced by MLST sequencing.

The diagram in Figure 3.4 was created using START2, one such analysis tool, which allows the input of unique MLST schemes, and more importantly, the processing of the data into dendrograms according to the sequence relatedness of the alleles (Jolley *et al*. 2001). This is important because although all alleles are numbered, these numbers are not allocated according to relatedness, but simply added in order of discovery. Therefore, a sequence-based dendrogram gives a more accurate representation of the relatedness between species. The MLST types of sequenced genomes were added to the analysis as a reference with which to compare the isolates in this study.

A number of conclusions may be drawn from the dendrogram produced in Figure 3.4. Firstly, the UHL set falls into two ST groups, which also correspond to two GC groups. This shows that at the time of collection, two main strains of *A. baumannii* were likely circulating within the UHL environment, with ST-207 and ST-208 being prevalent in the years 2007 and 2008, respectively. Although some of the strains have secondary, incomplete integrons, these groups show a good correlation with integron Type, suggesting that integrons could be a good marker for the clonal spread of strains around hospitals. However, this may only be the case in localised time and places, as is the case with the UHL strains. Whilst there is a suggestion of some isolated clusters, such as the strains carrying integron type XII, I or III, however, there are exceptions in each of these cases. This could suggest that integrons are not strictly bound to their strain type, but are probably exchanged, via horizontal gene transfer.

Figure 3.4 Phylogeny of strains and the integron Types carried



A neighbour-joining cladogram showing relatedness of strains used in this study based on the sequences of MLST alleles. Isolates are labelled according to name, isolate group, sequence type, allele designations and the integron Type carried. Global Clone designations are indicated by shaded boxes for GC types according to the legend. Unshaded names indicate strains where GC groups could not be allocated due to inconclusive PCR amplification of products

Conversely, when looking at the larger collection of EUR isolates that were collected over a larger span of time and geographically diverse locations, it is shown that correlation between ST and integron Type is less clearly defined.

3.2.3. Prevalence of strong P_c and weak P_{int} promoters *A. baumannii* class 1 integrons

In order to gain a more complete picture of integron activity in *A. baumannii*, the integrases for all the integrons were sequenced, and from this the type of the P_c promoter that affects gene cassette expression could be established. There are a number of different P_c types known, that have different strengths of expression on the gene cassettes, determined by the homology of their -35 and -10 sequences to the σ^{70} promoter sequence (Lévesque et al. 1994, Papagiannitsis, Tzouvelekis & Miriagou 2009). During the last five years, it has been discovered that the P_c strength is inversely related to the efficacy of the integrase (Jové et al. 2010, Guerin et al. 2011). This is because the P_c promoter is actually encoded in the 5' region of the protein-coding sequence of the nucleotides in the *intI1* ORF, therefore a change to one may affect the other. This inverse relationship means that a strong P_c promoter sacrifices strong gene expression for a more weakly active integrase, with a difference in recombination efficiency up to 51-fold between P_cW (weak) and P_cS (strong) promoters. Conversely, the P_cS was found to express the genes on the cassette array at a 30 fold higher rate than the P_cW promoter (Jové et al. 2010).

Therefore, P_c promoter type prevalence was investigated in *A. baumannii* integrons, through sequencing of isolate collection and analysis of available online sequences. Table 3.1 shows that in the majority of cases, integrases were complete without any mutations or deletions, and 70 % of the strains tested contained the P_cS promoter. Three strains (4.4 %) were found to have a *P*2 promoter, caused by a GGG insertion in the LexA binding site. Two of them were associated with a P_cS promoter. Cases where the P_c type could not be reported included gene cassette arrays where there was truncation at the 5' end, resulting in loss of *intI1*, or other cases where it was not possible to amplify the relevant region of *intI1* due to unknown reasons.

Table 3.1 Frequency of P_c promoter variants found in isolates

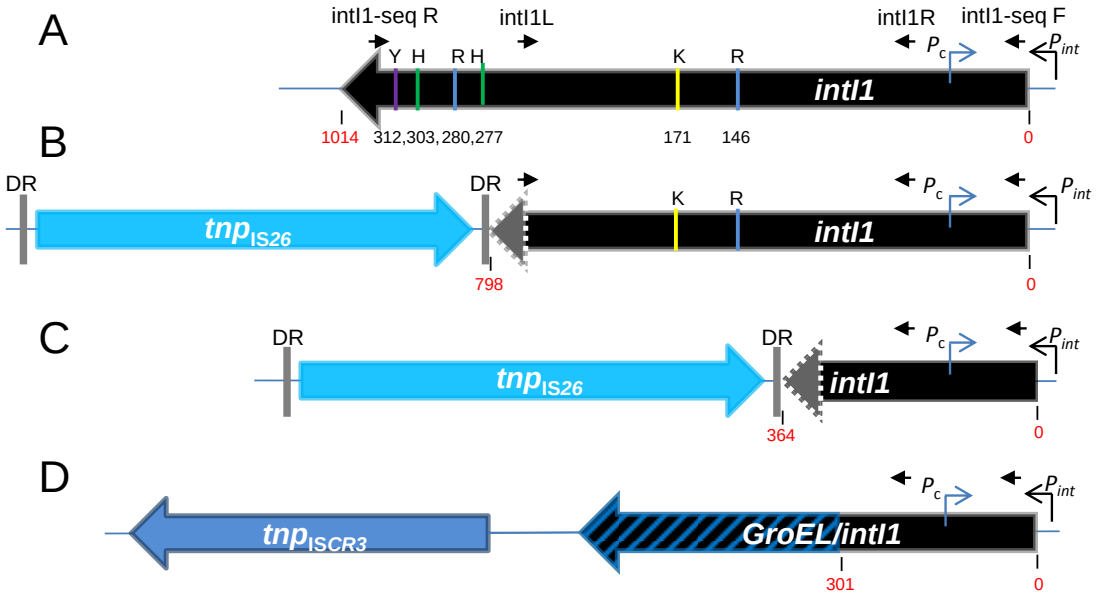
***P*c type**	**EUR (40)**	**UHL (12)**	**Ref Genomes (22)**	**Total (68)**
***P*cS**	27	12	9	48 (70%)
***P*2 PcS**	2	0	0	2 (2.9%)
***P*cH2**	0	0	4	4 (5.9%)
***P*2 *P*cW**	1	0	0	1 (1.5%)
***P*cW**	0	0	3	3 (4.4%)
Unknown or *P*c absent	10	0	0	10 (14.7)

Table showing the frequency of P_c promoters identified in this assay, with the promoters listed in decreasing order of strength (and therefore, increasing strength of integrase expression). The numbers underneath each column heading denote the total number of integrases analysed for P_c sequence for each group.

3.2.4. Prevalence of truncated integrases in *A. baumannii*

The sequence analysis of the integrases also identified several isolates in which the integrase was truncated at the 3' end by IS*26,* an IS*6* family transposase. This event was detected in strains MDR-TJ (Type XII, In439), 1656-2 (Type XIV, In266), 1656-2 (Type XV, In1056), TYTH-1 (Type XII, In439), and subsequently four of the clinical isolates: A380 (Type XII, In 439), A401 (Type XII, In439), 1401 (Type XIV, In266) and 1403 (Type XIV, In266). Figure 3.5 outlines the sections of the integrase gene that were deleted in these cases, when compared to an intact integrase from *A. baumannii* strain AYE. All the cases showed deletions of regions vital to integrase activity.

Figure 3.5 Schematic showing amino acid residues crucial for integrase function in *intI1*



Schematic showing a common insertion by IS*26* in *A. baumannii* that results in the truncation of the integrase gene, *intI1*, likely resulting in inactivation.

A) Complete, fully-functioning integrase, with amino acids crucial for DNA-binding marked by vertical lines in their relevant position. Relevant regions in the gene coding for conserved amino acids in tyrosine recombinases are arginine (R, blue), lysine (K, yellow), histidine (H, green) and tyrosine (Y, purple). Schematic based on ABAYE3575 from *A. baumannii* strain AYE

B) Common in-del event in *A. baumannii* by IS*26* (see text for details).

C) In-del event in *A. baumannii* strain 1656-2, at another locus, with all of the main DNA-binding residues of the *intI1* gene deleted.

D) Further example of non-functional integrase versions found in *A. baumannii*. Schematic of *groEL/intI1* gene fusion present in two copies in AYE (one in In622, and another gene ABAYE3633, which has a partial cassette of unknown function). The region depicted in black belongs to the integrase, and the segment belonging to GroEL is shown in a blue arrow with diagonal stripes.

Primers are indicated with black arrows. The P_c and P_{int} promoters are indicated by blue and black bent arrows, respectively. IS element direct repeats are marked denoted by grey vertical lines. Integrase gene truncation is denoted by a dashed arrow at the 3' end. The length of the *intI1* genes that are conserved with the full *intI1* in A are marked in red, and denote length in nucleotides. The positions of the conserved amino acids are labelled in black.

3.2.5. Localisation of all integrons to the chromosome and detection incomplete integrons through Southern blotting

3.2.5.1. Localisation of all integrons in this study to the chromosome

Once integrons were detected in the isolate collection, it was important to establish the location of the genetic structures in the strain. To localise them to either the chromosome or plasmids, Southern blotting was undertaken on preparations of DNA from the 45 integron-positive strains, and one integron-negative strain (as a negative control; see Chapter 2 for details). Two other methods were attempted, but the most successful and informative method to locate the integrons was through digesting isolated plasmid and genomic DNA with AleI, an enzyme which often cuts at the 3' end of the *intI1* gene, and which was calculated to give 95 % genomic DNA products under 25 kb when analysed on the AYE, AB0057 and ACICU genomes (data not shown). This therefore facilitated the separation of the DNA bands under regular gel electrophoresis conditions, as well as enabling the separation of integrons onto distinct bands in cases where there were multiple integrons present in one strain, and the AleI site was present.

The extraction of the natural low-copy number resistance plasmids present within the strains was most efficiently facilitated by the use of commercial plasmid midi-prep kits. However, since the plasmids were digested for the purposes of this experiment, an exact number of plasmids for each isolate could not be established.

For the Southern blotting, genomic and plasmid DNA were probed for the *intI1*, and then *qacEΔ1- sul1* regions sequentially (see Figure 3.6, below).

Figure 3.6 Schematic showing the binding locations of the two Southern blot hybridisation probes

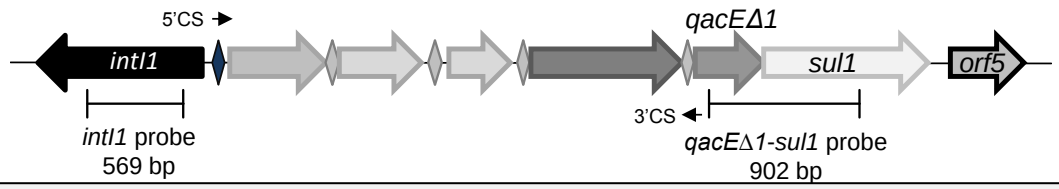
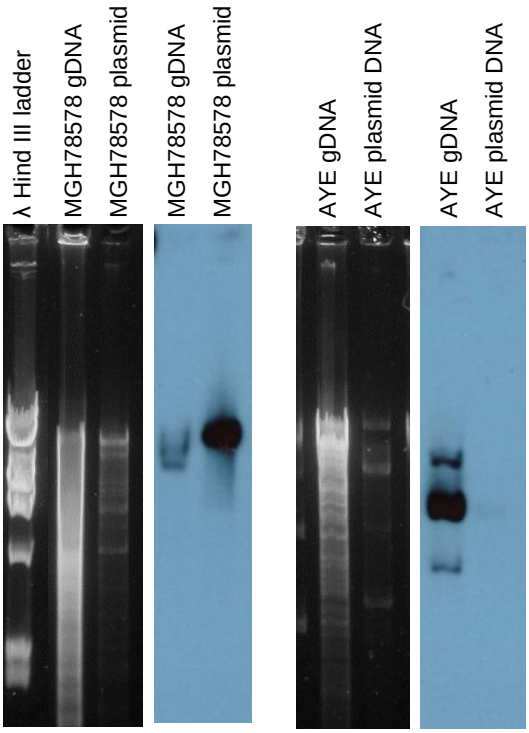


Diagram showing conserved regions from which DIG-labelled Southern hybridisation probes were amplified, with product size below.

Figure 3.7 Detection of integrons on gDNA or plasmid DNA preparations

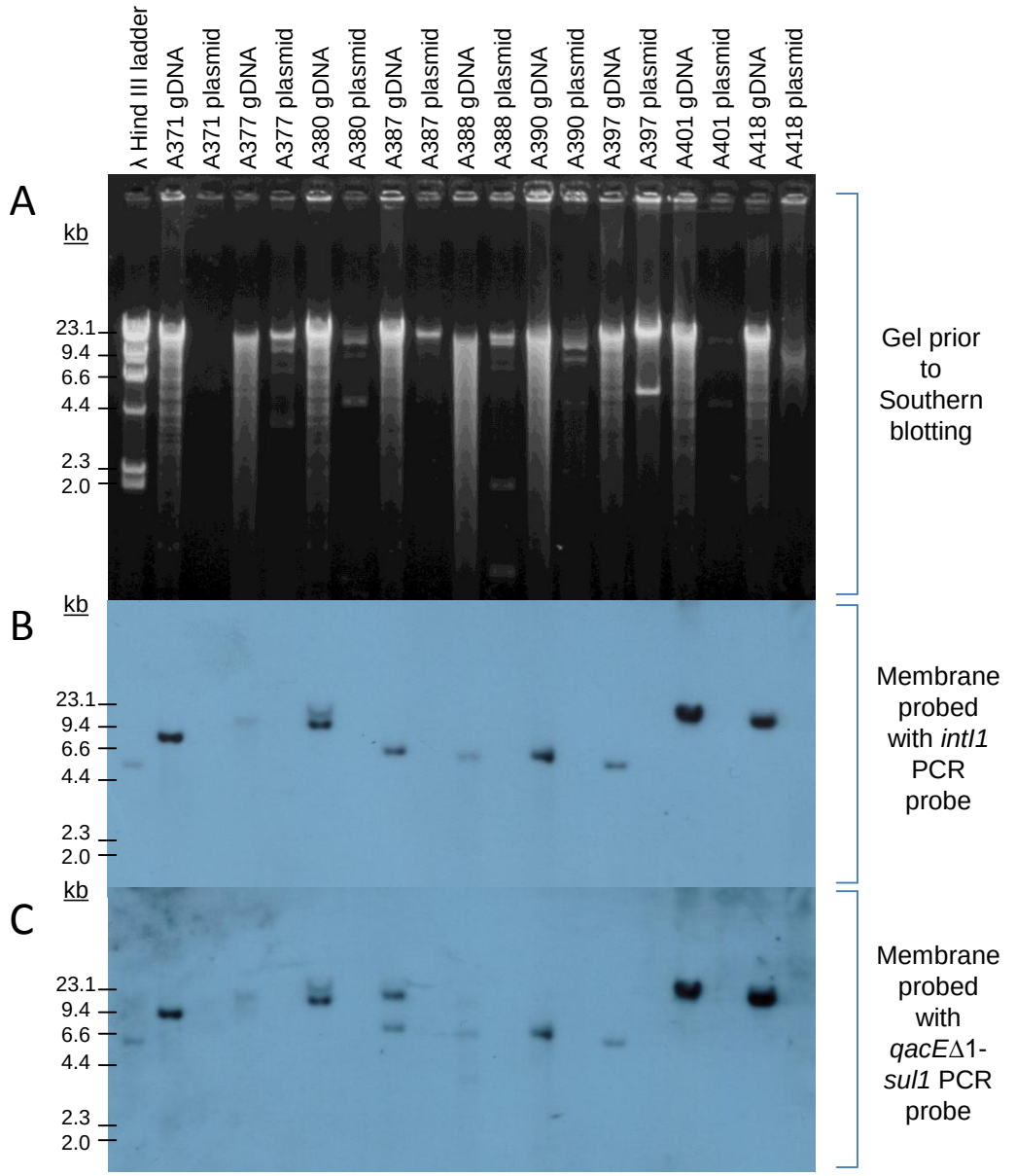


Results of experiments assessing the contribution of plasmid DNA in gDNA preparation during Southern blotting with the *intI1* PCR probe. Comparison of a strain with a known plasmid-borne integron (*K. pneumoniae* MGH 78578) and a known strain carrying integrons on the chromosome only (*A. baumannii* AYE). All DNA was digested with AleI prior to loading onto the gel

In order to account for the presence of plasmid DNA in the genomic DNA, and conversely also the possible presence of sheared genomic DNA in the plasmid DNA, initial testing of a positive and negative control strain was completed. Figure 3.7 shows the results of this experiment. It can clearly be seen that in the case of the *K. pneumoniae* strain MGH 78578 which contains a plasmid-borne integron, that there is the presence of some plasmid DNA within the genomic DNA preparation. However, it can be seen that the intensity of the band in the lane containing the plasmid DNA, the band is orders of magnitudes more intense, leading to the conclusion that the integron must be plasmid-borne. Conversely, in the strain *A. baumannii* AYE, which is known to carry four integrases (two of which GroEL-phage integrases, sharing a 113 bp homology with the *intI1* probe, Figure 3.5, D), there are only significant bands in the lane containing genomic DNA.

In this way, it was possible to confirm that the DNA preparations would give minimal, signal from the opposite source of DNA. This made the results reliable to interpret whether a positive band was allocated on either a chromosome or plasmid. Once this was confirmed, the technique was then applied to all 45 isolates that had been confirmed to be PCR-positive for at least one part of a class 1 integron, and one strain which was initially integron-

Figure 3.8 Example of gel and Southern blot for the detection of integrons



Images showing a gel prior to DNA transfer to Southern blotting (A); the photographic film produced when the subsequent membrane has been hybridised to a probe binding to *intI1* (B), and after stripping and re-hybridising with *qacE*Δ1-*sul1* region (C). Note the second band with the latter probe in A387

The λ HindIII ladder was mixed with approx 250 ng of 1380 gDNA as a positive control. All DNA samples were digested with AleI prior to loading onto the gel.

negative by PCR, but which later was found to have some binding by the *intI1* probe. All of the 46 strains that were positive for integron-type structures had these genetic elements located on the chromosome only.

An example of a gel and subsequent blots (Figure 3.8) clearly show that positive bands in the blots are only present in lanes where digested genomic DNA has been present, and that in strains where plasmids were present, they were present in a high enough copy number that would yield strong hybridisation from a probe (if any homology to the probe was present).

3.2.5.2. Detection of high numbers of incomplete integrons with deletions in the 5' conserved region

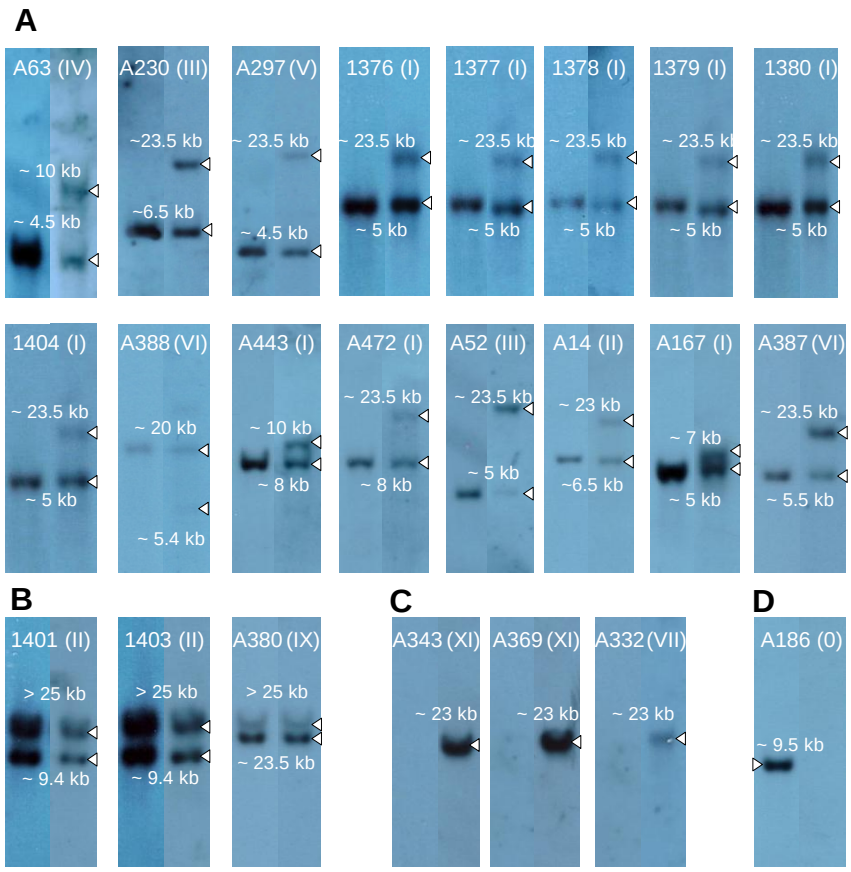
By separately probing the membrane for each of the conserved ends of the integron, this method also facilitated the detection of incomplete integron structures. All the strains that were included in the Southern blot analysis had been previously confirmed by PCR to contain at least one part of a class 1 integron; however, the results in this study enabled the discovery some unexpected integron configurations.

Two isolates (1401, 1403) were found to have two hybridising bands with both probes in the Southern blotting analysis, whereas only one band could be identified via PCR (Figure 3.9, B). When these strains were interrogated by traditional chromosome walking using HincII, it was found that both strains 1401 and 1403 contained the inverted integron previously found in sequenced strain 1656-2 (type XIV in Figure 3.1).

Perhaps the most intriguing finding was that 16 of the 46 strains tested by Southern blotting (34.8 %) had a second, incomplete integron that contained only the *qacEΔ1-sul1* region (Figure 3.9, A). Although the structures of all of these incomplete integrons have not yet been fully identified, these results suggest that there are a significant amount of cryptic integrons that are present within *A. baumannii* that are not easily detected by conventional PCR-screening methods.

Furthermore, due to the evidence we have seen of IS elements interrupting integrons, it is not unreasonable to conclude that these structures were originally full integrons, and due to insertion and deletion events, the 5' end has been lost.

Figure 3.9 Southern blot detection of multiple or incomplete integrons



Highlighted bands of interest in cases where one band was seen with the *intI1* probe, and two or more with the *qacEΔ1-sul1* probe (A); both probes showed two bands (B); one band with the *qacEΔ1-sul1* probe only (C); and one band with the *intI1* probe only (D). In each case, the relevant section of the photographic film shows the first hybridisation with the *intI1* probe on the left, and directly on the right, the same region of the film that matches to the same part of the lane when hybridised to the second probe binding to the *qacEΔ1-sul1* region, after stripping of the first probe and rehybridisation. The name of the strain where the bands were observed are noted above each picture, with the fully identified integron Type number in brackets.

The two strains that had been established by the two-step chromosome walking method to have only the 3' end of the class 1 integron, interrupted by an IS*Aba1* element (Figure 3.1, type XVIII) were also confirmed by the Southern blotting to lack a 5' conserved region (Figure 3.9, C), compounding the theory that the integrase gene may be under negative selection in *A. baumannii*.

In some cases, further integron structures may be present in strains but not detected due to an absence of suitably spaced AleI site. In two cases (A401 and A418) where two integrons were detected by 5'CS/3'CS PCR, only one hybridising band was seen on both the *intI1* and *qacE*Δ*1- sul1* blots. It could be that both integrons were on one AleI fragment, or that the integrons were present on separate digestion products of very similar size that were travelling as a doublet, indistinguishable by gel electrophoresis. The former could be caused by one of the integrases having a truncation or an SNP that prevented the fragment from being digested. However, the blots show that although all genomic DNA samples loaded onto the gel were standardised to roughly 500 ng each, the intensity of these bands containing two integrons was roughly twice as strong (Figure 3.8, lanes for A401 and A418 gDNA). It could be that other very strong hybridising bands may be harbouring more than one copy of the probe target region on the same digestion band.

Only one strain was found to be positive for *intI1* only (A186, Figure 3.9, D). This was an unexpected because it was initially included as a negative control in the Southern blotting assay, since it was PCR-negative for both *intI1* and *qacE*Δ*1- sul1*. This could be due to SNPs changes in the primer binding sites, but with significant homology remaining in the central part of the amplicon region in order to bind to the 569 bp *intI1* probe. It is possible that the *intI1* gene developed these SNPs because it is under selection to accumulate mutations that may attenuate its function as a resolving enzyme. This effect could also be due to truncation of the integrase within the amplicon region, but with sufficient amount of region remaining for some binding (as in Figure 3.5, C or D). This gives a total of 23 unusual structured integrons out of a total of 46 strains tested (50 %), indicating a very strong pressure for integron rearrangements and fluctuations in this set of *A. baumannii* clinical isolates.

3.3. Discussion

3.3.1. Integron typing and frequency as evidence for horizontal gene transfer

The integrons found in this study demonstrate a wide range of gene cassette configurations and their relative frequencies also provide some clue about integron acquisition and maintenance. Integron Types I, II, and III are some of the most commonly known integron types in *A. baumannii* (Fournier et al. 2006, Iacono et al. 2008, Turton et al. 2005, Liu et al. 2014), so it is not surprising to see them as the dominant types found within the tested isolates. Type XII is also widespread amongst *A. baumannii* and is present in six of the currently sequenced and fully annotated strains (TYTH-1, TCDC-AB0715, MDR-TJ, MDR-ZJ06, BJAB07104 and BJAB0868). Moreover, this array is frequently found in pathogens including *K. pneumoniae*, *B. cenocepacia, S. maltophilia* and *E. cloacae*.

Although all the integron Types found in this study have been previously reported in other *A. baumannii* strains at least once, even the rarer gene cassette arrays have also been found in other species. For example, integron Type V is present in *S. enterica* sub. *Typhimurium*-associated plasmids pSRC125 and pSRC26 as part of a transposon Tn*6025* (Cain et al. 2010). Since the integron was confirmed to be present on the chromosome in A297, it is possible that the whole transposon was transferred and became incorporated into the *A. baumannii* DNA. The integron array Type VIII has previously been annotated in only one *A. baumannii* strain, AO-8866, but has also been found once in *E. coli*, and another uncultured bacterium plasmid, pSp7 (Karah et al. 2011, Tennstedt et al. 2003). The discovery of integrons with identical sequences in other species and mobile genetic structures such as plasmids demonstrates the likelihood of horizontal gene transfer as an important mechanism in the dissemination of integrons in the pathogenic strain gene pool.

It could be suggested that the sample size of isolates has limitations and biases. All the *A. baumannii* isolates in our laboratory collection were included in this study, and in order to increase the number of strains, the information

from sequenced strains available from NCBI were also added to the analysis. However, it could be argued that all of these strains, including the sequenced isolates may have been biased in their choice, as interesting, or particularly pathogenic isolates may have been preferentially chosen for further study or kept for archiving. However, this project aimed particularly at analysing the spread of antibiotic resistance via integrons in clinical isolates that are pathogenic humans, so the results from this chapter are still relevant to addressing this question. Furthermore, the establishment of both GC and MLST typing systems showed that the strains came from a diverse array of backgrounds, and were not genetically homologous.

The construction of a dendrogram using MLST sequence data allowed the direct visualisation of the relatedness between the strains and their integrons. This demonstrated that there is no significant correlation between ST and integron Type, unless strains are collected from a localised area or time (or both, which was the case in the UHL collection). This suggests that over a longer time span, such as years, integrons can be mobilised between strains, although integrons may be a useful marker to detect clonal expansion in localised areas within a limited time period. This is consistent with the findings in the study by Turton, et al. (2005), who found that although in the broad sense isolate genotype and integron type were not strictly related, there was significant correlation in cases that were known to be clonal. For example, all ST-208 isolates contained integron Type II, even if they were isolated from different countries. However, it was seen amongst the UHL isolates that out of the four strains that fell into the ST-208 MLST, two of them had an extra XIV Type integron in addition to the Type II that was present in all of that genotype. This could suggest that even in the short time frame in which these strains were isolated, loss or gain of extra integrons could have occurred, suggesting that the genome of *A. baumannii* in hospitals can be very fluid, and exchange or loss events may occur over a matter of months. In addition, it is evident from Figure 3.1 that IS elements and other MGEs play a major role in shaping these changes.

The finding of truncation of the integrase or the 5’ conserved region independently in several different MLSTs, and the variety of integrase

truncations suggests that integrase attenuation is advantageous to a range of *A. baumannii* strains. Furthermore, this diversity suggests that these truncations could have arisen a number of times independently in different strains, with possible subsequent clonal expansion or horizontal gene transfer.

In recent years, evidence has emerged about how integrons may move as an intact array and integrase, utilising flanking MITE structures, dubbed the 'integrase mobilisation unit' (IMU) by Poirel, et al (2009). Their study showed that the regions flanking integrons contain MITE-like sequences which are able to mobilise the whole IMU structure. Since this initial study in *E. cloaceae*, another study has also shown this mechanism in action in *A. baumannii* (Domingues, Nielsen & da Silva 2011). These studies illustrate another means whereby integrons may be transferred to *A. baumannii* from other sources, in order to produce the patterns seen in Figure 3.4.

The diversity of the strains and mobility of the integrons was measured through the use of MLST and GC typing. However, it could be suggested that the typing systems used may have some limitations, since they both rely on PCR-based methods and therefore on appropriately designed primers which bind consistently to all strains. Due to the decreasing cost of genome sequencing, the number of available sequenced strains has greatly increased since the development of both MLST and the GC typing technique. This means that during their inception, fewer strains were available, and hence with both techniques, the design of the original primers was sometimes limited. As a result, not all the strains could be successfully amplified to yield PCR products with the original primers. The redesigning of two of the primer sets in this study contributes towards the refinement of these techniques, and widens the number of strains to which this technique can be applied.

3.3.2. Prevalence of P_C types suggest selection for less efficient integrases in *A. baumannii*

This study showed that 50 % of the integrons found in the *A. baumannii* isolate collection had some kind of integron disruption. In addition, it was shown

that even in those strains with a complete integrase gene, the nucleotide sequence for the integrases that were most prevalent gave rise to the least efficient integrase activity (as well as the highest level of P_c promoter strength). In comparison with this data set, a survey of the integrase sequences in the currently available *A. baumannii* reference genome sequences shows that out of 23 hits by BLAST for the region, 16 are P_cS (69.6 %), four are P_cH2 (17.4 %), and three are P_cW (13.0 %).; All three P_cW configurations detected were present in one strain, AYE. One of those integrases is truncated by an ISCR element, and the other is linked to a non-functional GroEL-*intI1* fusion gene, so technically AYE has only one fully functioning integrase of higher recombination efficiency. By contrast, analysis of all available integrase sequences across many species by Jové et al. (2010) found that P_cW was the most prevalent, with a frequency of 41.7 %, compared with 24 % prevalence of the P_cS promoter. This shows that in *A. baumannii*, the P_cS promoter is disproportionately more common when compared to other species, possibly because it is the configuration that gives the most attenuated integrase activity.

The most common P_c types found in the isolate collection used in this study were P_cS and P_cH2. Both of these promoter types actually result in the same amino acid sequence for the integrase, producing a less-efficient integrase protein, as mentioned above. This suggests that it is more advantageous for *A. baumannii* strains to retain promoter regions which favour increased gene expression, rather than integrase activity. This can be seen as another way in which *A. baumannii* strains that are able to reduce integrase activity within their cells become more prevalent and hence more successful.

3.3.3. Incomplete integrons reveal shortcomings of 5'CS/3'CS PCR and importance of Southern blotting in study of integron epidemiology

A significant number of incomplete integrases and integrons were detected in this study, where either there was a truncation in the integrase gene or loss of the whole 5' conserved region, suggesting that in *Acinetobacter*, this region might be under negative selection. Truncations in the *intI1* gene were revealed by sequenced genome analysis and subsequent PCR assays. Southern blotting revealed a high frequency of integron structures in *A.*

baumannii completely lacking the 5' conserved region which are undetectable by conventional integron-targeting PCR assays.

The absence or truncation of the integrase gene was frequently mediated by the insertion of an IS element, a phenomenon that was first described by Miriagou, et al. (2005). This insertion-deletion (in-del) event by IS*26* resulted in the deletion of a crucial DNA-binding region in the 3' end of the *intI1* gene, known as box II (Nunes-Duby et al. 1998). An essential tyrosine residue which gives its name for the class of tyrosine recombinases to which integrases belong, is also removed by this deletion event. In a study by Collis, et al. (1998), which studied the importance of these residues to integrase activity, found that a Y312L mutation resulted in the decrease of integrase activity to 0.02 % of wild type levels, compared to R146Q and H277L (3.2 % and 1 % of wild type levels, respectively (Collis et al. 1998). This study demonstrates that the carboxy-terminal tyrosine is a vital active site for integrase functioning, and it is reasonable to conclude that integrases with this region truncated are therefore inactive. This is yet more evidence that integrases carry a strong fitness cost for *A. baumannii*, above that seen in other Gram-negative pathogens.

Importantly, this IS*26* deletion event does not hinder the expression of the advantageous antibiotic-resistance genes carried by the integron. By confining the deletion to only the 3' end of the *intI1* gene, the vital 5' end, containing the P_c promoter, is preserved intact, allowing the expression of any downstream antibiotic-resistance genes. This retains the positive features of the integron, whilst eliminating the negative, fitness-affecting factors. This contrasts sharply with the studies on classically regulated integrons, in which usually the 3' conserved region is missing from incomplete integrons (Dawes et al. 2010).

Moreover, these in-del events may easily be missed because they are not encompassed by the commonly used 5'CS/3'CS or intI1L/intI1R primers (see Figure 3.5, Levesque et al. 1995, Ploy et al. 2000). Since this study uses a probe made by the PCR amplicon of the latter primers for the class 1 integrase, these events cannot even be detected by the Southern blotting in this study

(result not shown). The two instances in this study where this occurred were only found using IS*26* specific primers designed on the sequenced genomes.

This finding shows that in order to avoid false-positives, a larger area of the integrase can be amplified in addition to the routine intI1L/intI1R primers. The primers intI1-seq-F/intI1-seq-R were designed from an alignment of thirteen *A. baumannii* integrases, and chosen to be in conserved regions. They were designed with the aim to amplify integrases from all isolates. Since the reverse primer binds after the important carboxy-terminal tyrosine-coding residues, a positive amplicon produced by these primer pairs would be a sign of a complete integrase. This can give a simple and quick screening technique that would indicate that the integrase was likely to be functional or not, and whether further analysis was required.

It is important to note that all of the plasmid-borne integrons contain an integrase with an IS*26*-mediated truncation. This means that there are no complete integrase genes present on plasmids in *A. baumannii*, and therefore not present in more than one copy number, in stark contrast with other Gram negative pathogens. It is evident from this information that a fully-functioning integrase carries a heavy fitness cost in *A. baumannii*, and that those strains that had acquired an integron on a plasmid could not maintain it unless the integrase was neutralised, or perhaps lost the integron or the carrying plasmid altogether.

Whilst it is true that some *A. baumannii* strains contain more than one integron structure on the chromosome, in the majority of cases, at least one of the integrases is either truncated such as in AYE (ABAYE3625), MDR-ZJ06 (ABZJ_p00001), MDR-TJ (ABTJ_01323), A380, A401, or deleted completely, as in the strains Figure 3.9, C. In addition, integron Type XIV appears to have had the 5’ and 3’ end structures arranged in reverse positions, likely due to a recombination event mediated by a high number of IS*26* element transposase genes that are present in this locus (see schematic in Figure 3.1).

IS elements becoming incorporated into genetic structures are not uncommon, and these kinds of phenomena have been recently discussed by Harmer, et al. (2014). Such elements are constantly inserting and excising in

genomes, and by this process affecting the evolution of strains (Harmer, Moran & Hall 2014) through the generation of new configurations of integrons that favour maintenance in *A. baumannii*, such as an inactive integrase, which may then proliferate in the gene pool through clonal expansion or HGT.

3.3.4. Integron localisation related to fitness cost

None of the integrons found in this study were located on plasmids. It was ensured that high quality plasmids were extracted (where present), and a sufficient amount (~500 ng) digested and loaded onto the gel for optimal Southern blot probe binding (see Figure 3.8, A), therefore these results are deemed to be specific, and not missing any integrons due to false negatives.

This finding coincides with the currently available genome sequences and literature which also show a dearth of integrons in *A. baumannii* plasmids. Only four integrons have been located on *A. baumannii* plasmids, in MDR-ZJ (Type I, In561), BJB07104 (Type XII, In439), BJAB0868 (Type XII, In439) and NM55 (In301, Zhou et al. 2011, Zhu et al. 2013, Opazo et al. 2012). In each case, the integrons were found to have a truncated integrase due to an IS*26* insertion-deletion event which would have resulted in the likely inactivation as described in Figure 3.5. This means that to date, there have been no fully functioning integrases reported on any *A. baumannii* plasmid. This data demonstrates how it may be disadvantageous for *A. baumannii* to carry multiple copies of the integrase, and that in cases where integrons are present on possibly multi-copy plasmids, only those integrons are maintained on plasmids which have a defective integrase.

Integrase activity may have a negative effect on *A. baumannii* strains because the Moraxellaceae lack the LexA repressor of the integrase. This could result in constitutive expression of the integrase gene, which could cause harmful genomic instability due to illegitimate recombination events.

It has been known since 1994 that the integrase gene can recombine secondary, non-canonical sites (Recchia, Stokes & Hall 1994). The core conserved site of the *attC* (formerly 59 base elements) is only seven bases

long, and defined as GTTRRRY, with recombination occurring between the G and first T base. With such a short and variable sequence, there is a significant chance that very similar sequences may be found at other locations, creating non-canonical recognition sites for the otherwise site-specific integrase. Although recombination at these secondary sites occurs at a lower frequency than at the optimal integron recombination sites, their occurrence may nonetheless cause problems for a strain if inserted at vital house-keeping locations.

In fact, *in silico* genome sequence analysis by Harms, et al. (2013), showed that *A. baumannii* had a much higher number of possible secondary core sequences for recombination than compared to other common Gram-negative pathogens, such as *P. aeruginosa*, *S. enterica*, *K. pneumoniae* and *E. coli* (which are all LexA-positive). The available evidence suggests that integrons are a relatively recent acquisition in *A. baumannii* through horizontal gene transfer by contact with LexA-positive integron-carrying strains. This might mean that the *A. baumannii* genome has not had time to eliminate such similar non-canonical sites, and hence evolve a natural resistance against such illegitimate recombination events. In either case, the evidence from this study shows that integrase carriage has a higher fitness cost to *A. baumannii*, even without the absence of the LexA-mediated repression. Previous studies have shown that there is still some expression of the integrase under LexA repression, albeit at a lower level, when tested in *E. coli* and *V. cholerae* (Guerin et al. 2009). The study by Harms, et al. (2013) also shows that even though other Gram-negatives have lower numbers of possible non-canonical recognition sites, they are still present at a minimum of about 4,500 sites per chromosome; therefore, the integrase is likely to also exert a fitness cost to some extents in LexA-positive species.

This study found that IS elements caused truncation or deletion events at the 5’ end of the integron in all of the truncation events found. In particular, on the rare occasions when integrons were present on plasmids (which are potentially multi-copy), IS element-mediated truncation of the integrase was found in every case. Further analysis of the remaining integrons may increase this number or elucidate other mechanisms of integron rearrangements.

On the basis of all the evidence presented in this chapter the conclusion must be drawn that due to a heavy fitness cost of the integrase, *A. baumannii* is unable to stably maintain more than one copy of such a gene. These data suggest that loss of functionality in the integrase may confer a survival advantage and an increased chance to become a successful pathogen. Current knowledge suggests that this fitness cost of the integrase is most likely caused by unwanted secondary recombination events mediated by the integrase, an enzyme that is unregulated in such a LexA-negative species as *A. baumannii*. Therefore, integron activity in *A. baumannii* must be significantly different to that of other Gram-negatives, and thus warrants further investigation.

3.4. Future work

The work discussed in this chapter shows many pieces of evidence that point towards a conclusion of a cost-benefit issue of integrases in *A. baumannii*. With further work, this hypothesis could be extended and clarified:

1. Further examination of the incomplete integrons found by Southern blotting though chromosome walking using different restriction enzymes would elucidate the structure of other incomplete integrons, and may provide further information about mechanisms facilitating 5' region loss of integrons in *A. baumannii*.

2. Further chromosome walking procedures on strain A186 would also help to establish what kind of integrase structure is present on this strain, which is negative for *intI1* by PCR, but positive by Southern blotting to the same PCR product.

3. Southern blotting procedures for the strains found to be integron-negative by PCR may also lead to the discovery of further atypical integrons, such as in A186.

4. Screen the integron-positive strains for MITE structures to see how hight the frequency of MITE-mediated mobility of integrons may be in *A. baumannii*.

Chapter 4. Analysis of integrase activity under artificial induction

4.1. Introduction

In the experiments laid out in the previous chapter, it was noted that a significant number of integrons found in *A. baumannii* contained deletions in either the integrase gene or in the whole of the 5' conserved region of the integron. This led to the conclusion that the integrase must be exerting a significant fitness cost, resulting in its negative selection in *A. baumannii*. These findings are in line with those of Cambray et al. (2011), who hypothesised that the ancestral form of integrons arose under LexA regulation, and that a lack of LexA control significantly correlated with inactivation of the integrase gene. The remainder of this thesis looked towards experimental investigations to further quantify the exact fitness cost IntI1 may have, through comparing integrase activity in both *E. coli* and *A. baumannii* backgrounds, under an inducible (this chapter) and its native (Chapter 5) promoter.

Assessment of integrase activity and its impact on the host cell is a crucial aspect of integron studies. The limitations of previous experiments include the fact that the integrase gene is normally naturally present in one or a few copies per cell, and that in natural states, expression of *intI1* is low. This means that in many cases, excision/integration events happen at a very low frequency. Studies detecting these natural events consequently rely on complex or multi-step procedures such as nested PCR, which is prone to amplicon contamination (Hocquet et al. 2012). A simple solution to circumvent this involves cloning *intI1* and a target gene cassette onto a plasmid, which would result in a higher copy number of both and therefore increased number of excision events at more easily detectable levels (Yang et al. 2009). Having a higher number of copies facilitates the detection of transient intermediates involved in excision/insertion events with only one round of PCR. However, recombinases are known to be involved in illegitimate recombination, therefore it is not always possible to maintain such a gene at a high copy number. To combat this, some studies have inserted integrases at a single copy number on

the chromosome (Starikova et al. 2012). An alternative, outlined in this chapter, is cloning the integrase gene at a low or medium copy number plasmid under the control of a repressor.

Investigation of a potentially harmful enzyme such as IntI1 under experimental conditions also relies upon having control over the expression of the *intI1* gene. There are a range of inducible promoters on plasmids available, which can activate gene expression by the addition of tetracycline (Blau, Rossi 1999) or arabinose (Guzman et al. 1995). However, one of the oldest methods for induction is via IPTG, a molecular homologue to allolactose, which activates transcription from the *lac* operon promoter, P_{lac}. Additionally, this promoter can also be repressed by the presence of $LacI^{q}$, allowing tighter regulation of genes. This feature is particularly useful for cloning integrase genes, since it allows the gene to be expressed at an even lower level than the number of copies of the carrying plasmid per cell. It also means that the gene can be simply upregulated at any time by the simple addition of IPTG, in a concentration-dependent manner, that has been shown to be effective in *A. baumannii* (Damier-Piolle et al. 2008).

Furthermore, the maintenance of *A. baumannii*-compatible plasmids has been fraught with difficulty. Whilst some papers report that broad-host range origin of replication signals from plasmids such as pRO1614 (for example, on pFLP2) are maintainable within *A. baumannii* (Kumar et al. 2010), others consider pFLP2 to be a suicide plasmid within *A. baumannii* (Carruthers et al. 2013).

The only plasmids that have been consistently reported as stably maintained in *A. baumannii* strains are ones based on pWH1266, an *E. coli – A. baumannii* shuttle plasmid, constructed from the fusion of pWH1277, a cryptic plasmid from *A. lwoffii*, and pBR322, a commonly-used *E. coli* cloning vector (Hunger et al. 1990). pWH1277 contains an area identified as a putative *oriR* region that would be recognised by *Acinetobacter* species, and in addition, a putative toxin-antitoxin (TA) system that may encourage the maintenance of the plasmid. Use of this plasmid has been successfully reported as transformable into *A. baumannii* strains such as ATCC19606, ATCC17978, and several others

from independent laboratories (Ravasi et al. 2011, Wilharm et al. 2013, Saroj et al. 2012). Due to this success, the plasmid has been modified a number of times to confer alternative antibiotic resistance markers (other than ampicillin and tetracycline on the original pWH1266), thus expanding the range of applications. For example, pWH1266 has been converted to kanamycin and gentamicin resistant versions (Wilharm et al. 2013, Camarena et al. 2010).

Alternatively, Aranda, et al. (2010) have taken an approach by which they have used PCR amplification to clone the relevant origin of replication identified in pWH1266 into their desired plasmid, producing expression plasmids pET-RA, pAT-RA and pOT-RA (Aranda et al. 2010, Aranda et al. 2011). This latter method has been employed in this study in order to convert the integrase-bearing plasmid to be compatible with *A. baumannii*. This study therefore further confirms a simple method in which many experimental plasmids can be converted to *A. baumannii*-compatible forms, expanding the number of assays that can be completed in this species.

Previous studies of integrons in *A. baumannii* have focused more on descriptive epidemiology, rather than analysing gene cassette movement (Liu et al. 2014, Karah et al. 2011, Gallego, Towner 2001, Turton et al. 2005). Those few studies that have analysed integron activity and related fitness costs of the integrase in *Acinetobacter* have used the natural transformable, closely related *Acinetobacter baylyi* ADP1. These studies have been vital for elucidating the differences in integron activity between different species and have identified a fitness cost in harbouring a functional integrase in *A. baylyi* (Starikova et al. 2012). This PhD study builds on this knowledge, and also tests the fitness cost hypothesis in clinical isolates of *A. baumannii*. As mentioned in the previous chapter, statistical analysis of the genome sequence for non-canonical *attC* sites in a range of Gram-negative pathogens revealed that *Acinetobacter* would have a higher frequency of DNA damage events caused by illegitimate recombination mediated by the class 1 integrase (Harms, Starikova & Johnsen 2013). The construction of the plasmids outlined in this chapter with an *intI1* gene under an inducible promoter system facilitated the investigation of this postulation in a clinical isolate of *A. baumannii*, as well as two *E. coli* strains.

The first step for the investigation of IntI1 activity was to construct an inducible system by which *intI1* could be carried under a selectively and accurately induced promoter in easily replicable conditions in a laboratory setting. With this end in mind, an IPTG-inducible promoter-carrying expression vector pQE-80L (Qiagen) was employed as the basis of this unit. This plasmid contains a $LacI^q$ repressor, which prevents genes coupled to the P_{lac} promoter from being expressed constitutively at high levels, and allows easy de-repression by the addition of low-concentrations of IPTG to the culture. Additionally, the QIAexpress series of plasmids were originally produced from the pDS family (Bujard et al. 1987), which means that they have a low copy number, roughly 10 – 20 copies per cell (although the exact copy number of the pQE series plasmids have not yet been established). This is an important factor when cloning genes that code for proteins that could be potentially detrimental to a cell, as it means that even if the gene of interest is expressed constitutively to some degree, it is less likely to carry a heavy fitness cost to the cell in comparison to the same gene expressed on a high copy number plasmid. In this way, there is a reduced chance that plasmids containing potentially harmful genes would be lost, or that cells containing such structures would be too unhealthy to survive.

The class 1 integrase gene ABAYE3575 was chosen from *A. baumannii* AYE strain for cloning into the pQE-80L plasmid, as it was established to be a full and complete *intI1* gene coding for all amino acid residues necessary for integrase function. In addition, this gene was also found to be present in an identical sequence by BLAST analysis in many other Gram-negative pathogens such as *E. coli* and *P. aeruginosa*, suggesting that this gene could confer functional activity in many species.

4.2. Results

4.2.1. Construction of plasmids containing an inducible promoter for the investigation of integrase activity

The integrase gene ABAYE3575was cloned into the EcoRI and HindIII sites of the pQE-80L plasmid using a PCR amplicon of the *intI1* gene using primers PR1719 and PR1569. This process simultaneously removed unnecessary protein expression His-tags from the recipient pQE-80L plasmid that may interfere with the integrase activity if fused to the protein, and also meant that when the *intI1* gene-containing amplicon was ligated into the digested vector, it would be inserted in optimal position for expression under the P_{lac} promoter (see Figure 4.1). This process meant designing the forward primer PR1719 to contain the start codon of the integrase gene, which also simultaneously removed the natural P_{int} promoter and the LexA-binding box that is present upstream in the native promoter. Since the plasmid also contained the gene coding for the $LacI^q$ repressor, this meant that the integrase could be expressed in an IPTG concentration-dependant manner.

Figure 4.1 Construction of P_{lac}-coupled *intI1* gene on an IPTG-inducible plasmid, pQE-80L

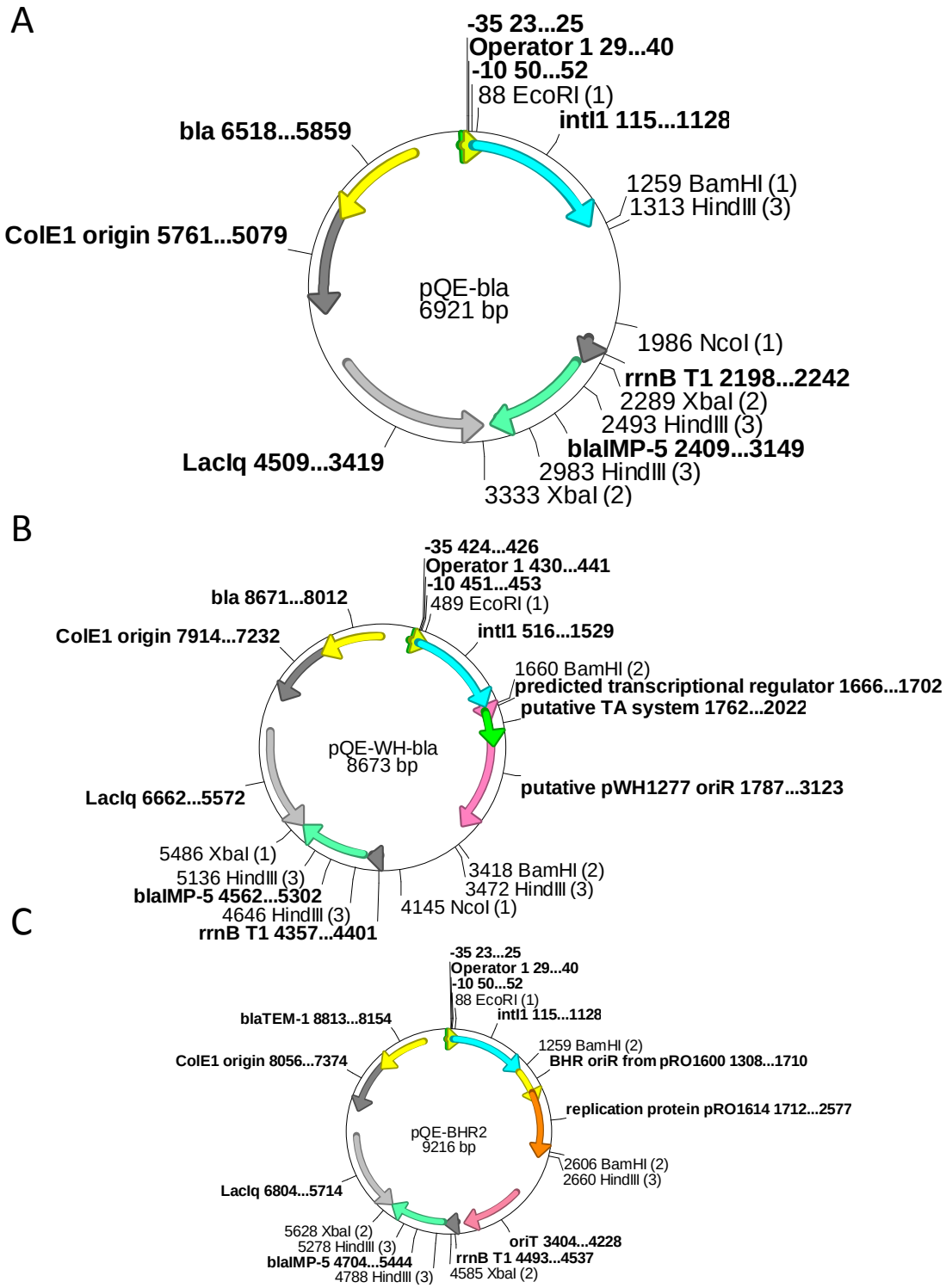
Nucleotide sequence of the start of the *intI1* gene and the region of pQE-80L directly upstream of the MCS site, once the digested fragments were ligated (see text for details). The start codon of the *intI1* gene is indicated by a double underline. The -35 and -10 sequences of the promoter P_{lac} are indicated. The restriction enzyme site EcoRI which was used in the directional cloning of the insert is underlined. The RBS region is shown by the shaded box. The sequence of the intI1-F3 forward primer used to create the *intI1*-containing amplicon is shown by the turquoise highlighting

In order to test whether the expressed IntI1 was capable of functioning in recognising *att* recombination sites for the excision of intervening regions, a single-gene cassette-containing integron was also cloned within the same plasmid containing the integrase gene. The integron containing the bla_{IMP-5} gene from the clinical isolate *A. baumannii* 65FFC was chosen, since it was present as the sole gene on an integron, and would provide a selectable resistance to ceftazidime. This gene cassette was first amplified by PCR using the 5'CS and 3'CS primers (with added XbaI sites), meaning that the native integrase gene and the 3' conserved region was not amplified, but the *attI* and *attC* sites vital for integrase-mediated recombination would be present in the amplicon. After digestion of both the plasmid and the amplicon with XbaI, the ligated products formed pQE-bla (Figure 4.2, A), which was used in initial tests in *E. coli* DH5α to assay integrase excision activity. By having both the *intI1* and target gene cassette on the same plasmid, it could be ensured that the two genes would be in a 1:1 molar ratio, and avoided the requirement for double antibiotic selection, which might place additional stresses on the cell, possibly affecting the results of any subsequent assays. Figure 4.2 C shows a version with pRO1614 origin of replication for compatibility with other species, such as *K. pneumoniae* or *P. aeruginosa*.

4.2.2. Optimisation of plasmids for transfer for *A. baumannii* carriage through addition of an *A. baumannii*-compatible *oriR*

Once the initial tests for IntI1 activity were completed, the experiments were planned to be replicated in *A. baumannii*, with a view to establishing whether the *intI1* gene is capable of being expressed and functioning in a similar manner and level as in *E. coli*. However, it was found that the ColE1 origin of replication was not compatible with *A. baumannii*, and an additional section of an origin of transfer would be required to be added to the plasmid, in order to be maintained in *A. baumannii*. This region was obtained from the pWH1266 plasmid, which is an *E. coli-A. baumannii* shuttle vector described in the introduction. A 1752 bp size amplicon containing this *oriR* and putative TA region was amplified by PCR using primers PR3136/PR3137, and ligated into the plasmid after both were digested with BamHI.

Figure 4.2 Plasmids used in integrase activity assays



A) pQE-bla, a plasmid created to study the integrase activity by coupling *intI1* to the IPTG-inducible P_{lac} promoter

B) pQE-WH-bla, a plasmid derived from pQE-bla, with the insertion of a region from pWH1266 that allows maintenance in *A. baumannii* strains

C) pQE-BHR2, also derived from pQE-bla, with the insertion of the broad host-range origin of replication from pRO1600, facilitating maintenance in strains of species such as *P. aeruginosa* and *K. pneumoniae*

The new plasmid formed, pQE-WH-bla (Figure 4.2, B), was able to be transformed into the *A. baumannii* strain A457 by electroporation. This strain, which was originally isolated in Estonia, was chosen due to its high susceptibility to a range of antibiotics, including ampicillin. It was confirmed to be *A. baumannii* by *rpoB* and MLST sequencing.

Lastly, during the process of converting the pQE-bla plasmid into an *A. baumannii*-compatible version, a number of other broad host-range origins of replication were also inserted into the plasmid in order to test their compatibility in *A. baumannii*. The pRO1614 origin of replication was amplified by PCR from pFLP2 using primers PR2546/PR2614 to produce a 1341 bp product containing both the *oriR* sequence, and a gene coding for a replication protein. Although the resultant plasmid pQE-BHR2 was not compatible for carriage by *A. baumannii*, the introduction of this origin of replication would allow maintenance by other Gram-negative species, such as *K. pneumoniae* and *P. aeruginosa*. Replacement of the ampicillin resistance gene with a gene coding for a suitable antibiotic resistance gene may facilitate further integrase activity studies in a wider range of known MDR pathogens.

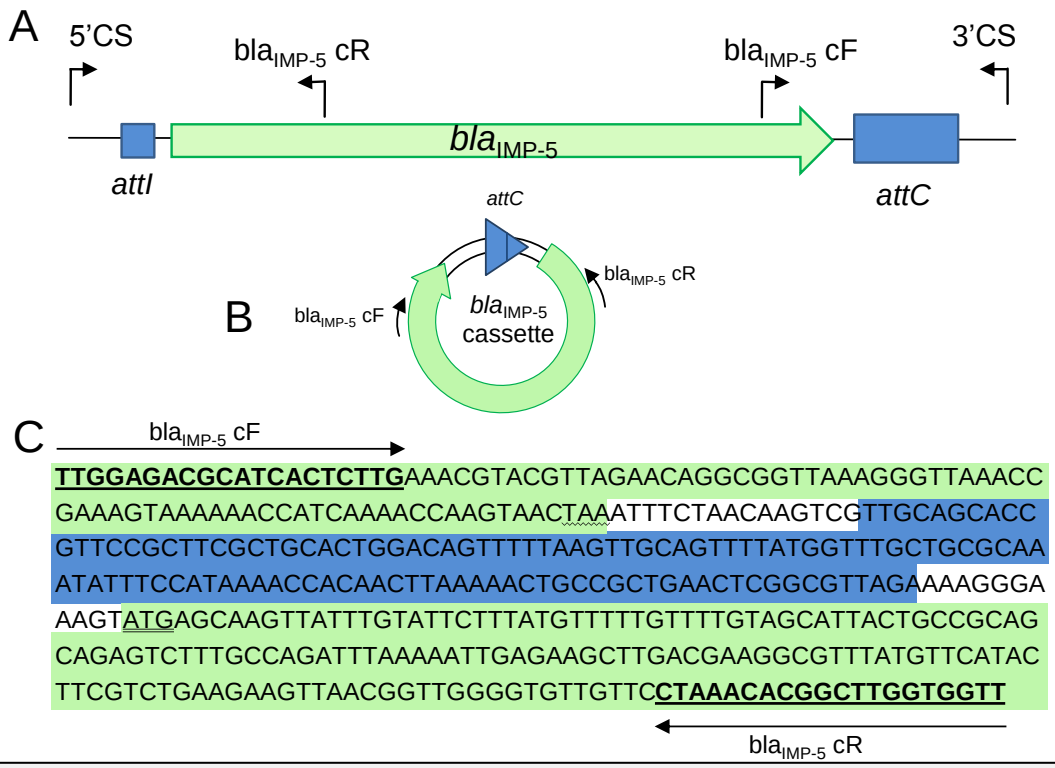
4.2.3. Validation of integrase activity in *E. coli* DH5α and *A. baumannii* A457 through detection of circular gene cassettes

The activity of the integrase was detected through a PCR assay where primers were directed outwards from the bla_{IMP-5} cassette (Figure 4.3), and thus would only produce an amplicon when the cassette was in its circular form after excision from the integron due to the action of the integrase (Collis, Hall 1992b). This type of assay has been commonly used in integron studies (Hocquet et al. 2012), and can be simply performed on a crude DNA preparation (Yang et al. 2009), which facilitates the processing of a larger number of samples from many time points.

Validation tests were initially conducted to confirm that this assay was functioning according to expectations. First, a conventional PCR using GoTaq polymerase (Promega) was conducted on culture preparations with and without

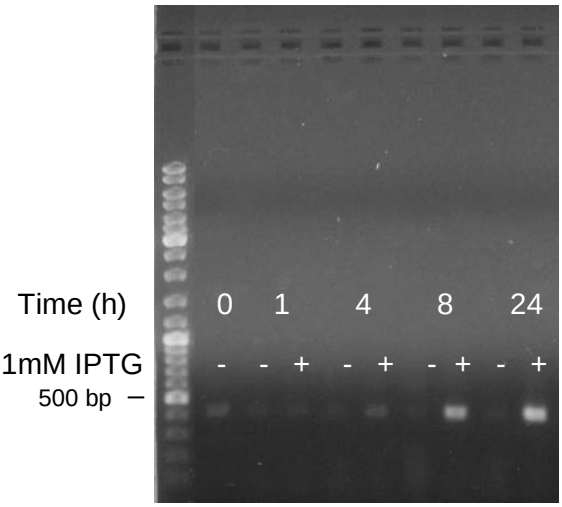
1.0 mM IPTG induction, taken at different time points, shown in Figure 4.4. It can be seen from the gel image that the detection of the circular cassette increases over time, as the number of cells also increases. In addition, there is no contamination between cultures or lanes, and that only one clear band is produced by this PCR, suggesting that it is highly specific, and no amplification of non-specific bands. This PCR product was sequenced to confirm that this was the junction of the circular form of the *bla*$_{IMP-5}$ cassette, and the results of the sequencing are shown in Figure 4.3, C.

Figure 4.3 Structure of *bla*$_{IMP-5}$ gene cassette in linear and circular



Structure of the bla$_{IMP-5}$ single cassette integron from *A. baumannii* 65FFC, from Coimbra, Portugal (Silva, Correia et al. 2002), in both its integron (A) and its free circular form (B, with arrows showing primer binding sites). Sequencing of the PCR product from the bla$_{IMP-5}$ circular primers is shown in C, with the location of the primers marked with bold letters and underline, while the *attC* site is shown in blue. The coding region for the end and beginning of the bla$_{IMP-5}$ gene is shaded green, with the start codon is marked with a double underline, and the stop codon with waved underline.

Figure 4.4 Gel image showing increased rate of detection of circular form of *bla*$_{IMP-5}$ gene cassette



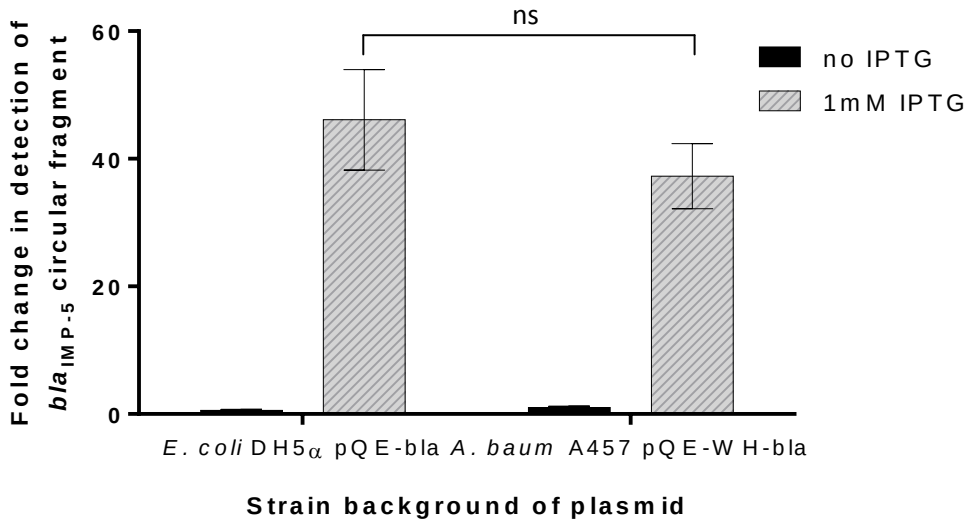
Gel showing PCR results of crude DNA preparation from a culture of pQE-bla in *E. coli* DH5α, taken at time points shown, with and without induction of 1 mM IPTG as indicated by a – or + sign. IPTG was added after one hour of growth, so at time 0, there is only one preparation of cells completed. The marker is Gene Ruler 1 kb (bp). The PCR product was amplified using the bla$_{IMP-5}$-cF and bla$_{IMP-5}$-cR primers as outlined in the following figure, with a 403 bp expected amplicon size.

Most importantly, the intensity of the PCR band is visibly stronger in the culture with the addition of IPTG. Whilst every step was taken to ensure equal amounts of DNA were added to each PCR reaction, this assay is not quantitative. However, the results of this experiment confirmed the viability of the assay, and therefore, the assay was subsequently progressed to investigation by quantitative methods, specifically qPCR, as described in the next section.

4.2.4. PCR detection of circular *bla*$_{IMP-5}$ shows integrase activity reaches maximal levels after IPTG induction

The levels of the circular cassette were analysed through a qPCR assay that allowed relative quantification between different conditions and backgrounds. In order to correct for sample to sample variation, the results were normalised to an internal region of ColE1, the origin of replication sequence for the pQE-based plasmids, which would have an identical copy number to the *intI1* and *bla*$_{IMP-5}$ gene.

Figure 4.5 Fold change in detection of circular gene cassette *bla*$_{IMP-5}$ after 8 hours of growth, with and without IPTG induction



Bar graph showing the fold change in levels of circular bla$_{IMP-5}$, when an IPTG-inducible integrase is artificially induced on pQE-bla or pQE-WH-bla. Frequency of integrase activity is detected by the qPCR when normalised to ColE1 also present on the plasmid. Error bars show SEM from three independent replicates. No significant difference was observed between the plasmid in *E. coli* DH5α and *A. baumannii* A457 (Student's unpaired *t*-test, $P > 0.05$)

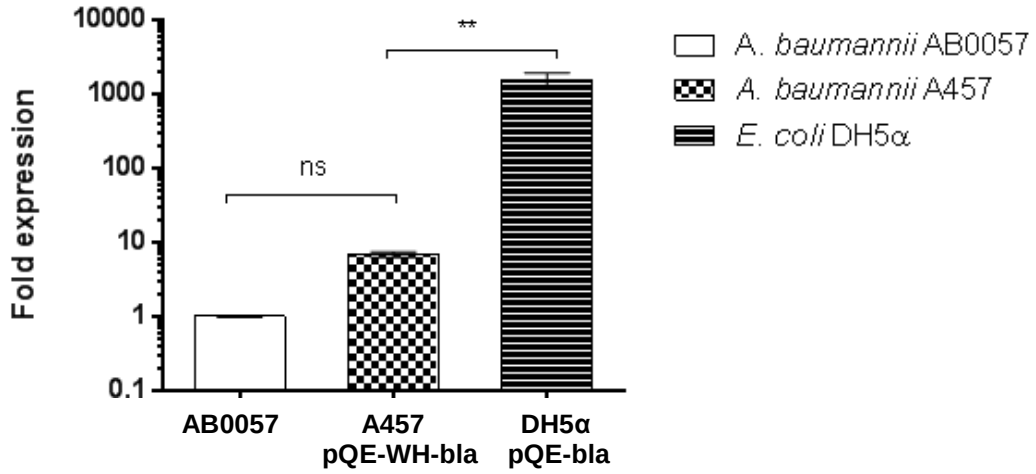
Whilst the initial tests had been completed in *E. coli*, it was not known whether similar activity would be detectable in an *A. baumannii* background. The relative detection of the circular *bla*$_{IMP-5}$ cassette in both *E. coli* DH5α and *A. baumannii* A457 is shown in Figure 4.5. It can be seen that there is a significant increase in the levels of the circular cassette during IPTG induction in both strains, and although the levels are higher in *E. coli*, this is not significantly different to the levels detected in *A. baumannii*. This could be due to the integrase expression exceedin the copy number of the *bla*$_{IMP-5}$ gene, therefore limitation the maximum amount of integrase activity detectable by this assay.

4.2.5. Expression of *intI1* from pQE-bla plasmids are significantly higher than natural wild-type *A. baumannii* strains, and subject to leaky expression

In order to confirm that the increase in detection of the circular cassette was due to an increase in integrase activity, the change in levels of *intI1* mRNA transcription were analysed through qRT-PCR. For this work, housekeeping gene 16S rRNA (16S rDNA) which is stably expressed, was used as a reference gene for normalisation.

Figure 4.6 below shows that the level of expression for the integrase gene was significantly higher in the strains containing pQE-bla or pQE-WH-bla, even in the absence of IPTG, relative to the baseline levels detected in the wild type *A. baumannii* strain AB0057, which is known to carry only one chromosomal integron.

Figure 4.6 Fold change in integrase expression after 8 hours of growth, without any induction methods

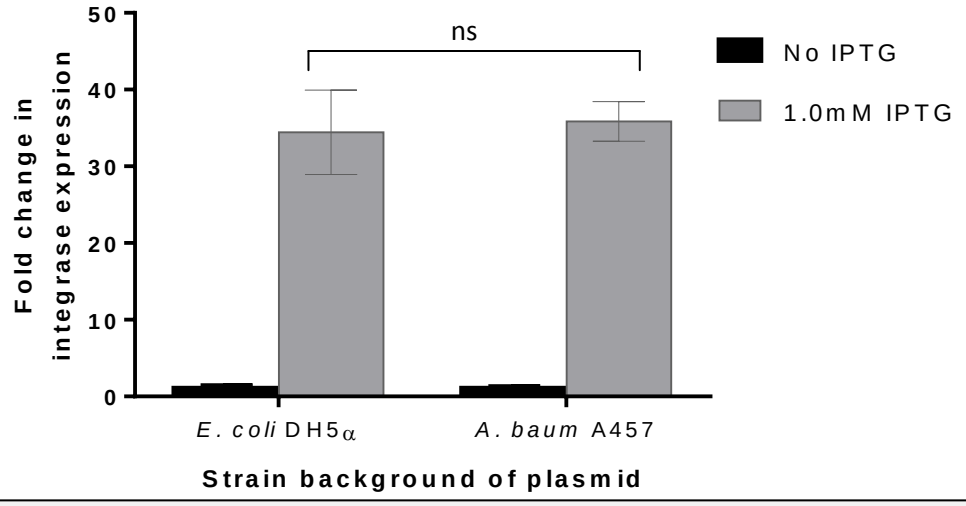


Bar graph showing relative difference in integrase expression from qRT-PCR data between a wild-type *A. baumannii* clinical isolate AB0057 containing only one chromosomal integrase, and two strains (*A. baumannii* A457 and *E. coli* DH5α) transformed with pQE-bla based plasmids (plasmids are labelled where present). Results normalised to 16S rRNA for comparison. Error bars show SEM from three independent replicates. Statistical analysis was completed by using a one-way ANOVA; ns = not significant; ** = $P \leq 0.01$

The expression of *intI1* under normal conditions was expected to be low in AB0057, but the dramatically larger level of expression in the two strains

containing the integrase-carrying plasmids shows that the plasmids have leaky expression. It is not unusual for inducible promoters to have such leaky expression, since in biological systems true 100 % repression is often difficult to achieve. However, the repression of the integrase was at a high enough level so that transformants of these plasmids could be selected.

Figure 4.7 Fold change in integrase expression of pQE-bla carrying plasmids after 8 hours of growth, with and without IPTG induction



Bar graph showing the relative change in integrase expression after the addition of 1.0 mM IPTG in two strains carrying the IPTG-inducible integrase containing plasmids (pQE-bla in DH5α and pQE-WH-bla in A457). Results normalised to 16S rRNA for comparison Error bars show SEM from three independent replicates. Both strains when exposed to IPTG were compared by statistical analysis using Student's unpaired *t*-test, and were not significant: $P > 0.05$.

In addition, the effect of IPTG induction to increase the expression of *intI1* was also investigated by qRT-PCR. It can be seen that whilst the baseline level of integrase expression without IPTG induction was shown to be significantly different in Figure 4.6, the addition of IPTG causes upregulation of the integrase by a similar factor (Figure 4.7). This is somewhat unexpected, since the detection of the circular bla_{IMP-5} cassette was lower at the same time point in *A. baumannii* A457 when compared to *E. coli* DH5α.

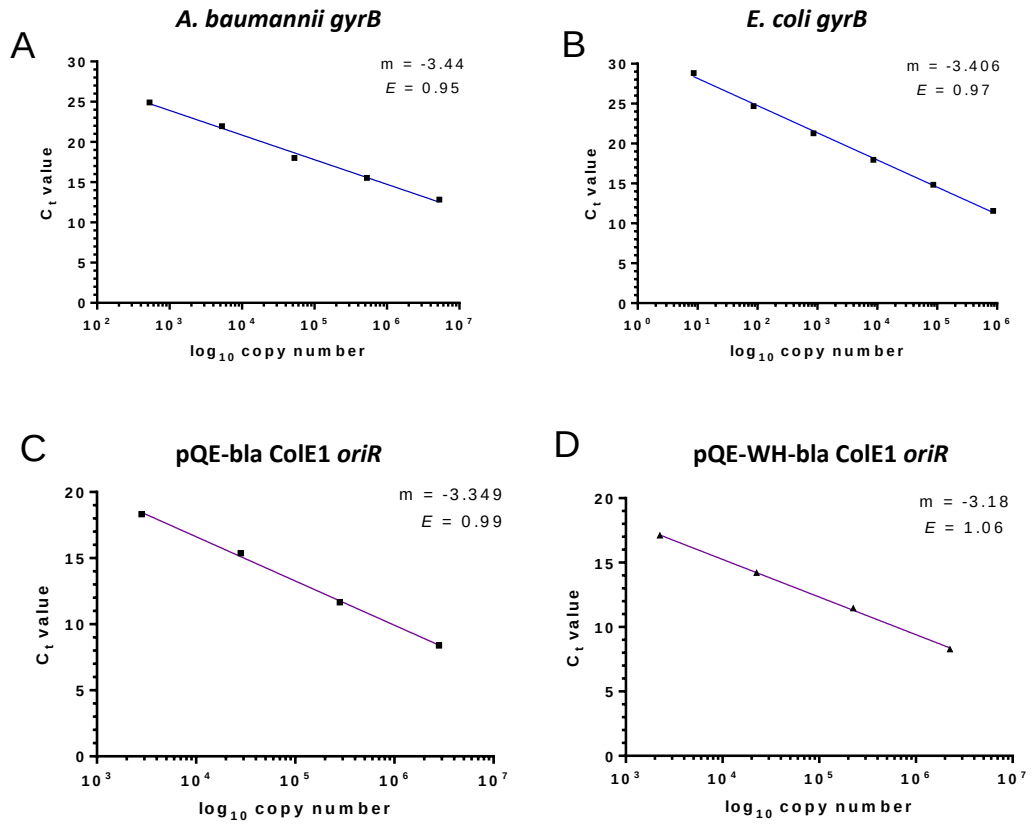
Nevertheless, this data also shows that it is possible for *intI1* to be expressed within an *A. baumannii* cell to fold and construct a functioning

integrase, and that therefore, recombination, whether site-specific or illegitimate, can occur.

4.2.6. Absolute plasmid copy number estimation shows low copy number carriage for all the plasmids in different backgrounds

Using a qPCR assay has recently gained popularity in establishing plasmid copy number (PCN) in bacterial strains (Lee et al. 2006, Skulj et al. 2008). In this study, an absolute quantification method was used, in which 10-fold serial dilutions of the relevant plasmid and genomic DNA were tested by a qPCR assay to create standard curves of the single-copy genes used to determine PCN in this study – ColE1 *oriR* for the pQE-bla plasmids and *gyrB* for the chromosome (with individual species-specific primer sets).

Figure 4.8 Standard curves for ColE1 *oriR* and *gyrB*



Graphs showing line of best fit between the C_t value and $\log_{10}$ of the copy number of a single copy gene either on the plasmid or the chromosome. Standard curves were drawn using serial 10-fold dilutions of genomic (A and B) or plasmid (C and D) DNA, and a line of best fit was added.

Table 4.1 Estimated plasmid copy number by absolute quantification

Strain and plasmid	$C_t \times E$		Copies (copies/µl)		PCN
	ColE1	*gyrB*	ColE1	*gyrB*	
***E. coli* DH5α pQE-bla**	16.04	20.75	1.49×10^4	1.5×10^3	9.57
***E. coli* DH5α pQE-WH-bla**	17.19	22.01	2.16×10^3	6.4×10^2	3.29
***A. baumannii* A457 pQE-WH-bla**	15.90	23.02	5.98×10^3	2.0×10^3	7.76

Table showing an example result of calculations. The C_t values were put into GraphPad Prism which interpolates the actual copy number from the line of best fit on the graph. The average plasmid copy number is given in the right hand column calculated from three biological replicates, with the standard deviation indicated.

In each case, a known quantity of purified DNA was used to create ten-fold serial dilutions, and from the concentrations, the copy number of the DNA was calculated according to the following equation:

$$\text{DNA (copy)} = \frac{6.02 \times 10^{23}\ \text{(copy/mol)} \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660\ \text{(g/mol/dp)}}$$

This calculation allowed the construction of the graphs in Figure 4.8, facilitating the calculation of DNA copy number from the C_t values of the cultures. To create the serial dilutions plasmids pQE-bla (6921 bp) and pQE-WH-bla (8679 bp) were tested separately for greater accuracy of the reading. For the testing of *gyrB* primers, the genomic DNA of the wild type (plasmid-free) *E. coli* DH5α and *A. baumannii* A457 were used. Their sizes were estimated from the size of sequenced genomes of similar strains. In the case of *E. coli*, the size of the closely related MG1655 strain was used (4,641,652 bp) and for *A. baumannii*, a conservative estimate based on the average size of several strains was used (4,050,000), since the strain used here had an unknown sequence size.

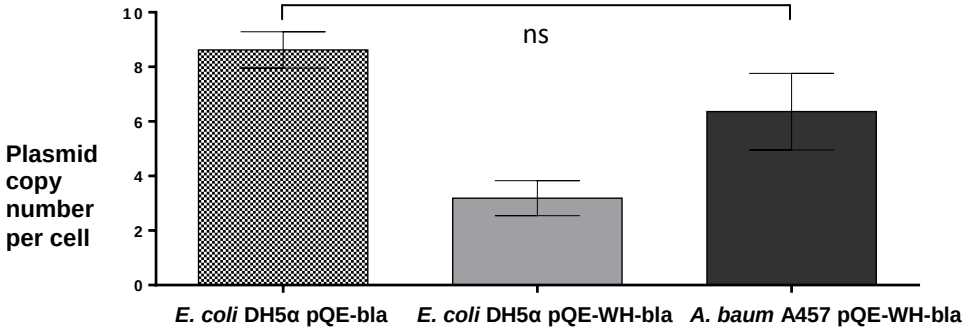
In each case, the efficiency of the qPCR was calculated for each primer pair and template using the slope value obtained from the graph, according to the equation below:

$$E = 10^{-1/\text{slope}} - 1$$

Samples from cultures of *E. coli* DH5α containing either pQE-bla or pQE-WH-bla as well as *A. baumannii* A457 pQE-WH-bla were taken after 8 h of subculture. A crude DNA preparation was used as a template for qPCR.

The C_t values were adjusted according to the efficiency before reading across the graph to see the corresponding DNA copy number. The GraphPad Prism software was used to interpolate the results, which is able to take accurate readings across from the line of best fit on the graph. The copy number of the plasmids calculated on the basis of these results is shown in Figure 4.9. It can be seen that the copy number of pQE-WH-bla in *A. baumannii* A457 is similar to the copy number of pQE-bla in *E. coli* DH5α at 8 hours after subculture. In addition, the carriage of pQE-WH-bla is significantly lower in DH5α, which could suggest some unknown fitness costs in an *E. coli* background.

Figure 4.9 Average copy number of plasmids after 8 hours of subculture



Bar graph showing the average copy number of the pQE-bla plasmids in the strain backgrounds as indicated, when normalised to single chromosomal copy gene gyrB. Error bars show SEM calculated from three independent replicates. Statistical analysis was completed with a one-way ANOVA, and no significant differences between all three values could be found.

4.2.7. The overexpression of *intI1* causes significant fitness costs in all backgrounds tested, with most severe effects shown in *A. baumannii* or *recA* mutants, suggests DNA damage has occured

To assess whether this illegitimate recombination could be detected as a fitness cost affecting the growth of cells transformed with the pQE-based plasmids, the growth curves of three strains bearing these plasmids were

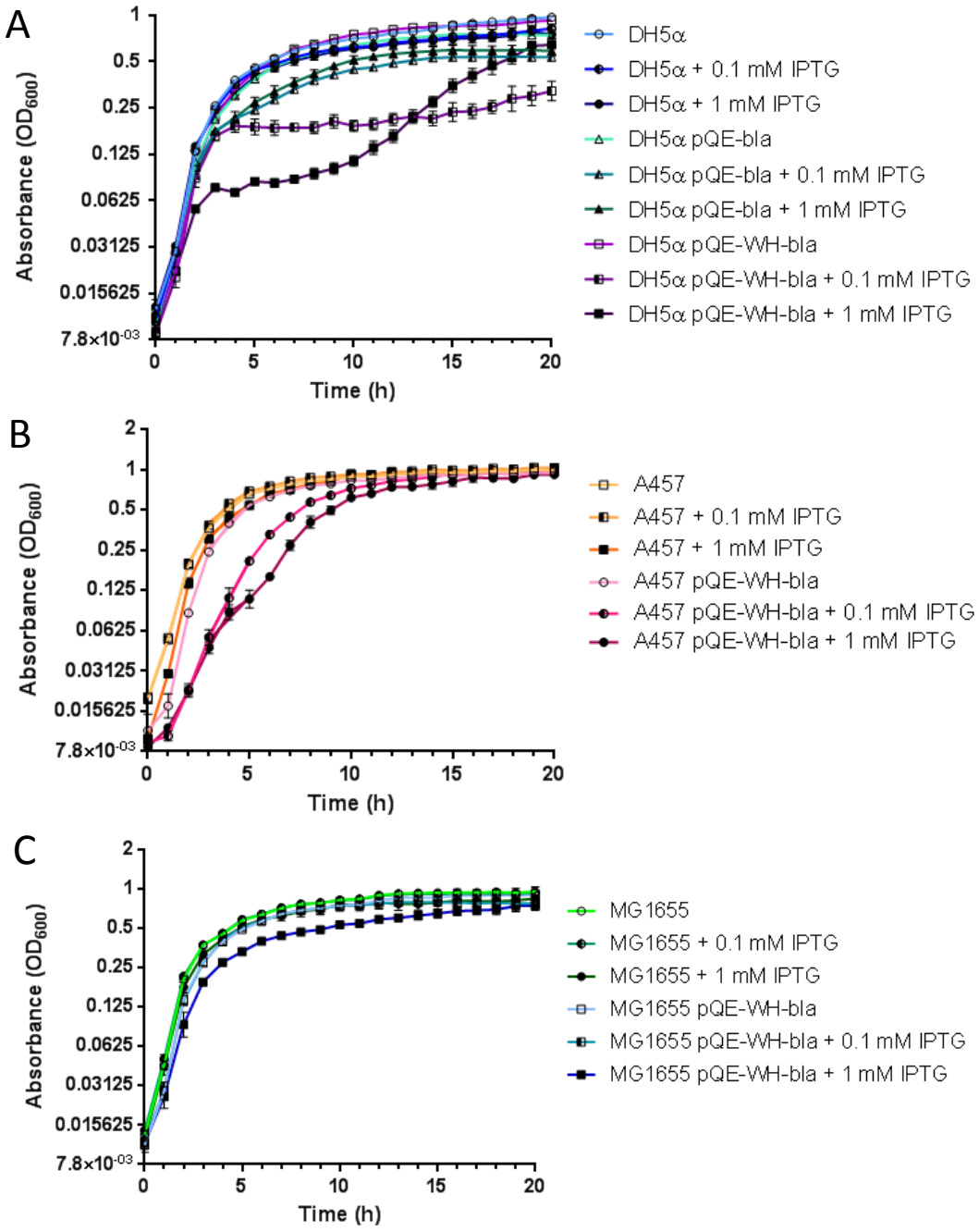
analysed. Given the previous evidence regarding fitness costs of the integrase, it would be expected that expression of IntI1 would be a greater burden for *A. baumannii* than for *E. coli*. To this end, we compared the growth curves of *A. baumannii* strain A457 with two closely related *E. coli* strains, with and without plasmid and IPTG induction (Figure 4.10). In all cases, growth media was supplemented with appropriate concentrations of ampicillin in order to ensure the maintenance of the pQE-bla plasmid.

Although the initial experiments detecting the circularised form of the bla_{IMP-5} gene cassette had been conducted within the laboratory strain *E. coli* DH5α, this strain has been engineered to contain a *recA* mutation, in order to increase stability of inserted DNA. This mutation reduces the rate of homologous recombination by 10,000 fold compared to the wild type *E. coli* K12. However, this also means that cells with this mutation have an impaired response to DNA damage, and so strains with this genotype tend to be less fit and can accumulate many mutations (Casali 2003). For this reason, it would be expected that the carriage of a DNA recombinase would have a significant impact on the fitness of *E. coli* DH5α strain in comparison to other strains.

The very closely related *E. coli* MG1655 was chosen as a comparison background strain. MG1655 and DH5α are both derived from the original *E. coli* model strain K-12, but MG1655 is much more closely related to K12, differing only through the curing of the F plasmid and lambda phage. Therefore, MG1655 has not had any of the genetic modifications, such as the *recA* mutation, and would provide a relevant comparison to DH5α, with the majority of the remaining genes being isogenic. With a functioning RecA, MG1655 would also be able to activate LexA-mediated pathways for DNA repair.

In addition, *E. coli* is known to have a lower number of non-canonical *attI* sites when compared to *A. baumannii* (Harms, Starikova & Johnsen 2013). This means that MG1655 would make a useful comparison to *A. baumannii* A457, since a healthy *E. coli* strain would be expected to have a better fitness than *A. baumannii* when carrying an integrase.

Figure 4.10 Growth curves of strains with and without pQEbla - based plasmids and IPTG induction showing absolute fitness cost of intI1 expression



Growth curves showing the effects of IPTG on the cells with and without the pQE-bla plasmids. The effects of the plasmids were tested on two strains of *E. coli* – DH5α (A) and MG1655 (C) – and one strain of A*. baumannii*, A457 (B) In all cases, cells without IPTG induction are shown with clear shapes, cells with 0.1 mM IPTG are shown in half shaded shapes, and 1 mM IPTG are shown with filled shapes. Standard error bars are shown and were calculated from eight replicates

Although the effect of integrase overexpression by IPTG affects all the species tested at a statistically significant level, it can be seen from the growth curves that MG1655 is the least affected strain, showing a visible inhibition of growth only at the highest concentration of IPTG tested. Indeed, without the presence of IPTG, there is no observable difference in growth for the wild type strain compared with the strain containing pQE-WH-bla. Conversely, in *A. baumannii* A457 and the *recA*-negative lab strain DH5α, the presence of the plasmid exerts an effect even without IPTG induction, and also at the lowest concentration tested, of 0.1 mM IPTG.

Table 4.2. Statistical analysis of growth curves

Control strain	Strain with plasmid	No IPTG	0.1 mM IPTG	1 mM IPTG
***E. coli* DH5α**	***E. coli* DH5α pQE-bla**	****	****	****
***E. coli* DH5α**	***E. coli* DH5α pQE-WH-bla**	ns	****	****
***A. baumannii* A457**	***A. baumannii* A457 pQE-WH-bla**	***	****	****
***E. coli* MG1655**	***E. coli* MG1655 pQE-WH-bla**	*	****	****

Statistical significance between growth curves of wild type and strains transformed with pQE-bla based plasmids under different IPTG concentrations.

Statistical analysis calculated by two-way ANOVA, comparing their area under curve.

ns = not significant, * = $P \leq 0.05$, *** = $P \leq 0.001$, **** = $P \leq 0.0001$

Significantly, when an effect of the overexpression of *intI1* with 1.0 mM IPTG is seen with strain MG1655, it is at a much later stage of growth, during the stationary phase. This could be due to the simple cost of producing the integrase protein at a high level. In comparison, the DH5α strain containing the overexpressed plasmid shows significant impairment of growth at an earlier phase, and the *A. baumannii* A457 strain shows impairment throughout the exponential phase and even the lag phase. The *recA*-deficient DH5α growth curve shows that the cells are not able to recover to similar fitness levels of the

wild-type cultures even within 20 hours. In comparison, it can be seen that after 15 hours, the growth of A457 and MG1655 strains with pQE-WH-bla are growing at similar levels to their non-plasmid containing counterparts. This is likely to be due to the fact that these latter two strains contain intact recombination machinery (*recA*), allowing them to resolve DNA damage.

These growth curve experiments confirm expectations that IntI1 likely exhibits a significant fitness cost in all the species examined, but to different extents, depending on IPTG concentration, the presence or absence of *recA*, and the relative frequency of non-canonical recognition sites for IntI1.

4.3. Discussion

4.3.1. pQE-bla plasmids are an efficient unit to investigate integrase activity

This work has built upon existing knowledge of $LacI^q$ repressed and IPTG-inducible systems in which a gene of interest (in this case, the *intI1* gene) is inserted into a vector in such a position to be expressed under the control of the P_{lac} promoter, in a low copy number plasmid. Furthermore, the pQE-WH-bla plasmid incorporates a key origin of replication required for maintenance in *A. baumannii* (Hunger et al. 1990), expanding the use of this plasmid to a species in which integrase activity has not been studied. This is an example of a plasmid system that brings together knowledge and features from many systems in order to create a unique construct in which the excision/integration activity of the integrase can be controlled and may easily be assessed under experimental conditions.

The assessment of the relative expression of the integrase and detection of the circular $bla_{IMP\text{-}5}$ cassette (incorporated into the plasmid), showed that the plasmid did not fully repress *intI1* to a maximal level. Nevertheless, the expression of *intI1* without IPTG occurred at a low enough level so that three strains of Gram-negative bacteria tested (including a clinical isolate) could be transformed without loss or rejection of the plasmid. In addition, there was a

noticeable difference in level of activation of the integrase after IPTG induction, in a concentration dependent manner, so the leaky expression was not a barrier to completing controlled integrase activity assays in the range of strains tested.

Since BLAST analysis had confirmed that the integrase cloned onto this plasmid is known to be carried in an identical sequence across many integrons containing diverse gene cassettes in several different species, it could be surmised from this information that this unit could be utilised for excision assays of many other gene cassettes (providing that the *attI* and *attC* sites are intact). With the simple addition of an origin of antibiotic resistance determinants that are compatible with MDR strains, the pQE-BHR2 (broad host range) version of the plasmid would also facilitate such similar investigations in other Gram-negative pathogens, such as *P. aeruginosa* or *K. pneumoniae* (Choi, Schweizer 2006, van Aartsen, Rajakumar 2011).

4.3.2. The *intI1* gene can be expressed in *A. baumannii* to produce a functional integrase

The lack of detectable gene circularisation in this species lead to questioning whether *intI1* can be expressed in *A. baumannii*. This study shows that an *A. baumannii* clinical isolate is fully capable of expressing the integrase gene to produce a functional integrase enzyme. This has not been previously reported in this species, for which the coverage of integrons has been largely confined to descriptive epidemiological studies documenting the frequency of different gene cassette arrays (Liu et al. 2014, Karah et al. 2011, Gallego, Towner 2001, Turton et al. 2005).

Previous studies analysing gene cassette rearrangement due to integrase activity were completed in *V. cholerae*, *P. aeruginosa* and *E. coli* (Guerin et al. 2009, Hocquet et al. 2012, Yang et al. 2009). Only recently have some studies attempted to investigate integron activity in *Acinetobacter*, using the closely-related *Acinetobacter baylyi* species as the model organism (Starikova et al. 2012, Domingues et al. 2012). *A. baylyi* is very closely related to *A. baumannii*, and is more amenable for transformation and genetic

modification facilitating a range of experimental assays (Vaneechoutte et al. 2006). In addition to a greater capacity for natural transformation, *A. baylyi* is also not a known human pathogen, whereas *A. baumannii* is the most commonly isolated species of *Acinetobacter* in human clinical isolates (Joly-Guillou 2005). However, these studies have shown experimentally that integrases are under negative selection in *A. baylyi*, and are likely to maintain mutations over time. These findings demonstrate that integron activity and movement is different in members of this genus compared to the previously studied species. The data presented in this chapter studied the human pathogen *A. baumannii* directly, and confirm that integron fitness costs can apply to both members of this genus. These findings therefore also support the use of *A. baylyi* as a good model organism for studies of *A. baumannii*, since these phenomena identified can be applicable to both species.

4.3.3. Integrase activity between pQE-bla and pQE-WH-bla plasmids in different strain backgrounds can be comparable for the purposes of these experiments

The results of the relative quantification assay for integrase expression show that although the amount of expression increases by a similar factor in both *A. baumannii* A457 and *E. coli* DH5α after the application of IPTG, the level of expression is significantly higher in *E. coli*. This could be due to the more optimal functioning of the P_{lac} apparatus in *E. coli*. However, the total amount of circular bla_{IMP-5} cassette detected was not significantly different between the two strains, which could mean that regardless of any difference in integrase expression, both strains had reached a limit in the amount of circular bla_{IMP-5} cassette that could be produced. This is because the copy number of the bla_{IMP-5} gene is a finite number, and therefore could be a limiting factor for integrase activity.

It is also possible that there could be differences between the pQE-bla and pQE-WH-bla plasmids. However, since the level of upregulation after IPTG induction is not statistically significantly different between the two strains, this would suggest that impairment of the integrase gene expression is unlikely to

be the cause. Alternatively, it could be that the plasmid was lost in some cells, and that A457 cells without the plasmid may have survived in a population where enough cells retained the plasmid to break down the antibiotic in the medium. The qPCR was normalised to the level of ColE1 detected, which would have been able to normalise for plasmid loss in the event that lost plasmids were broken down, but not if the plasmid DNA was maintained free in the growth medium. It is assumed that in the majority of cases, and exogenous plasmid would likely be degraded. The copy number of the cells was also tested after 20 hours, and was found to be significantly higher in *A. baumannii* (~60 copies per cell; data not shown), and this was thought to be due to presence of plasmid in the medium or dead cells.

The difference could also be due to the fact that *A. baumannii* has been calculated to have a greater number of non-canonical sites than *E. coli*, which the integrase may recognise (Harms, Starikova & Johnsen 2013). It is possible that the integrase enzyme may be undergoing competitive inhibition from alternative sites in the genome, preventing it from binding with the true *attI* and *attC* sites of the bla_{IMP-5} cassette. However, this hypothesis would require further testing of the pQE-WH-bla plasmid in *E. coli* DH5α to analyse whether the effect is due to this reason or another difference between the two plasmids.

The determination of absolute copy number of each plasmid per cell showed that plasmid carriage for all the pQE-based plasmids were relatively low. The levels of pQE-bla in *E. coli* DH5α and pQE-WH-bla in *A. baumannii* A457 was relatively similar, indicating that the results can be comparable. The copy number of pQE-WH-bla in *E. coli* DH5α however was lower. The establishment of the copy numbers were taken on crude DNA preparations of cultures at 8 hours after subculture, which was the time at which readings were taken for the other experiments in this study. However, the copy number of plasmids may change over time, as was determined by Skulj, et al. (2008). Their study of the PCN of pET3a in *E. coli* BL21 that 20 h was the time point at which PCN was at its maximum. However, this time point was not used because qPCR data may produce variable results if there has been a large accumulation of dead cells. This may be expected in this study because IntI1 was predicted to have deleterious and possibly lethal effects, so many cells

could have been affected and may have resulted in significant cell death. It is possible that strain to strain variation would mean that plasmid copy number may be different at different times in the strains used here, and further experiments at several time points would be required to confirm this.

The growth curve of both plasmids in DH5α (Figure 4.10, A) also showed differences in between the DH5α strains carrying the two different plasmids, suggesting that the addition of the pWH1266 origin of replication may be causing negative effects in an *E. coli* background. It could be that the *oriR* from pWH1266 may have been partially recognised by the *E. coli* cell and caused partitioning problems during cell replication. However, there are a number of other commonly used shuttle plasmids with two origins of replication (such as pFLP2) with no known negative effects. Furthermore, there is no evidence to suggest that the *E. coli* DH5α may recognise the pWH1266 *oriR*.

Alternatively, it is predicted by BLAST analysis that the pWH1266 *oriR*-containing region considered to be necessary for replication within *A. baumannii* could be part of a toxin-antitoxin (TA) system. If this system is not expressed properly in *E. coli* or results in a toxin that is more harmful to *E. coli* than *A. baumannii*, it could result in a lower copy number.

Every step was taken to ensure that addition of the pWH1266 *oriR* would not impact on the functioning of the plasmid in any unwanted way. The insertion of the pWH1266 *oriR* was chosen to be at a neutral location, at the BamHI site downstream of the integrase gene, which does not interrupt any other genes or regions, except the remnant region of chloramphenicol that was present on the pDS plasmid from which the pQE-Expressionist series were developed. This chloramphenicol gene was no longer intact or in use. At a position 933 bp downstream of the BamHI site, there is an intact *rrnB* T1 terminator, which is uninterrupted by the insertion of the pWH1266 *oriR*.

It is possible that since the insertion of the pWH1266 *oriR* increased the size of pQE-bla by 1758 bp, the resultant plasmid pQE-WH-bla was over 8.6 kb. In some instances, increases in size of plasmid can cause difficulties for the cell and can result in insert instability. However, most plasmids below 10 kb are still considered as small, and are not thought to exert negative effects (Ausubel

et al. 2003). However, the low copy number could indicate that the plasmid exerted a significant enough fitness cost that only cells maintaining the plasmid at a low copy number could survive. The fitness cost of the carriage of pWH1266 alone in DH5α would have to be tested through growth curves under the same conditions to analyse whether some feature inherent in the plasmid contributes to the differences in growth curves.

Whilst the pQE-bla- and pQE-WH-bla–containing versions of DH5α exhibited some differences in growth curves, the MG1655 strain did not exhibit any differences in between the wild type and those strains carrying the pQE-WH-bla plasmid, as predicted. For these reasons, the differences seen in the two plasmids in DH5α were concluded to be due to alternative factors independent of integrase activity; therefore, the results were accepted as a true reflection of the intended assays.

Finally, the empty pQE-80L plasmid which was originally used as a backbone for the pQE-bla plasmids was also tested under these same growth conditions in *E. coli* DH5α. However, since this plasmid was originally designed to be a protein expression plasmid, it contains six His-tags, which are known to carry a heavy fitness burden for cells. Therefore, the results of these growth curves were not considered homologous to the plasmids constructed in this study, and the data were consequently not considered comparable from which reliable conclusions could be drawn. For this reason, these results have not been shown here.

4.3.4. The *intI1* overexpression exerts a fitness cost that is likely to be due to illegitimate recombination at secondary, non-canonical sites

The fitness cost of a class 1 integrase in *A. baylyi* has already been investigated by Starikova, et al. (2012), who showed that the maintenance of an integrase even in a single copy on the chromosome can exert a fitness cost. Furthermore, over time, *A. baylyi* strains containing plasmids with integrases tended to accumulate mutations in the *intI1* gene that render the integrase inactive. In addition, a further study from this group demonstrated that

Acinetobacter species have a higher number of non-canonical recombination sites that may be recognised by the integrase enzyme (Harms, Starikova & Johnsen 2013).

For these reasons, the strains chosen to be tested for fitness cost via growth curves were selected to provide experimental examples of how DNA damage could be incurred by IntI1 in different SOS-response system backgrounds, and assessing whether the presence of absence of *recA* had any effect in resolving DNA damage from any illegitimate recombination events. Growth curves were chosen as a phenotypic marker for these fitness costs on the basis that they allow qualitative and quantitative results at different stages of growth. It could be said that growth curves only allow a very crude approximation for absolute fitness cost, and rely upon the assumption that fitness differences can be expressed as different growth rates. However, this kind of assay is appropriate for the study of DNA-damaging conditions and responses, since growth requires multiple rounds of DNA replication followed by cell division; therefore high fidelity replication of DNA during cell division process is vital for efficient growth. This means that any effects of the integrase on DNA damage would be most evident during the latent and logarithmic phases of growth. This is indeed the results that were seen in these experiments, as both *A. baumannii* strains containing the pQE-WH-bla plasmid showed delayed latency and slower growth rate during the logarithmic phase, and *E. coli* DH5α even showed a lower maximum growth at the stationary phase.

DH5α was observed to have significant growth impairment when carrying the pQE-bla or pQE-WH-bla plasmids, even without induction. This was expected in the event that the integrase was causing DNA damage through illegitimate recombination at alternative sites, since this lab strain has been engineered to contain a *recA* mutation, which means it is less able to activate responses to cope with DNA damage. The ancestral strain MG1655 from which DH5α is derived was confirmed to not exhibit such a cost for carrying an integrase-expressing plasmid, since it contains a fully functional *recA* and recombination mechanism. This suggests that the fitness cost of IntI1 is highly likely to be due to DNA-damage.

Conversely, the *A. baumannii* A457 strain tested with the pQE-WH plasmid was significantly impaired in the logarithmic phase of growth, and then later recovered to maximal growth at stationary levels. This could have been due to the DNA damage caused by the action of the integrase, which the cells were able to repair, since this clinical isolate contains a fully functioning recombination mechanism (intact *recA*), and putative error-prone polymerases, such as *umuC* (Aranda et al. 2014). However, this depends on whether IntI1 is fully able to recombine a section of DNA at non-canonical sites. It is thought IntI1 resolves the excision junction to leave two separate pieces of double stranded DNA (dsDNA), however RecA activity is normally triggered by the presense of single stranded DNA (ssDNA). RecA is able to hold the ssDNA alongside a matching piece of dsDNA, and it is able to locate the matching segment through conformational proofreading. Whilst complete excision of DNA segments may not trigger RecA activity, it could be that non-canonical sites may not be similar enough to allow the integrase to complete full excision. It could also be that excision of vital sections of DNA for the maintenance of the choromosome may have been excised, and are thus causes secondary single stranded lesions that RecA is then able to resolve.

Alternatively, it is also known that RecA is involved in incorporation of exogenous DNA, particularly in strains that are known to be naturally competent. It could be that during the generation of random DNA damage from the integrase, *A. baumannii* cells with damaged or missing sections from their chromosome are able to uptake “healthy” DNA from neighbouring live or dead cells, with which to repair their own DNA. In addition, since RecA is known to have a central role in activating other DNA repair pathways such as RecBCD and RecF, it could also be the case that RecA may be required to activate these other pathways, rather than directly resolving chromosomal damage itself.

The results of these fitness tests support the hypothesis that the integrase is capable of causing events that are harmful to the host cell, including possibly illegitimate recombination, regardless of species. Integrase activity is independent of RecA, and a strain that is deficient in RecA activity is

therefore still able to express a functioning integrase, but is less able to repair any subsequent DNA damage that may be produced.

The reason for a significant difference in DNA damage in the latent and logarithmic phases is thought to be because the *A. baumannii* genome has an approximately 1.5-fold higher number of non-canonical recognition sites that the integrase may recombine. This was calculated by Harms, Starikova and Johnsen (2013), as they showed that *A. baumannii* had on average 1.212–1.227 ratio of observed to expected number of *attI* sites per chromosome. In comparison, *E. coli* had a lower ratio of only 0.794–0.833 of *attI* sites. This is a difference of roughly 1.5-fold greater number of sites in *A. baumannii*. Although this ratio is lower in *E. coli*, this number still represents potentially up to 9,000 alternative *attI* sites per chromosome (Harms, Starikova & Johnsen 2013). This correlates with the fact that growth in both the *E. coli* strains tested is inhibited to some degree, not just the *A. baumannii* strain. However, these experiments do not show primary evidence for the DNA-damage itself, rather the secondary effects of growth inhibition or delay. Regardless of the mechanism, this data demonstrates that the over-expression of the integrase does cause significant fitness costs, which could lead to their negative selection.

The negative effects of integrases may be diminished in other Gram-negative pathogens (such as *E. coli*, *P. aeruginosa* and *K. pneumoniae*), since they have a LexA-mediated SOS mechanism, which naturally represses the class 1 integrase (Guerin et al. 2009). However, it is also likely that integrons first evolved in species such as these, and that therefore, over time, strains with a significantly high number of non-canonical sites would have been selected out from the gene pool. Conversely, many *Acinetobacter* species are isolated from environmental habitats, and even *A. baumannii* itself was originally considered to have been a soil organism (Baumann 1968). It is possible that *A. baumannii* only came in contact with integrons relatively recently, after becoming exposed to other integron-carrying pathogens and their mobile genetic elements in hospital or healthcare environments in the last 50 years, the time in which it has become an emerging human pathogen. It is likely that *A. baumannii* has not yet had sufficient time to undergo significant selection to reduce the number of non-canonical recognition sites for integrases in their genome. The propensity for

Acinetobacters to uptake foreign DNA in such environments may have resulted in the acquisition of a range of foreign MGEs into *A. baumannii*, for which this genus may not have been adapted to, subsequently resulting in a conflict of cost-benefit for this species. The data presented in this chapter demonstrate experimentally how this phenomenon may explain the high frequency of 5' end truncated integrons in *A. baumannii*, outlined in Chapter 3.

4.4. Future work

This chapter has outlined how the overexpression of *intI1* can have fitness costs detectable in growth curves. Potential future experiments that could be conducted in order to cement these findings and further improve the pQE-bla plasmids are outlined below.

1) Repeat initial tests of pQE-bla in *E. coli* DH5α with pQE-WH-bla, to confirm that the two plasmids behave in an identical way in DH5α, and that the insertion of the pWH1266 origin of replication does not affect the integrase activation unit plasmid in any way.

2) Construct a pQE-80L plasmid without His-tags to test as a control for the growth curves, alongside a pWH1266 control, to further validate the results from the growth curves.

3) Test pQE-bla plasmids with other integron gene cassette arrays, to analyse the frequency of excision of gene cassettes further away from the *attI*.

4) The cloning of the integrase gene under its natural promoter could be tested in a *recA* and LexA-positive control strain such as MG1655, and compare its functioning in *A. baumannii*, specifically the upregulation of *intI1* expression under DNA-damaging conditions, in order to compare it with the data produced in this chapter from the IPTG-inducible system. This could give some interesting results regarding the level of upregulation under the natural activation system, vs the artificially constructed activation system outlined here.

5) Replacement of the integrase gene on pQE-WH-bla vector with an alternative gene that is unlikely to be under negative selection in *A. baumannii*, for example the chloramphenicol resistance gene *cat*. This would help to answer the question of whether IntI1 is specifically causing DNA damage, or whether the burden of overexpressing any gene and producing high levels of a protein are responsible for the differences in growth rates.

6) Replacement of the bla_{TEM-1} gene on the pQE-BHR2 plasmid with an antibiotic resistance gene suitable for maintenance in multidrug-resistant isolates, such as *aacA4* (apramycin) or *hph* (hygromycin).

Chapter 5. Assessment of the role of $UmuD_{Ab}$ in integrase activity and DNA-damage response in *A. baumannii*

5.1. Introduction

In the previous chapters, the frequently observed occurrence of disrupted integron structures and IntI1-associated fitness costs in *A. baumannii* were outlined. The experimental data laid out in Chapter 4 showed that overexpression of the integrase can cause detectable fitness costs that are higher in *A. baumannii* than *E. coli*, likely due to a higher number of predicted non-canonical recombination sites, and also linked to the lack of the classical LexA-mediated repression in *A. baumannii* that is normally found in the majority of other Gram-negative pathogens. As a consequence, it is also unlikely that induction of a DNA-damage response would activate integron activity in *A. baumannii*, as shown in other species (Hocquet et al. 2012, Guerin et al. 2011).

The regulation of enzymes that result in MGE mobilization and acquisition of antibiotic resistance genes in response to DNA-damage stress is now well established in *E. coli* and other Gram-negative bacteria (Petrosino et al. 2009, Gillings 2013, Hocquet et al. 2012). Since the SOS-response pathway in *A. baumannii* appears to differ from these model strains, it is important to investigate whether MGE mobilization can be activated in the same way in *A. baumannii*. The classical SOS system present in most Gram-negatives is dependent on a LexA-mediated pathway. LexA is a protein that is a well-established global regulator of around 30 to 40 genes in *E. coli* (Butala, Žgur-Bertok & Busby 2009, Courcelle et al. 2001), including integrase expression (Guerin et al. 2011). Following DNA damage or replication stress, RecA is converted into its activated form RecA*, and this latter protein triggers autocatalysis of LexA, facilitating the derepression of many genes, some of which code for error-prone polymerases, that are thought to help the cell repair any DNA damage. Due to absence of LexA-homologues in the *Acinetobacter genus*, it is unclear whether a similar regulatory mechanism exists in this genus.

LexA is a vital part of the classical SOS response, where under non DNA-damaging conditions, it is bound in a homodimer form to specific recognition sites known as “LexA boxes”. These boxes are usually in promoter regions, and the binding of the homodimer complex represses the expression of downstream genes. The presence of single-stranded DNA (ssDNA) formed from DNA-damaging events, activates RecA, which in turn promotes the self-cleavage of LexA. After self-cleavage, the LexA segments dissociate from the binding box in the promoter region, and the previously repressed genes can be expressed. The cleavage of LexA occurs at the residues Ser119 and Lys156 (Little 1991). This means that a fully functional LexA requires two domains, one for DNA-binding (Lewis et al. 1994), and a self-cleaving peptidase region to facilitate autocatalysis (Paetzel, Dalbey & Strynadka 2002).

The pathway for the classical SOS-system also includes the activation of a number of other enzymes. A single-strand break often results in subsequent double-strand damage, producing DNA lesions which could stall replication (Cox et al. 2000). In order to reactivate the replication fork, the *E. coli* SOS system brings about several processes to overcome DNA damage, including the upregulation of error-prone polymerases. These polymerases, including DinB, $UmuD'_2C$ (DNA polymerases IV and V, respectively) and PolB bring about mutagenesis-mediated repair of stalled replication forks (Wagner et al. 1999, Tang et al. 1998, Napolitano et al. 2000). They are able to bypass DNA lesions, allowing replication to recommence, but often at the expense of replication fidelity, resulting in the introduction of point mutations or frame shift errors. For this reason, these error-prone enzymes are usually very tightly regulated (Courcelle et al. 2001). However, the benefits of tolerating these mutations outweigh the costs, since otherwise a stall in the replication fork would result in arrested growth and division. Following lesion repair, the absence of single-stranded DNA means that RecA is no longer activated, and levels of whole LexA homodimers also rise to return to their baseline levels.

The Umu proteins were discovered after treatment with UV resulted in a higher mutation rate compared with background levels. Deletion of *umuDA*, *B* and *C* returned the mutation rate to spontaneous levels (Kato, Shinoura 1977). The proteins are thought to function together in different forms when activated.

Under normal circumstances, UmuD forms homodimers that bind with UmuC as part of DNA damage checkpoint control, delaying cell division, to allow time for DNA repair (Nohmi et al. 1988). Under DNA-damaging conditions, activated RecA* also facilitates the autolysis of $UmuD_2$, which when bound to UmuC, produces the functional polymerase V $UmuD'_2C$. The resulting enzyme allows translesion DNA synthesis and consequent DNA repair (Tang et al. 2000). PolV lacks 3' - 5' exonuclease activity, meaning that it is error-prone, and it is thought that the Umu enzymes evolved to have such a trait in order to increase genetic variation for evolutionary selection (Radman 1999).

However, many of the experiments on Umu proteins were performed in the classical model organism, *E. coli*. Recently, there has been a growing interest in the equivalent proteins in *A. baumannii*, UmuC and $UmuD_{Ab}$ (Aranda et al. 2014, Hare et al. 2012a). This is because, since the discovery that *A. baumannii* doesn't have a classical LexA-mediated SOS response, alternative repair mechanisms have been investigated in this species (Norton, Spilkia & Godoy 2013, Robinson et al. 2010). According to BLASTp searches, $UmuD_{Ab}$ is the closest match to LexA in *A. baumannii*. However, $UmuD_{Ab}$ is significantly different from its *E. coli* counterpart UmuD, sharing only 60 % identity at the amino acid level, and is also quite distinct to LexA itself. Firstly, the two "UmuD" proteins differ in size, with $UmuD_{Ab}$ being 59 amino acids larger than UmuD resulting in a size difference of 18.85 kDa compared to 15.66 kDa respectively In comparison, LexA is 22.36 kDA, and the nucleotide lengths of the genes are more similar, at 609 bp for *lexA* and 612 bp for *$umuD_{Ab}$*. However, LexA and $UmuD_{Ab}$ only share 37 % identity overall at the amino acid level (Hare et al. 2012a), and this similarity is restricted to the self-cleaving peptidase site at the carboxy-terminus. $UmuD_{Ab}$ doesn't have a known DNA-binding region at the amino-terminus. However, it has been shown by Hare, Perkins & Gregg-Jolly (2006) that $UmuD_{Ab}$ is involved in the transcriptional regulation of a DNA damage related *ddrR* gene which is upstream of *$umuD_{Ab}$*. Aranda *et al*. (2013) later showed through an electromobility shift assay (EMSA) that purified $UmuD_{Ab}$ did in fact bind to a shared palindromic sequence upstream of both *$umuD_{Ab}$* and *ddrR*. This palindromic sequence was found upstream of other UmuC or $UmuD_{Ab}$ homologues within ATCC 17978 (which contains many

copies of these genes; see below) but no other genes had this sequence. Therefore it is likely that only the genes identified could be regulated by $UmuD_{Ab}$. In addition, most *A. baumannii* strains contain multiple copies of the UmuDC operons, which are most likely horizontally acquired, with four putative *umuC* and three *umuD* genes in *A. baumannii* ATCC 17978, compared to only one in *E. coli* (Norton, Spilkia & Godoy 2013, Hare et al. 2012b).

These findings highlight the ways in which $UmuD_{Ab}$ differs in comparison with homologues found in other Gram negative pathogens. It exhibits some traits that are in common with LexA, and others that it shares with UmuD. For this reason, the exact role of $UmuD_{Ab}$ in *A. baumannii* has been investigated by several studies (Aranda et al. 2014, Hare, Perkins & Gregg-Jolly 2006, Hare et al. 2012a). Whilst these studies have confirmed that $UmuD_{Ab}$ has a significant role in DNA-damage response, these studies have not investigated the possibility of involvement in activation of MGE mobility, such as integrons. This study outlines the start of such an investigation.

Since antibiotics such as quinolones can themselves cause bacterial DNA damage, clarification of the mechanism for how MGE enzymes can be activated is highly important for the understanding of how they contribute to the acquisition of antimicrobial resistance determinants. Such knowledge could help to influence the planning of clinical prescription guidelines in future. It is evident from a number of recent studies that many types of MGEs apart from integrons are also capable of being activated by the SOS response. These include the *V. cholerae* CTXφ prophage (Quinones, Kimsey & Waldor 2005), and the SXT integrative conjugative element also from *V. cholerae* (Beaber, Hochhut & Waldor 2004). This situation is of great concern, since it means that therapeutic antimicrobials may in fact be encouraging the spread of antibiotic resistance within and across all important pathogenic bacterial species.

The focus of this chapter is using experimental assays designed to investigate what role $UmuD_{Ab}$ may have in the DNA-damage response of *A. baumannii*, specifically in the context of increased integrase activity. Specifically, this chapter includes an analysis of the effects DNA-damaging conditions on integron activity in three wild-type *A. baumannii* reference strains,

and how the expression of $UmuD_{Ab}$, *intI1* and *recA* may change. These investigations aimed to establish whether $UmuD_{Ab}$ was acting as a LexA homologue in *A. baumannii*. Two mutants that do not produce the $UmuD_{Ab}$ protein were created, and DNA-damaging conditions were induced through media supplementation with mitomycin C, a DNA-damaging agent often used in cancer chemotherapy, which also affects bacteria through its highly potent DNA crosslinking action. The effects of these conditions on relative gene expression was compared to the same conditions in the absence of mitomycin C. Finally, the mutants were complemented using a modified version of the *E. coli – A. baumannii* shuttle plasmid pWH1266, in order to analyse the restoration of the phenotype.

5.2. Results

5.2.1. DNA-damaging conditions did not result in increase of detection of circular gene cassettes

Based on the results of recent studies that exposure to DNA-damaging conditions resulted in increased integrase expression and gene cassette movement (Guerin et al. 2009, Hocquet et al. 2012), three reference *A. baumannii* strains, AB0057, AYE and ACICU were treated with similar conditions. These strains were chosen because they are fully sequenced, so the full details of their integron structures and DNA-damage response genes are available. In addition, these strains are in common use in labs focussed on *Acinetobacter* studies, and subsequently they are well studied model isolates. The subcultures were assayed by qPCR for the presence of circular gene cassettes from the natural integrons. For these experiments, mitomycin C was chosen to create DNA-damaging conditions, since it is an established trigger for the SOS response, and has been used in to trigger integrase expression in integron studies, such as in Guerin, et al. (2009) and Hocquet, et al (2012). Three different concentrations of mitomycin C were tested (3, 6 and 9 µg/ml) after four hours of induction, the circular form of the gene cassettes *aacC1*, *orfX*, *orfC*, *aadA1a*, *dfrIA* and/or bla_{VEB-1}, were sought according to which gene cassettes were carried by each strain. In addition, two antibiotics were also

tested, 0.4 µg/ml ciprofloxacin and 10 µg/ml trimethoprim, which had been used successfully by Guerin et al. (2009) to induce integrase activity. Since these excision events are likely to be at very low frequencies, qPCR was used to detect these products, alongside negative controls of integron-negative species. However, none of these experiments yielded detection of circularised gene cassettes (data not shown).

According to some studies, the rate of excision under natural circumstances is so low, that circular products are only detectable using nested PCR (Hocquet et al. 2012). This has also been shown to be true for some other MGEs, such as ICE*Kp1* (Lin et al. 2008). For this reason, nested PCR was also attempted for the detection of the *aacC1* gene in mitomycin C-induced AYE and AB0057, however, this type of PCR yielded inconsistent products, and when sequenced, none of the amplicons produced had a sequence matching an expected circular product. As the first set of primers for *aacC1* gene produced non-specific amplicons, a second set of primers annealing to alternative locations in this gene corresponding to a larger product were also designed, but these too did not result in detection of circular *aacC1* cassette. Therefore, it was concluded that the excision of the gene cassette due to integrase activity was occurring at an extremely low level, beyond the levels detected by both qPCR and nested PCR methods used in this study.

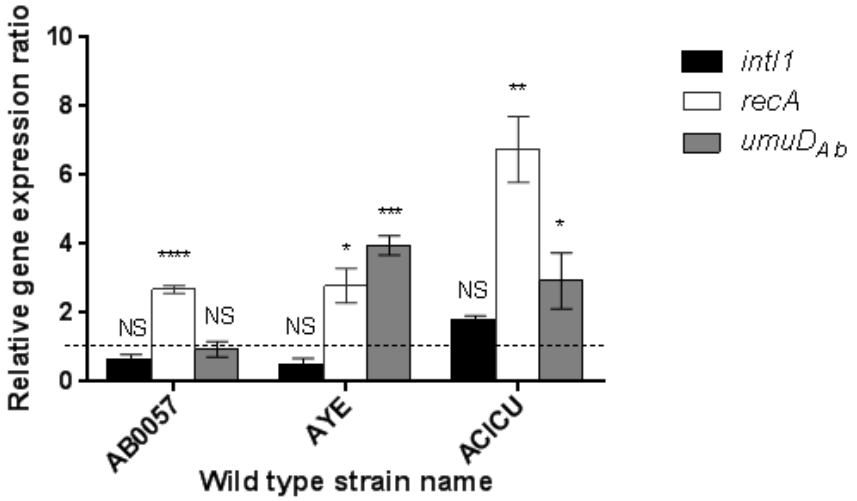
5.2.2. DNA-damaging conditions do not significantly increase *intI1* expression in *A. baumannii*

Assuming that there were no experimental errors, these results suggested that the integrase was either not functional in *A. baumannii* or not induced under DNA-damaging conditions. Since the previous chapter had proved that *intI1* could be expressed to produce a fully functioning integrase protein in an *A. baumannii* strain, it was hypothesised that a lack of circular cassettes were due to the experimental conditions not inducing increased *intI1* expression to produce detectable levels of gene cassette excision. In order to investigate this, qRT-PCR was conducted for three strains, to analyse the relative expression of *intI1*, and also those of the SOS-response-associated *recA*, and the putative *lexA* homologue *$umuD_{Ab}$*, in response to DNA-damaging

conditions. Data from three strains was analysed in order to help account for strain-to-strain variation. The results are shown in Figure 5.1.

It can be seen from the graph that there is statistically no significant change in the expression of *intI1* after exposure to DNA-damaging mitomycin C, compared with baseline expression levels in all of the strains tested. This could explain why there was no increase in detection of the circular gene cassettes in the initial experiments.

Figure 5.1 Relative change in expression of integron and DNA damage-related genes after 4 h of exposure to 6 µg/ml mitomycin C



Bar graph showing change in expression of integron-related genes during exposure to 6 µg/ml mitomycin C, as determined by qRT-PCR, in *A. baumannii* reference strains AB0057, AYE and ACICU. Error bars show SEM from three biological replicate experiments. Dashed line is used as a comparator for the baseline level expression of each gene. Two-tailed Student's *t*-tests using 1.0 (dotted line) as comparator; NS – not significant: *P* >0.05; *: *P* <0.05; **: *P* <0.01; ***: *P* <0.001.

5.2.3. Construction of *umuD$_{Ab}$* mutant alleles for suicide plasmid-mediated allelic exchange

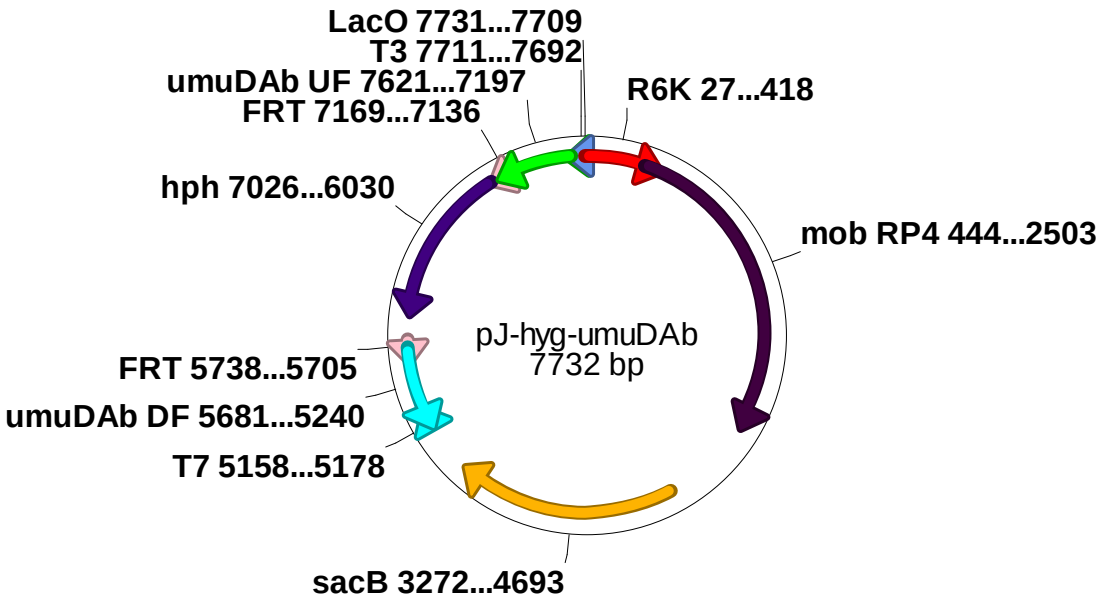
Mutants of *umuD$_{Ab}$* were created to investigate its role in DNA-damage response. They were made by the suicide vector allele exchange method as outlined in the Materials and Methods section. Briefly, the pDS132-based plasmid pJTOOL-3 was used as the vector, which had previously been successfully used by Rieck et al. (2012) for mutant creation in *A. baumannii*. This plasmid contains an *R6K* origin of replication, which requires the phage

lambda π protein for replication, in such strains as *E. coli* S17-1. Since *A. baumannii* lacks the π protein, the plasmid acts as a suicide vector in this species.

After conjugation via the *mob RP4* origin of transfer into *A. baumannii*, the plasmid can be either incorporated into the chromosome, or be lost. This means that selection for antibiotic resistance conferred by the plasmid would result in the isolation of recipient strains in which the plasmid has chromosomally integrated, due to a targeted allelic recombination event. In this case, an SOE product, containing flanking regions of the *umuD$_{Ab}$* gene fused to the sides of the hygromycin resistance gene *hph* was created, in order to target the allelic replacement of *umuD$_{Ab}$*

The use of pJTOOL-3 allowed the ligation of this SOE PCR product into the PstI site of the plasmid (van Aartsen, Rajakumar 2011). This restriction site was chosen since it was absent from the SOE product sequence, and only present once in the MCS of pJTOOL-3, and were incorporated into the 5' end of the primers used to amplify the final SOE product (PR3002/PR3005).

Figure 5.2 Schematic of pJ-hyg-umuD$_{Ab}$, used to create Δ*umuD$_{Ab}$* mutants in *A. baumannii* AYE and AB0057



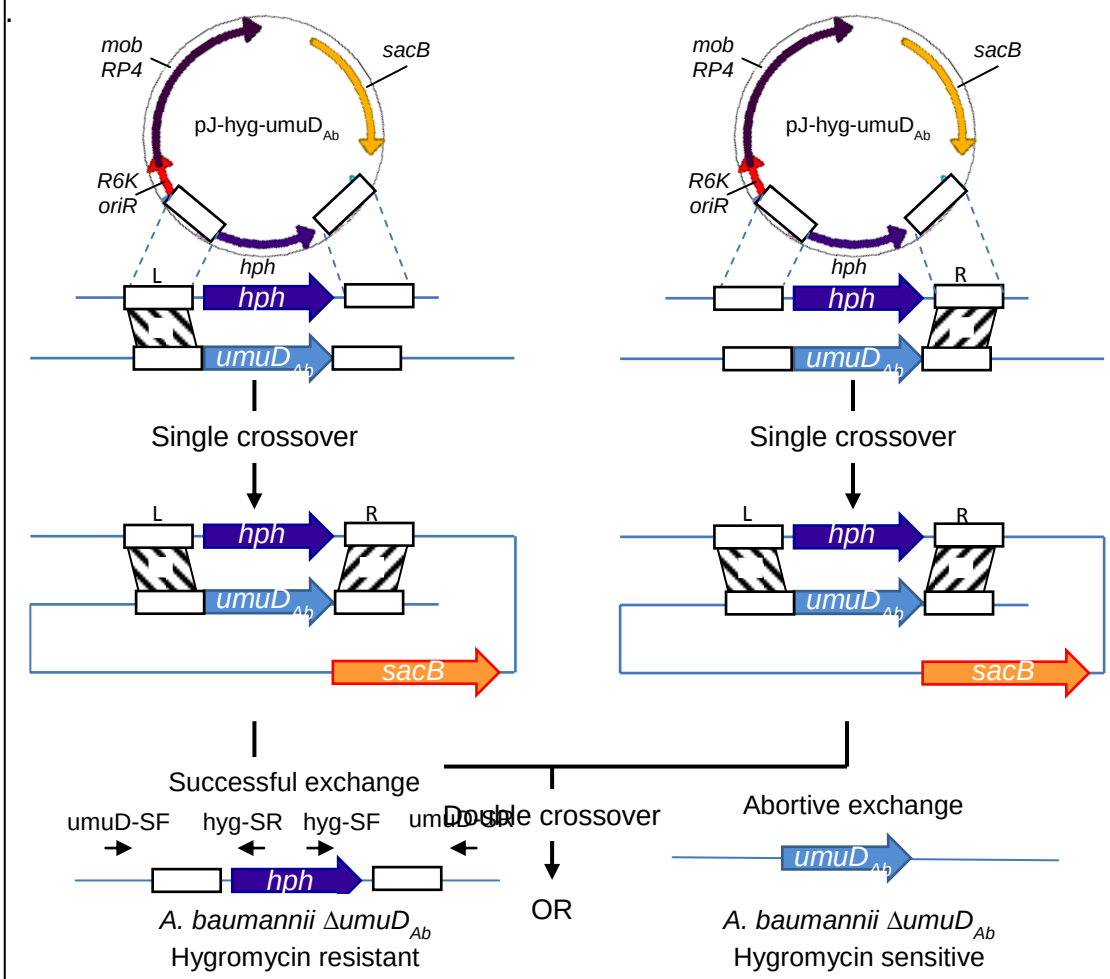
Schematic showing the structure of pJ-hyg-umuD$_{Ab}$, used for allelic exchange. UmuD$_{Ab}$ UF and DF represent upstream and downstream flank, respectively.

The pJTOOL-3 plasmid backbone also contains a *sacB* gene, which codes for the exo-enzyme levansucrase, which gives cells containing this gene sensitivity to sucrose, thereby facilitating a simple method of counterselection. In this way, single crossover (SCO) recombinants that have undergone a further recombination event to create a double crossover are easily detectable. Using LA media supplemented with 10 % sucrose, double crossover mutants in two *A. baumannii* strains were selected.

In all cases, putative single and double crossovers were confirmed by PCR using primers in flanking regions of the chromosome coupled with primers facing outwards from the hygromycin resistance gene cassette (umuD-SF/hyg-SR, PR3075/ PR3313 and hyg-SF/umuD-SR, PR3314/PR3076; Figure 5.4). On the basis of these results, $DCO2_{AB0057}$ and $DCO3_{AYE}$ were chosen for further studies in the subsequent experiments (Figure 5.4).

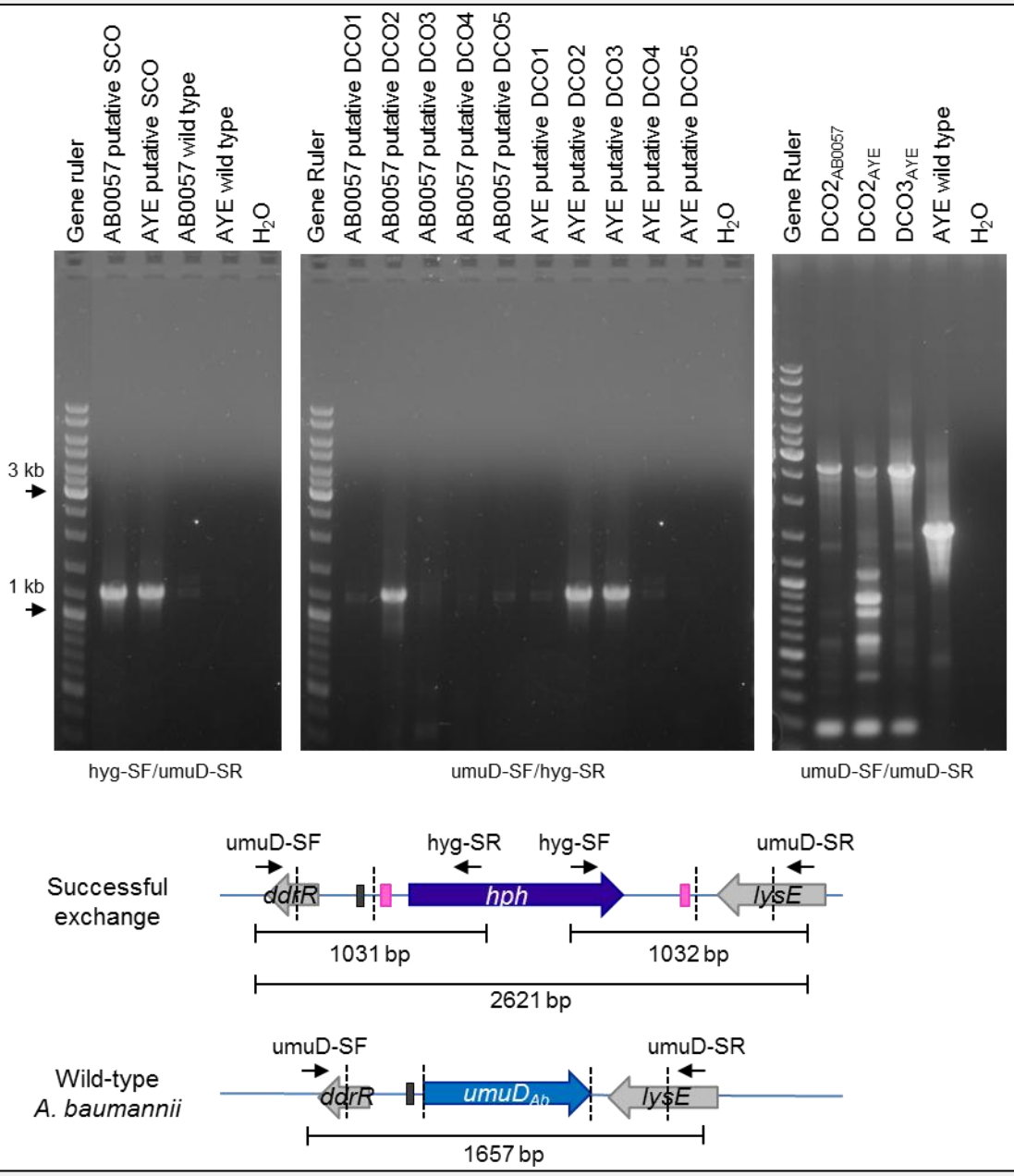
The plasmid pJ-hyg-$umuD_{Ab}$ used for the allelic exchange also contained FRT sites flanking the hygromycin gene. This would allow Flp recombinase-mediated site-specific excision of the gene cassette, leaving only a single FRT scar. However, this was not completed during this project, since at that time, an *A. baumannii*-compatible pFLP2 plasmid was not available, and the hygromycin gene cassette was not deemed to confer any phenotypic difference to the strains.

Figure 5.3 Schematic representation of the mechanism for allelic exchange



Firstly, merodiploids formed from loss of the free plasmid by recombination on one of the homologous flanking regions. Through antibiotic selection this selects for clones harbouring a chromosomally-integrated suicide plasmid. This can be from either a left (L) or right (R)-sided single-crossover homologous recombination. Both possibilities are shown in this diagram. After counter selection on LA sucrose plates, putative double-crossover mutants are identified by a hygromycin resistant, sucrose-sensitive, phenotype and verified by colony PCR analysis. This integrated plasmid may then undergo a second homologous recombination even on the opposing side flank, in order to create a mutant, or the plasmid may be lost altogether, and the single crossover strain would then revert to the original wild type. The primers used to confirm the incorporation of the plasmid at the correct locus and the occurrence of the allelic exchange are denoted by black arrows.

Figure 5.4 PCR assays conducted for the confirmation of mutants

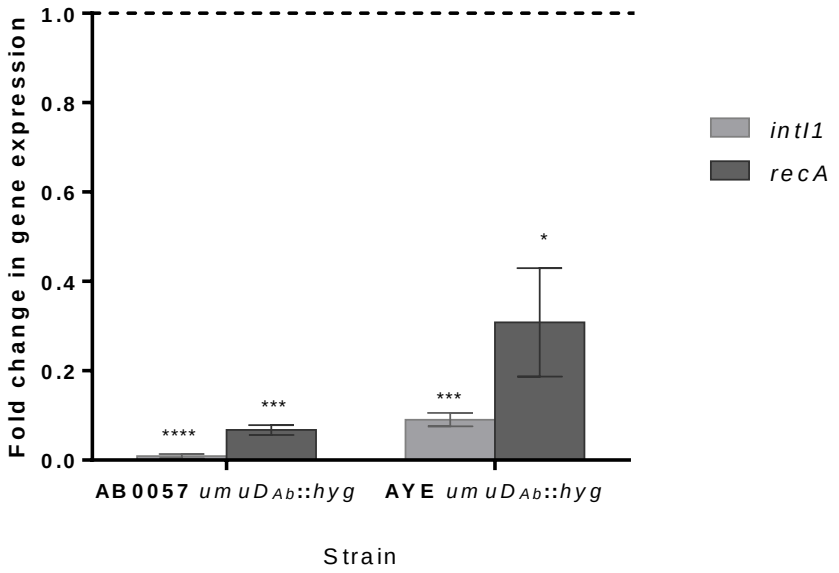


Gel images showing the results of PCR assays conducted to confirm successful allelic exchange events. Schematics of the expected sizes for PCR products of all the PCRs are shown below the gel pictures, with primers indicated by black arrows, FRT sites by pink boxes, palindromic DNA binding region by black boxes and the flanking regions involved in the allelic exchange indicated by dashed vertical lines.

5.2.4. DNA-damaging conditions result in down-regulation of *recA* and *intI1* in Δ*umuD*$_{Ab}$ mutants

Once the mutants were detected, they were also exposed to the same DNA-damaging conditions using mitomycin C, to analyse whether there were any changes to *intI1* or *recA* expression. It can be seen that after exposure to DNA damaging agents, both *intI1* and *recA* are down-regulated in both strains.

Figure 5.5 Changes in relative gene expression after exposure of Δ*umuD*$_{Ab}$ mutants to DNA-damaging conditions



Changes in gene expression of *intI1* and *recA* after induction of DNA-damaging conditions (4 hours of exposure to 6 µg/ml mitomycin C) in Δ*umuD*$_{Ab}$ mutants of both AB0057 and AYE. Bar graph shows the mean and error bars are SEM from three independent replicates. In the case of each gene, the baseline of 1 (dashed line) is taken as the normal expression of that gene under non-DNA damaging conditions. Significance calculated through unpaired Student's *t*-test using 1.0 – dotted line – as comparator; NS = not significant: *P* >0.05; **: *P* <0.01; ***: *P* <0.001.

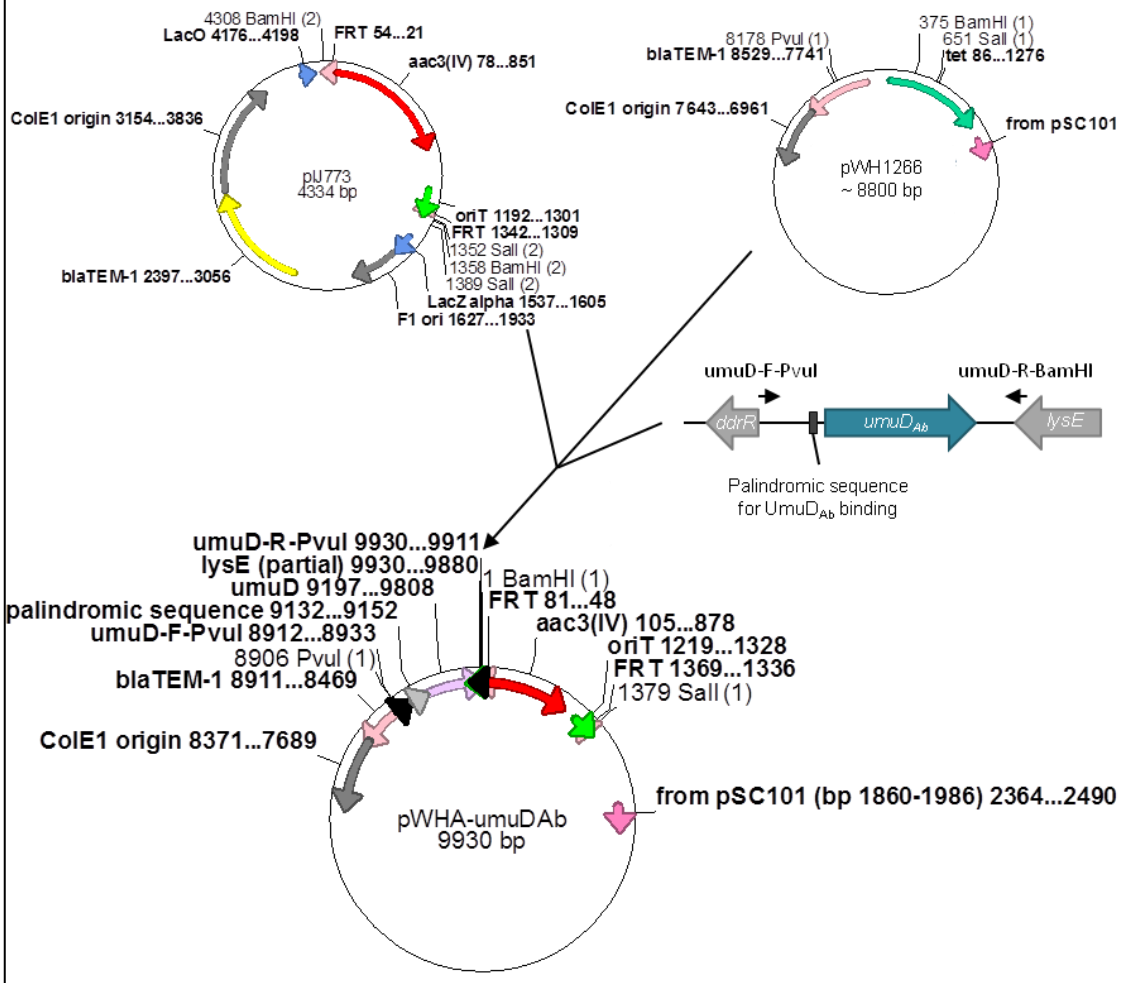
This is an unusual result because a study by Aranda, et al. (2013) showed that $UmuD_{Ab}$ was not required for expression of RecA, and that $UmuD_{Ab}$ did not bind to the region upstream of *recA*. However, coupled with the results from the wild-type strains, where $UmuD_{Ab}$ is still present and *recA* expression was upregulated at roughly four-fold under DNA-damaging

conditions, this data may suggest that $UmuD_{Ab}$ may be involved in induction of RecA in another manner under DNA-damaging conditions.

5.2.5. Construction of complementation plasmid and transformation

In order to complement the Δ*umuD*$_{Ab}$ mutants, the gene was reintroduced into the strains under its natural promoter on a plasmid. The backbone of this plasmid was based on the *E. coli-A. baumannii* shuttle plasmid pWH1266 described in the previous chapter. However, since both the *A. baumannii* strains that were mutated in this study were multidrug resistant, the addition of an apramycin gene cassette to this plasmid was required. The 1378 bp BamHI-SalI fragment excised from pIJ773, a section of DNA which contains *aac3-IV*, a gene that confers resistance to apramycin was selected. Furthermore, this fragment also bears an RP4 origin of transfer, facilitating conjugation via *E. coli* S17-1 donor strain (Gust et al. 2003). An amplicon containing the *umuD*$_{Ab}$ gene, 285 bp upstream and 122 bp downstream of the gene was also amplified by PCR from *A. baumannii* AYE using primers umuD-F-PvuI/umuD-R-BamHI (3409/3474). This region also contained the palindromic sequence upstream of *umuD*$_{Ab}$, required for DNA binding of $UmuD_{Ab}$, as identified by Aranda, et al (2013). No nucleotides from the start of *ddrR* were included in this amplicon, and although promoter or ribosome binding sequences were not found in the intergenic 285 bp region through bioinformatics software, it was assumed that these structures would be contained within the upstream region of the gene (see Figure 5.6). Moreover, the amplified PCR product is a region that has an identical sequence in both *A. baumannii* strains AB0057 and AYE, which were used in this study. The PCR product was digested with PvuI and BamHI, and the pWH1266 plasmid was also digested with PvuI and SalI. These sections of DNA were then ligated in a three-way reaction with the BamHI-SalI digestion fragment from pIJ773 to produce pWHA-$umuD_{Ab}$, estimated to be a 9.9 kb plasmid. A schematic of the construction of this plasmid is shown in Figure 5.6.

Figure 5.6 Diagram showing the construction of pWHA-umuD$_{Ab}$



Schematic representation of the elements used to construct pWHA-umuD$_{Ab}$, a plasmid used for complementation of the *umuD$_{Ab}$* gene in Δ*umuD$_{Ab}$* mutants.

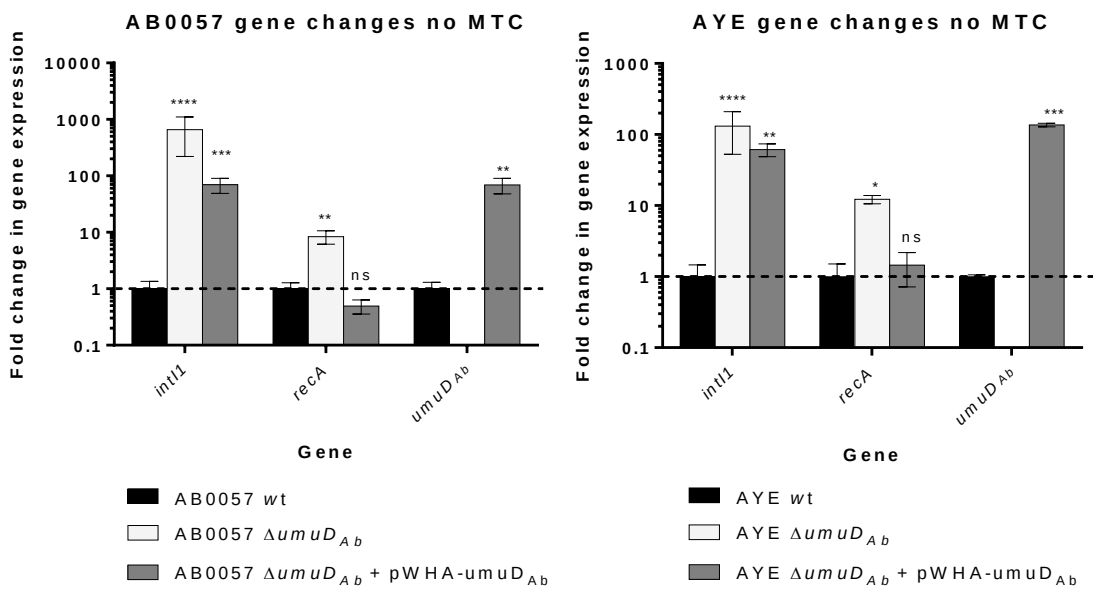
The plasmid was transferred to both strains via conjugation, with triparental mating using an *E. coli* strain with the helper plasmid pRK2013. Successful transconjugants were selected for using medium containing apramycin at 60 µg/ml and were confirmed to contain the plasmid by PCR of the origin of replication for pWH1266 (pWHoriR-F-BamHI/pWHoriR-R-BamHI; PR3136/PR3137).

5.2.6. Deletion of *umuD$_{Ab}$* results in increased expression of *intI1*

To assess the impact UmuD$_{Ab}$ may have on integrase activity, a comparison of changes in gene expression between the wild-type, mutant and

complemented strains for both AB0057 and AYE were analysed though qPCR. The relative expression of *intI1* and also DNA-damage related *recA* were analysed in each case, and normalised to the wild type levels, which were taken as 1 for each gene (Figure 5.7). These experiments were completed under identical conditions as the previous experiments, in order to establish what effect the absence of *umuD$_{Ab}$* has, whilst keeping all other variables the same. It is evident from these results that without *umuD$_{Ab}$* the expression of *intI1* dramatically increases. Paradoxically, overexpression of *umuD$_{Ab}$* from the pWHA-umuD$_{Ab}$ plasmid also results in an upregulation of the *intI1* expression, compared to wild-type levels, albeit to a lesser extent.

Figure 5.7 Changes in relative gene expression after deletion and complementation of *umuD$_{Ab}$*



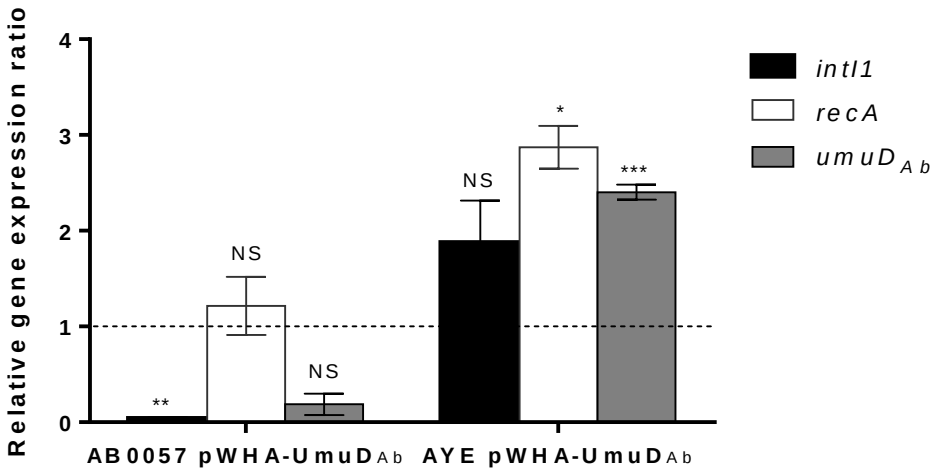
Graphs showing the effects of changes in *intI1*, *recA* and *umuD$_{Ab}$* after deletion of the *umuD$_{Ab}$*, and complementation of UmuD$_{Ab}$ using the pWHA-umuD$_{Ab}$ plasmid. RNA extraction was completed 5 hours after subculture of overnight culture. Bar graph shows the mean and SEM from the three independent replicates. In the case of each gene, the baseline of 1 (dashed line) is taken as the normal expression of that gene under normal conditions of the wild type. Significance calculated through unpaired Student's *t*-test using 1.0 – dotted line – as comparator; NS – not significant: *P* >0.05; *: *P* <0.05; **: *P* <0.01; ***: *P* <0.001.

Despite the high levels of detected integrase expression, the excision of gene cassettes from both integrons (*aacC1* and *aadA1*) were still not detectable from either mutant, under normal or DNA-damaging conditions (data not

shown). This could be that since each gene only has one copy per cell, that the excision rate was still not occurring at a high enough frequency to be detectable by conventional qPCR methods.

In the absence of UmuD$_{Ab}$, it can also be seen that *recA* expression is upregulated in both strains. Since UmuD$_{Ab}$ and RecA are both likely to be involved in stress-response in *A. baumannii*, it could be that *recA* is upregulated in order to compensate for the lack of UmuD$_{Ab}$. Following complementation by pWHA-umuD$_{Ab}$, the levels of *recA* return to similar amounts as expressed in the wild-type strains.

Figure 5.8 Changes in relative gene expression after DNA-damaging conditions during *umuD$_{Ab}$* overexpression



Graphs showing the effects of changes in *intI1*, *recA* and *umuD$_{Ab}$* after overexpression of *umuD$_{Ab}$* from the pWHA-umuD$_{Ab}$ plasmid, during DNA-damaging conditions, induced by 4 hours of exposure to 6 µg/ml mitomycin C. Bar graph shows the mean and SEM from the three independent replicates. In the case of each gene, the baseline of 1 is taken as the normal expression of that gene under normal conditions of the wild type. Significance calculated through unpaired Student's *t*-test using 1.0 – dotted line – as comparator; NS = not significant: *P* >0.05; **: *P* <0.01; ***: *P* <0.001.

The amount of change for all the genes after the knocking out of *umuD$_{Ab}$* follows similar trends between the two strains, which illustrates that UmuD$_{Ab}$ has comparable effects in AB0057 and AYE. However, following the addition of mitomycin C and the induction of DNA-damaging conditions showed differing results in the two strains. It was shown that whilst in AB0057 pWHA-umuD$_{Ab}$, the *intI1* was significantly down-regulated following addition of mitomycin C, in

AYE pWHA-$umuD_{Ab}$ the change was not significant from the mitomycin C-free conditions. The changes in expression of *recA* and *$umuD_{Ab}$* also do not correlate between the two strains, but do correlate with their respective wild-type expression patterns. However, the relative changes are either not significant, or a low factor in all cases.

In conclusion, $umuD_{Ab}$ is shown to be exhibiting some features that are to be expected of a LexA homologue, but conversely also showing the reverse. Whilst $UmuD_{Ab}$ is clearly involved in DNA-damage response of *A. baumannii*, its exact role remains unclear.

5.3. Discussion

Bacterial stress responses are a vital part of the armoury for prokaryotes to modulate their gene expression to cope with unfavourable environments. These pathways, such as the SOS response, which originally were thought to be mainly linked to DNA damage repair, have been recently linked to increasing activity and movement of mobile genetic elements, such as integrons or ICEs (Guerin et al. 2009, Beaber, Hochhut & Waldor 2004), via the action of global regulator LexA. Since the *Acinetobacter* genus lacks a direct LexA homologue, a regulatory pathway for integrons has not yet been identified in this species. $UmuD_{Ab}$ has been investigated as a possible homologue, and studies have outlined its role in UV-mediated DNA damage (Aranda et al. 2014, Hare et al. 2012b).

Integron activity can be detected through the presence of circular gene cassettes, which are only produced by the site-specific recombination of the integrase (outlined in previous chapter). However, in wild-type conditions, this activity happens at too low a frequency for ease of detection, and consequently, nested PCR or other more sensitive methods are required (Hocquet et al. 2012). The amplification of gene cassettes using such methods proved to be problematic for wild-type *A. baumannii* strains, even under known integrase-inducing conditions (Guerin et al. 2009, Hocquet et al. 2012). Despite multiple attempts with varying decontamination and cleaning procedures at every step of the nested PCR, no true amplicons could be detected that matched to the

expected sequence of circular gene cassettes. Therefore, this chapter aimed to investigate whether *de novo* integrase activity was occurring in *A. baumannii*, through gene expression analysis on whether the integrase expression was functioning and at what level, and whether its expression could be affected by DNA-damaging conditions, or through the deletion of a putative repressor, $UmuD_{Ab}$.

5.3.1. The increase in expression of *$umuD_{Ab}$* and *recA* in response to DNA-damaging conditions does not increase to the same levels as *umuDC* and *recA* in *E. coli*

The increase in expression of *$umuD_{Ab}$* in response to DNA-damaging conditions was variable between the strains. Hare, et al. (2006) previously reported that there was no change in *$umuD_{Ab}$* expression in *A. baylyi* under DNA-damaging conditions. In this study, although there were two cases where significant change was detected, the overall change was less than three-fold on average in ACICU and four-fold in AYE. This is still relatively low when compared to levels reported for *E. coli*, which can show upregulation of 15- to 30-fold of the *umuDC* operon (Friedberg, Walker & Siede 1995). This could be suggestive that either the DNA-damage response isn't as strong in *A. baumannii*, or that $UmuD_{Ab}$ is not functioning in an identical manner to its *E. coli* counterpart.

Moreover, other studies on *$umuD_{Ab}$* in *A. baylyi* ADP1 have also concluded that expression of *$umuD_{Ab}$* is not dependent on DNA-damage or RecA (Hare, Perkins & Gregg-Jolly 2006).

The four-fold increase in *recA* expression could be significant enough to trigger the increased expression of enzymes involved in DNA damage repair pathway, of which RecA is normally the first responder. However, the overall level of *recA* induction is still significantly lower, compared to levels seen in *E. coli*, where *recA* can be upregulated up to 17-fold (Casaregola, D'Ari & Huisman 1982).

The changes in the expression of *$umuD_{Ab}$* differ between the strains, with a significant increase in AYE and ACICU, but no significant change in AB0057. This is unexpected because AB0057 and AYE share a 100 % identity in the *$umuD_{Ab}$* gene and upstream and downstream regions, whilst ACICU has a 98 % identity. However, it highlights the challenge of exploring this mechanism in the face of strain-to-strain variation. It could be that the DNA-damage response in *A. baumannii* involves a complex web of several other genes, and, as outlined in the introduction, the number of some genes such as *umuC* and *$umuD_{Ab}$* can vary between strains. Differences in features such as these may account for some variations.

The upregulation of *lexA* expression levels detected in *P. aeruginosa* by Hocquet, et al. (2012) after induction with 2 µg/ml mitomycin C for 1.5 hours shows similar levels of change as that seen in *$umuD_{Ab}$* in AYE and ACICU. However, this upregulation of *$umuD_{Ab}$* might also be expected if the protein was acting as part of an error-prone polymerase as UmuD in *E. coli*, since mitomycin C causes DNA damage. Therefore, these results are not conclusive in establishing the exact role of $UmuD_{Ab}$.

5.3.2. pWH1266-based plasmids can facilitate gene complementation in MDR *A. baumannii* strains carrying natural plasmids

Following standard protocols for suicide-vector based allelic exchange and replacement, two *$umuD_{Ab}$* mutants were created in strains AB0057 and AYE. Since both of these strains are multi-drug resistant isolates, and the hygromycin resistance gene had already been added as part of the allelic exchange procedure, there were limited options for the choice of antibiotics for the selection of the complemented strains. The use of theveterinary antibiotic apramycin facilitated the selection of a complementation plasmid. Prior to this study, pWH1266-based plasmids had been used in *A. baumannii* strains ATCC 17978, a range of nosocomial isolates and also *A. nosocomialis* M2 (Aranda et al. 2010, Wilharm et al. 2013, Saroj et al. 2012). This study shows that pWH1266 is also compatible with AB0057 and AYE, despite these strains already carrying one and four plasmids, respectively. The addition of an *oriT*

also means the plasmid can be transferred by conjugation, and electroporation was also attempted in AYE, although the efficiency was relatively low (~2 x 10^5 CFU/ml; not repeated).

5.3.3. Knockout of *$umuD_{Ab}$* in *A. baumannii* clinical isolates significantly increases *intI1* expression, but has minimal impact on *recA* expression

The effects on gene expression following the knockout of $UmuD_{Ab}$ from the cell system yielded some interesting results. The significantly large increase in *intI1* expression would be consistent with the theory that $UmuD_{Ab}$ was functioning as a repressor of the integrase gene. However, since the complementation of $UmuD_{Ab}$ also results in upregulation of the integrase gene, this result is inconclusive. In addition, under DNA-damaging conditions, *intI1* expression is actually down-regulated in the absence of *$umuD_{Ab}$*. It is possible that $UmuD_{Ab}$ is likely to be involved in a cascade of different genes that are activated during DNA-damaging conditions. These as-yet unidentified genes may function in such a way that their action in turn could result in the increased expression of *intI1*.

The increase in expression of *recA* in the Δ*$umuD_{Ab}$* mutant also suggests that the two proteins may interact in some way. If $UmuD_{Ab}$ was functioning as an error prone DNA polymerase, it could be that any stalled replication forks were not bypassed by $UmuD_{Ab}$, and that a greater amount of RecA was required in order to compensate and resolve any DNA damage (although these results were taken under non-DNA damaging conditions, so the amount of DNA damage expected would be relatively low). After the reintroduction and overexpression of $UmuD_{Ab}$ through pWHA-$umuD_{Ab}$, the levels of *recA* returned to levels not significantly different to the wild-type.

Since $UmuD_{Ab}$ was overexpressed at a level hundred-fold above normal, the cells ability to cope with DNA-damage may already have been at an optimal level; therefore little change in other stress-response genes would be required. Conversely, if $UmuD_{Ab}$ was acting in a manner homologous to LexA, it might be expected that during DNA-damaging conditions, a greater amount of RecA

would be required for activation of all of the $UmuD_{Ab}$. However, it is known that $UmuD_{Ab}$ does not require RecA for expression, unlike LexA (Norton, Spilkia & Godoy 2013). RecA is also not known to be activated by $UmuD_{Ab}$, and has been shown not to bind with the region upstream of *recA* (Aranda et al. 2013). However, the results shown in this study suggest that under DNA-damaging conditions when *$umuD_{Ab}$* is overepxressed, *recA* expression is downregulated.

It could be argued that the above information is not concordant with the expected behaviour of LexA, and that therefore $UmuD_{Ab}$ cannot be acting as a direct homologue. However, this doesn't explain why the frequency of integrase activity in wild-type *A. baumannii* strains would be below detectable levels. In the absence of a repressor, constitutive expression of a gene might be expected, yet this is not seen. It could be either that *A. baumannii* has an alternative, unknown method by which integrases can be respressed, or that conditions are not optimal for integrase expression.

The role of $UmuD_{Ab}$ has been investigated by a number of studies in recent years (Hare, Perkins & Gregg-Jolly 2006, Aranda et al. 2014, Hare et al. 2012a). Aranda et al. (2014) showed that whilst the absence of $UmuD_{Ab}$ did not seem to affect the growth rate of strains or their ability to cope with UV-induced DNA damage, the strains were less prone to spontaneous mutations that occur naturally in many bacteria to confer resistance to rifampicin or streptomycin. Specifically, they found that the strains deficient in *$umuD_{Ab}$* had a lower frequency of transition mutations, which is the specific type of mutation created by the *E. coli umuDC* complex (Reuven et al. 1999). This would suggest that $UmuD_{Ab}$ either functions as an error-prone DNA polymerase, or that it regulates the expression of another such enzyme.

Aranda et al. (2013) had shown that $UmuD_{Ab}$ could bind to a palindromic sequence upstream of its own gene sequence, and that of other $UmuD_{Ab}$ and UmuC homologues in the genome, suggesting that $UmuD_{Ab}$ was involved in the regulation of other genes. In addition, they found varying effects of *$umuD_{Ab}$* knock out on these genes, with some being upregulated, and others downregulated. This suggests that $UmuD_{Ab}$ could have dual-regulatory effects. Dual-regulatory effects have also previously been found in the LexA of

Rhodobacter sphaeroides, therefore, Aranda et al. concluded that this feature is another way in which $UmuD_{Ab}$ could function as a LexA homologue. However, these results may not apply to all *A. baumannii* strains, for example, AYE, AB0057 and ACICU do not have a *umuC* gene immediately downstream of *$umuD_{Ab}$*, but only one copy at another location on the genome. This may mean that the DNA damage response could also have strain-to-strain variation.

Although $UmuD_{Ab}$ binds to an identified palindromic sequence, there is no similarity between this sequence and that of the known LexA-binding box which is identifiable in many of the *A. baumannii*-carried integrons. The binding of $UmuD_{Ab}$ was found to be very specific to this palindromic sequence, and that a perfect symmetrical sequence was required for the binding of the protein (Aranda et al. 2013). Furthermore, $UmuD_{Ab}$ was not able to complement LexA-deficient *E. coli* strains (Norton, Spilkia & Godoy 2013). This means that although $UmuD_{Ab}$ has some DNA-binding activity, it is unlikely to be active in integron regulation.

5.3.4. Ambiguous role of $UmuD_{Ab}$ further supports atypical DNA-damage response in *A. baumannii*, possibly involving a competence regulon

Although members of the *Acinetobacter* genus code for a *umuD*-type gene that shares some similarity to that in *E. coli*, it is known that the DNA-damage response in *A. baumannii* differs significantly to other Gram-negatives. In addition to LexA, *Acinetobacter spp.* also lack other SOS response-associated enzymes, such as the error-prone DNA polymerase II (PolB or DinB), the stress response factor σ^{38}, in addition to cell division inhibitor SulA, and transporters FtsE and FtsX, which are normally upregulated during the classical SOS response (Robinson et al. 2010). These enzymes are conserved in the majority of all other Gammaproteobacteria, which demonstrates how *Acinetobacter* spp. is a unique genus within this group.

The four DNA-damage response genes that have been formally identified in *Acinetobacter* spp. include UmuC (truncated in comparison to homologues in *E. coli;* (Hare, Perkins & Gregg-Jolly 2006), $UmuD_{Ab}$ (still currently being investigated), DdrR (function still currently unknown, but strong

upregulation occurs during DNA-damage) and RecA (functionality similar to that of RecA in other Gram-negative bacteria, but lacks an identifiable SOS-box, and also the gene does not require the RecA protein for expression, unlike in other Gram-negatives, (Rauch et al. 1996, Gregg-Jolly, Ornston 1994). However, the microarray data from Aranda, et al. (2013) shows that 35 other genes were upregulated after exposure of ATCC 17978 to 0.5 µg/ml mitomycin C for two hours. Many of these genes have unknown function, and could therefore be part of an entirely new mechanism against DNA-damage.

It is evident to see from the results of both this study and others (Aranda et al. 2011, Norton, Spilkia & Godoy 2013, Aranda et al. 2013) that *A. baumannii* has an atypical stress response system, and that it should not be placed under the same umbrella as the other, common Gram-negative nosocomial pathogens. However, even in LexA-negative species, there are alternative genetic modulative mechanisms that facilitate bacteria to cope with DNA-damaging conditions. In contrast to the classical SOS response, which involves the upregulation of mutagenic enzymes, other LexA-negative species such as *Neisseria gonorrhoea*, *Legionella pneumophila* and *Streptococcus pneumoniae* create genetic diversity to cope with DNA damage by upregulating competence mechanisms in order to take up DNA from the environment (Charpentier et al. 2011, Prudhomme et al. 2006). These DNA fragments can then be recombined into the cell's genome to create variation. This is a logical response in an environment with a population with a high number of the same or a similar genetic population, since the uptake of sections of intact homologous DNA would facilitate the repair of lesions by homologous recombination, which can be facilitated by RecA. However, it also means that in mixed populations, foreign DNA from other species may also be taken up (Charpentier, Polard & Claverys 2012).

If *A. baumannii* were to have such a response to DNA damage, it could explain the rapid rise of this species as a major human pathogen, due to its swift acquisition of multi-drug resistance determinants. It could be that this uptake occurred very quickly following the entry of *A. baumannii* strains into hospital environments, where it would have come into contact with a variety of other Gram-negative pathogens, whilst simultaneously being exposed to sub-

inhibitory concentrations of antibiotics. However, analysis of microarray data from Aranda, et al. (2013) did not shown the upregulation of any known competence genes in ATCC 17978 after exposure to 0.5 µg/ml mitomycin C for two hours. It could be that the concentration or the exposure time of the drug was not optimal, since Charpentier et al. (2011) showed that the highest upregulation of competence genes in *L. pneumophila* occurred between 0.02 and 0.05 µg/ml mitomycin C. A range of concentrations and times could be tested on *A. baumannii* in order to establish whether a similar mechanism is in action for this species. It is also interesting to note that *L. pneumophila* has a gene with closer homology to $umuD_{Ab}$ than the *E. coli umuD* (Hare, Perkins & Gregg-Jolly 2006), suggesting that they may have similar DNA-damage response pathways, and meaning that some parallels could possibly be drawn between the two species.

Conversely, general reports have shown that many *A. baumannii* strains have low competence levels, with only a few exceptions (Ramirez et al. 2010). The most widely used naturally competent *Acinetobacter* species used are *A. baylyi* ADP1 (Vaneechoutte et al. 2006, Starikova et al. 2012, Hare, Perkins & Gregg-Jolly 2006) and *A. baumannii* M2 (Saroj et al. 2012, Gaddy, Actis 2009, Niu et al. 2008), the latter of which was subsequently redesignated to be *A. nosocomialis* (Philip Rather, personal communication). Previously, strains of *A. calcoaceticus* have also been used in natural transformation experiments (Rauch et al. 1996). The fact that the close relatives of *A. baumannii* are naturally competent, and that *A. baumannii* genomes also have a range of competence related genes, suggests that *A. baumannii* itselt was also naturally competent but may have recently lost this ability. Some evidence shows that *A. baumannii* DNA uptake is dependent on activation of motility mechanisms (T4P; (Wilharm et al. 2013), and other studies have demonstrated that competence is only an adaptive trait during active growth and early stationary phase (Utnes, et al. 2015). It may also be due to the fact that many *A. baumannii* clinical isolates have a genomic island or Tn*AbaR* element inserted into *comM* gene (Shaikh et al. 2009, Fournier et al. 2006), a gene which is thought to be involved in the competence regulon. It could be that this insertion is due to “selfish gene” activity, where an inserted segment of DNA can create a more genetically

stable environment in its recipient, by disabling the host's ability to uptake o incorporate foreign DNA. Further investigation of the *A. baumannii comM* gene is required in order to establish whether this could be the cause of the low competence in this species.

In addition, closer analysis of the other 35 genes found to be upregulated during DNA-damaging conditions by Aranda, et al. (2013), would help to establish exactly what kind of response mechanism is operational in *A. baumannii*. This thesis confirms the current knowledge of an atypical DNA-damage system in this species, and with further investigations, could lead on to discovering a potentially novel response mechanism.

The understanding of these kinds of stress responses is vital in learning how to handle and prevent the growing pandemic of MDR pathogens. The available evidence suggests that the use of sterilizing agents in hospitals such as alcohol (Edwards, Patel & Wareham 2007), UV light (Riley, Nardell 1989), disinfectants (McDonnell, Russell 1999, Aiello, Larson & Levy 2007) and antibiotics (Beaber, Hochhut & Waldor 2004, Guerin et al. 2009) actually promote persistence and mutagenesis of strains, as well as horizontal gene transfer, if not completed appropriately. An alternative pathway of treating infectious species whilst simultaneously attempting to prevent the encouragement of such antibiotic resistance has been put forward, by exploring the possibility of using RecA inhibitors alongside conventional therapies. Recent research has found potential candidates, some of which have been patented (Wigle et al. 2009, Sexton et al. 2010). By targeting RecA, this kind of therapy would allow the DNA-damaging agents to reach their full potential, through preventing bacterial recovery and adaptation following induction of their DNA-damage response mechanisms (Cirz et al. 2005, Lee et al. 2005). This is because RecA is involved in both the classical SOS-response as well as the induction of competence (Charpentier, Polard & Claverys 2012, Rauch et al. 1996). Some have questioned the use of such agents, since RecA is such a vital part of bacterial cell functioning, suggesting that resistance would quickly develop. However, the development of these RecA inhibitors in combination with other therapies may help to retain bacterial susceptibility to antibiotics and also reduce the selection of persister or resistant cells.

It is clear that the *A. baumannii* response to rectify DNA-damage is drastically different to other Gram-negative pathogens, and that this impacts on the maintenance, structure and evolution of acquired genetic elements. This study contributes to the growing knowledge regarding $UmuD_{Ab}$, and in time will help to establish the exact role of this protein in the *A. baumannii* DNA-damage response.

5.4. Future work

Although this chapter has made progress into the analysis of the role of $UmuD_{Ab}$ in integron activity, there are still further questions to be answered. Although some effects of this protein on *intI1* expression have been identified, it is still not known whether $UmuD_{Ab}$ can bind to the region upstream of *intI1*, and further questions regarding the DNA-damage response in *A. baumannii*. Methods for investigating these queries are outlined below.

1. Establish whether the MIC of strains are similar through disc diffusion assay.

2. Conduct EMSA experiments to determine whether $UmuD_{Ab}$ can bind to the LexA box in the 5' region of integron.

3. Ideally the experiments outlined in this chapter should also be repeated with other known DNA-damaging agents, such as ciprofloxacin or ultraviolet light, in order to test whether different sources and types of DNA damage elicit the same response.

4. Investigation of *A. baumannii* competence regulon, and induction of competence with different levels of exposure of different DNA-damaging agents, and comparison to currently known naturally competent *A. baylyi* or *A. nosocomialis* strains.

5. Convert pQE-WH-bla into an apramycin-resistant version, and then transform into the $\Delta umuD_{Ab}$ mutants. Compare the mutants' ability to cope with DNA damage compared with the wild-type strains (through growth curves as in Chapter 4). If $UmuD_{Ab}$ was functioning as an error-

prone polymerase, significant growth inhibition would be expected in the mutants.

6. Conduct a pull-down assay, in which the DNA sequence of the integrase promoter could be fixed to magnetic beads, and then the cell lysate of A. baumannii containing all the proteins of the cell can be passed through, so establish which, if any of the proteins could be binding to regulate integrase activity. The bound protein can then be eluted and analysed by western blotting or mass spectrometry.

Chapter 6. General Discussion and Final Conclusions

This thesis has examined in detail the features and genetic context of a particularly common class of mobile elements, integrons, and their contribution towards multi-drug resistance in *A. baumannii.* Integrons are small, semi-mobile genetic elements that have the ability to capture and selectively express different genes coding for antibiotic resistance, and can be easily spread between Gram-negative pathogens by HGT. Whilst it is clear that dissemination of such MGEs are encouraged by the widespread use of antibiotics, analysis of their variability and genetic location in this thesis suggested that in *A. baumannii*, integrons are under different selective pressures. This species has an atypical DNA-damage compared to other Gram-negative pathogens, from which it originally acquired integrons. As a result of a lack of regulation and adaptation to this new mobile genetic element means that inactivation of the integrase gene can confer a selective advantage. The current hypothesis is that since the integrase is a DNA recombinase enzyme, it could have deleterious effects if left unregulated. Using a combination of epidemiological survey on a substantial set of clinical isolates as well as experimental data to confirm this fitness cost, this study has given an insight into how horizontally acquired mobile genetic elements may change over time after acquisition into a new environment. By closely analysing the gene cassette array types of integrons carried, their structure and location on the genome, it was evident that irregular configurations conferring advantageous features to *A. baumannii* have become significantly more prevalent in the clinical isolate gene pool for this species than comparable Gram-negative pathogens. This study also highlighted the flaws of current class 1 integron-targeted PCR assays, furthermore, new tools for studying integrase activity in a range of strain backgrounds were created.

Detection of integron types through PCR mapping, chromosome walking and Southern blotting yielded a total of 68 class 1 integron structures in 58 isolates. Using these multiple detection methods allowed the discovery of 29 (40.3 %) integrons which are either incomplete, or have an *intI1* truncation. A

significant number of these disruptions were due to IS elements-mediated insertions or deletions. An in-del event caused by IS*26* was the cause for integrase truncations in many of the examples observed in this study, including all integrases located on *A. baumannii* plasmids. For those strains that do contain functioning integrases, they show a disproportionately large frequency of *intI1* alleles that give the lowest resolving efficiency, but the maximal strength P_c promoter for gene cassette expression in comparison to a cross-species analysis of integrases. Such a high frequency of irregular structures and a specific preference for promoter is evidence to suggest negative selection in action in current strains isolated from patients in local as well as European *A. baumannii* strains, and shows how loss of function in the integrase is likely conferring an adaptive trait. This case also highlights the important role that mobile genetic elements (such as IS in this case) have on shaping each other's structure and function.

It was also evident from these data that established PCR-based methods for detecting integrons would be liable to give high rates of both false-negative and false-positive results regarding integron presence or activity. A new set of *intI1*-targeting PCR primers outlined in chapter 3 give an alternative PCR assay to encompass a larger section of the integrase gene, which would only give a positive result when all the regions coding for the IntI1 functioning domains would be present. Through this new amplicon, the detection of false positive integrons which actually contain non-functional integrases may be circumvented. In addition, this thesis demonstrated through the use of Southern blotting that many incomplete integrons are being undetected by conventional 5'CS – 3'CS PCR assays. These undiscovered elements may still contribute to multidrug resistance phenotypes.

As the cost of whole genome sequencing continues to decrease, these problems would become easier to avoid, and the full picture of all integron types carried by different strains will become easier to determine. But presently, this study highlights the importance of using a combination of methods, such as Southern blotting and chromosome walking, which reveals significantly more information on integrons than PCR assays alone.

The high frequency of integrons with 5' end truncations in *A. baumannii* clinical isolates was consistent with a recently discussed theory regarding a fitness cost of IntI1 (Cambray et al. 2011, Harms, Starikova & Johnsen 2013). Based on current knowledge, the most biologically plausible explanation is that the integrase causes DNA lesions or causes lethal deletions of segments of the chromosome through illegitimate recombination at non-canonical sites. Previous studies have shown that negative selection of IntI1 is occurring in *A. baylyi* ADP1, a model organism closely related to *A. baumannii*, which could be due to this deleterious effect (Harms, Starikova & Johnsen 2013, Starikova et al. 2012).

Growth curves of strains with and without an overexpressed intI1 gene were used in this thesis to investigate this fitness cost in *A. baumannii*. The fitness cost experiments were completed using a novel *A. baumannii*-compatible plasmid bearing an integrase under the control of P_{lac}, which allowed the induction of the integrase easily by IPTG, in a concentration-dependent manner. The plasmid also contained a reporter cassette, the circular form of which could be easily detected by PCR, in order to quanitfy the extent of integrase activity. The integrase was shown to be biologically active in both an *A. baumannii* and *E. coli* strain. It was evident that strains carrying an overexpressed *intI1* had significant growth rate impairment, if they lacked *lexA* or *recA*. This could be indicative of illegitimate recombination events mediated by the integrase, since normal DNA damage repair pathways could not be activated, either from RecA or LexA. In addition, since RecA is required for LexA activation, any *recA* mutant strain would also be unable to activate LexA mediated repair pathways.

Furthermore, the *A. baumannii* genome was calculated to have roughly 1.5-fold higher number of alternative non-canonical recognition sites (Harms, Starikova & Johnsen 2013), which could explain the greater fitness cost observed in the *A. baumannii* clinical isolate compared to the equivalent *E. coli* strain tested.The findings in this thesis confirm that overexpression of the integrase gene can create a fitness cost.

These plasmids show how it is possible to overexpress integrases in a controlled manner, in order to investigate integrase activity or fitness cost. Through modification of these plasmids to contain appropriate antibiotic resistance genes (such as those conferring resistance against hygromycin or apramycin), this system may facilitate the investigation of integrase activity in a wider range of other Gram-negative pathogens including some MDR strains. Furthermore, by placing the integrase even under its natural promoter on a plasmid, analysis of integrase activity under the natural conditions could be facilitated. Since by placing the integrase on a plasmid, it would be present at a higher copy number, the necessity for nested PCR and all its shortcomings could be circumvented.

Preliminary qPCR assays looking at integron activity in wild-type *A. baumannii* clinical isolates showed that IntI1-mediated gene cassette excision was occurring at a frequency lower than detectable after 40 cycles of qPCR (< 1.1×10^{-9} per cell). Since the previous studies suggested that *A. baumannii* was capable of expressing a biologically functional integrase, it was concluded that this lack of detectable integrase activity was due to absence of appropriate conditions that result in the expression of *intI1*. Since other studies have shown that DNA-damaging conditions trigger the classical “SOS response”, which in turn activates the expression of a range of genes, including *intI1* (Guerin et al. 2011, Hocquet et al. 2012), *A. baumannii* clinical isolates were also exposed to these conditions, to test whether increase in integrase activity could be induced. Whilst quantitative real-time PCR analysis showed that although there was some level of *intI1* upregulation, none of the three strains tested had any significant change in *intI1* levels after 4 hours of exposure to DNA-damaging conditions (6 µg/ml mitomycin C).

The low level of integrase activity under natural conditions may be explained if *A. baumannii* had a homologous repressor of integrases, LexA, which is found in most other Gram-negative pathogens. The closest match for this protein is $UmuD_{Ab}$, an enigmatic protein that shares some features with LexA and some with *E. coli* UmuD. $UmuD_{Ab}$ was investigated through knock out studies, and development of a conjugable plasmid that can be maintained within MDR *A. baumannii* strains for complementation. Whilst the absence of

$UmuD_{Ab}$ through mutational deletion does result in a significant upregulation of *intI1,* complementation of the mutant with *umuD*$_{Ab}$ on a low copy number plasmid also results in significant increase in *intI1* levels, albeit to a much lower extent. Since these results do not confirm $UmuD_{Ab}$ as a strict regulator of *intI1* in the way that LexA is, they suggest that integrase activity in *A. baumannii* could be constitutive. This may explain the heavy fitness cost that resulted in such a high number of deletion and truncation events in the 5' region of the integrons in the clinical isolate collection. In combination with future studies, this thesis will help to elucidate the exact role of $UmuD_{Ab}$ in integron activity and the wider DNA-damage response.

$UmuD_{Ab}$ has been shown to bind to a palindromic region upstream of its own gene and a DNA-damage response gene, *ddr* (Aranda et al. 2013). Use of EMSA to establish whether $UmuD_{Ab}$ is capable of binding to the P_{int} region (as LexA does) would determine whether $UmuD_{Ab}$ could have any regulatory effect on *intI1* expression. In addition, conversion of the pQE-WH-bla into an apramycin-resistant version could facilitate its transfer to the Δ*umuD*$_{Ab}$ mutants made in Chapter 5. Analysis of differences in growth curves between wild-type and Δ*umuD*$_{Ab}$ strains' ability to grow with the overexpressed integrase may also yield some further information regarding the role of $UmuD_{Ab}$.

In conclusion, these results demonstrate that integrons function and can change in different ways, depending on the host's genetic background. In particular, this study highlights how the influence of the internal bacterial defence mechanisms may impact on the maintenance, structure and evolution of mobile genetic elements. Moreover, the regions of integrons that cause fitness costs can be easily ameliorated, whilst retaining the advantageous features, as seen in strains with truncated integrases or strong gene cassette promoters. Whilst these results suggest that gene cassettes in the integrons of *A. baumannii* may have less of a propensity to rearrange to more favourable configurations in adaptation to changing environments, they nevertheless contribute to its MDR phenotype, and form a vital part of the armory of *A. baumannii* in its development as a successful pathogen. It is also clear from this study, that there is an alternative DNA-damage response pathway in action in *A. baumannii*, which could possibly include activation of competence pathways.

Therefore, the evidence in this thesis suggests that general use of antibiotics is likely to trigger and continue to stimulate the development and acquisition of antibiotic resistance, regardless of the type of DNA-damage response. Future setting of prescription guidelines should take these findings into account when planning antibiotic use, since restriction of certain classes of DNA-damaging antibiotics may help to slow the rates of SOS response-mediated genetic mobilisation events, as well as reducing uptake of foreign DNA via horizontal gene transfer through induction of competence pathways. By bringing this information to light, this thesis adds to the growing evidence that adherence to antibiotic prescription guidelines world-wide would be the most effective way to combat the global problem of antibiotic resistance. Only through a full understanding of these mechanisms and appropriate responses can we increase the chances of gaining the upper hand with bacteria in this evolutionary arms race.

References

Aarestrup, F.M., Seyfarth, A.M., Emborg, H.D., Pedersen, K., Hendriksen, R.S. & Bager, F. 2001, "Effect of abolishment of the use of antimicrobial agents for growth promotion on occurrence of antimicrobial resistance in fecal enterococci from food animals in Denmark", *Antimicrobial Agents and Chemotherapy,* vol. 45, no. 7, pp. 2054-2059.

Adams, M.D., Goglin, K., Molyneaux, N., Hujer, K.M., Lavender, H., Jamison, J.J., MacDonald, I.J., Martin, K.M., Russo, T. & Campagnari, A.A. 2008, "Comparative genome sequence analysis of multidrug-resistant *Acinetobacter baumannii*", *Journal of Bacteriology,* vol. 190, no. 24, pp. 8053.

Aiello, A.E., Larson, E.L. & Levy, S.B. 2007, "Consumer antibacterial soaps: effective or just risky?", *Clinical infectious diseases,* vol. 45 Suppl 2, pp. S137-47.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. 1990, "Basic local alignment search tool", *Journal of Molecular Biology,* vol. 215, no. 3, pp. 403-410.

Álvarez-Pérez, S., Lievens, B., Jacquemyn, H. & Herrera, C.M. 2013, "*Acinetobacter nectaris* sp. nov. and *Acinetobacter boissieri* sp. nov., two novel bacterial species isolated from floral nectar of wild Mediterranean insect-pollinated plants", *International Journal of Systematic and Evolutionary Microbiology,* vol. 63, no. 4, pp. 1532-1539.

Anandham, R., Weon, H., Kim, S., Kim, Y., Kim, B. & Kwon, S. 2010, "*Acinetobacter brisouii* sp. nov., isolated from a wetland in Korea", *The Journal of Microbiology,* vol. 48, no. 1, pp. 36-39.

Andersson, D.I. 2006, "The biological cost of mutational antibiotic resistance: any practical conclusions?", *Current Opinion in Microbiology,* vol. 9, no. 5, pp. 461-465.

Aranda, J., Bardina, C., Beceiro, A., Rumbo, S., Cabral, M.P., Barbé, J. & Bou, G. 2011, "*Acinetobacter baumannii* RecA protein in repair of DNA damage, antimicrobial resistance, general stress response, and virulence", *Journal of Bacteriology,* vol. 193, no. 15, pp. 3740-3747.

Aranda, J., Poza, M., Pardo, B., Rumbo, S., Rumbo, C., Parreira, J., Rodríguez-Velo, P. & Bou, G. 2010, "A rapid and simple method for constructing stable mutants of *Acinetobacter baumannii*", *BMC Microbiology,* vol. 10, no. 279, pp. 1-12.

Aranda, J., López, M., Leiva, E., Magán, A., Adler, B., Bou, G. & Barbé, J. 2014, "Role of *Acinetobacter baumannii* UmuD homologs in antibiotic resistance acquired through DNA-damage-induced mutagenesis", *Antimicrobial Agents and Chemotherapy,* vol. 58, no. 3, pp. 1771-1773.

Aranda, J., Poza, M., Shingu-Vazquez, M., Cortes, P., Boyce, J.D., Adler, B., Barbe, J. & Bou, G. 2013, "Identification of a DNA-damage-inducible regulon in *Acinetobacter baumannii*", *Journal of Bacteriology,* vol. 195, no. 24, pp. 5577-5582.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, K. (eds) 2003, Vectors Derived from Plasmids *Current Protocols in Molecular Biology*, Chapter 1, Section II.

Barton, B.M., Harding, G.P. & Zuccarelli, A.J. 1995, "A general method for detecting and sizing large plasmids", *Analytical Biochemistry,* vol. 226, no. 2, pp. 235-240.

Bartual, S.G., Seifert, H., Hippler, C., Luzon, M.A.D., Wisplinghoff, H. & Rodríguez-Valera, F. 2005, "Development of a multilocus sequence typing scheme for characterization of clinical isolates of*Acinetobacter baumannii*", *Journal of Clinical Microbiology,* vol. 43, no. 9, pp. 4382-4390.

Baumann, P. 1968, "Isolation of *Acinetobacter* from soil and water", *Journal of Bacteriology,* vol. 96, no. 1, pp. 39-42.

Baumann, P., Doudoroff, M. & Stanier, R. 1968, "A study of the *Moraxella* group II. oxidative-negative species (genus *Acinetobacter*)", *Journal of Bacteriology,* vol. 95, no. 5, pp. 1520-1541.

Beaber, J.W., Hochhut, B. & Waldor, M.K. 2004, "SOS response promotes horizontal dissemination of antibiotic resistance genes", *Nature,* vol. 427, no. 6969, pp. 72-74.

Bennett, P.M. 2004, "Genome plasticity" in *Genomics, Proteomics, and Clinical Bacteriology - Methods and Reviews*, eds. N. Woodford & A.P. Johnson, Springer, , pp. 71-113.

Berlau, J., Aucken, H., Malnick, H. & Pitt, T. 1999, "Distribution of *Acinetobacter* species on skin of healthy humans", *European Journal of Clinical Microbiology and Infectious Diseases,* vol. 18, no. 3, pp. 179-183.

Bikandi, J., San Millán, R., Rementeria, A. & Garaizar, J. 2004, "*In silico*analysis of complete bacterial genomes: PCR, AFLP–PCR and endonuclease restriction", *Bioinformatics,* vol. 20, no. 5, pp. 798-799.

Black, C.G., Fyfe, J.A. & Davies, J.K. 1998, "Absence of an SOS-like system in *Neisseria gonorrhoeae*", *Gene,* vol. 208, no. 1, pp. 61-66.

Blau, H.M. & Rossi, F.M. 1999, "Tet B or not tet B: advances in tetracycline-inducible gene expression", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 96, no. 3, pp. 797-799.

Blot, S., Vandewoude, K. & Colardyn, F. 2003, "Nosocomial bacteremia involving *Acinetobacter baumannii* in critically ill patients: a matched cohort study", *Intensive Care Medicine,* vol. 29, no. 3, pp. 471-475.

Bou, G., Cervero, G., Dominguez, M., Quereda, C. & Martínez-Beltrán, J. 2000, "PCR-based DNA fingerprinting (REP-PCR, AP-PCR) and pulsed-field gel electrophoresis characterization of a nosocomial outbreak caused by imipenem-and meropenem-resistant *Acinetobacter baumannii*", *Clinical Microbiology and Infection,* vol. 6, no. 12, pp. 635-643.

Bou, G. & Martínez-Beltrán, J. 2000, "Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC β-lactamase in *Acinetobacter baumannii*", *Antimicrobial Agents and Chemotherapy,* vol. 44, no. 2, pp. 428-432.

Bouvet, P.J.M. & Grimont, P.A.D. 1986, "Taxonomy of the genus *Acinetobacter* with the recognition of *Acinetobacter baumannii* sp. nov.,*Acinetobacter haemolyticus* sp. nov.,*Acinetobacter johnsonii* sp. nov., and *Acinetobacter junii* sp. nov. and emended descriptions of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*", *International Journal of Systematic Bacteriology,* vol. 36, no. 2, pp. 228.

Brisou, J. & Prévot, A.R. 1954, "Studies on bacterial taxonomy. X. The revision of species under *Acromobacter* group", *Annales de l'Institut Pasteur,* vol. 86, no. 6, pp. 722-728.

Bujard, H., Gentz, R., Lanzer, M., Stueber, D., Mueller, M., Ibrahimi, I., Haeuptle, M. & Dobberstein, B. 1987, "A T5 promoter-based transcription-translation system for the analysis of proteins *in vitro* and *in vivo*", *Methods in Enzymology,* vol. 155, pp. 416-433.

Butala, M., Žgur-Bertok, D. & Busby, S.J. 2009, "The bacterial LexA transcriptional repressor", *Cellular and Molecular Life Sciences,* vol. 66, no. 1, pp. 82-93.

Cain, A.K., Liu, X., Djordjevic, S.P. & Hall, R.M. 2010, "Transposons Related to Tn*1696*in IncHI2 Plasmids in Multiply Antibiotic Resistant *Salmonella enterica* serovar Typhimurium from australian animals", *Microbial Drug Resistance,* vol. 16, no. 3, pp. 197-202.

Camarena, L., Bruno, V., Euskirchen, G., Poggio, S. & Snyder, M. 2010, "Molecular mechanisms of ethanol-induced pathogenesis revealed by RNA-sequencing", *PLoS pathogens,* vol. 6, no. 4, pp. e1000834.

Cambray, G., Sanchez-Alberola, N., Campoy, S., Guerin, É, Da Re, S., González-Zorn, B., Ploy, M.C., Barbé, J., Mazel, D. & Erill, I. 2011, "Prevalence of SOS-mediated control of integron integrase expression as an adaptive trait of chromosomal and mobile integrons", *Mobile DNA,* vol. 2, no. 6, pp. 1-15.

Cambray, G., Guerout, A. & Mazel, D. 2010, "Integrons", *Annual Review of Genetics,* vol. 44, pp. 141-166.

Carr, E.L., Kämpfer, P., Patel, B.K., Gürtler, V. & Seviour, R.J. 2003, "Seven novel species of *Acinetobacter* isolated from activated sludge", *International Journal of Systematic and Evolutionary Microbiology,* vol. 53, no. 4, pp. 953-963.

Carruthers, M.D., Nicholson, P.A., Tracy, E.N. & Munson Jr, R.S. 2013, "*Acinetobacter baumannii* utilizes a type VI secretion system for bacterial competition", *PloS One,* vol. 8, no. 3, pp. e59388.

Casali, N. 2003, "*Escherichia coli* host strains" in *Escherichia coli Plasmid Vectors* Springer, pp. 27-48.

Casaregola, S., D'Ari, R. & Huisman, O. 1982, "Quantitative evaluation of *recA* gene expression in *Escherichia coli*", *Molecular and General Genetics,* vol. 185, no. 3, pp. 430-439.

Catalano, M., Quelle, L., Jeric, P., Di Martino, A. & Maimone, S. 1999, "Survival of *Acinetobacter baumannii* on bed rails during an outbreak and during sporadic cases", *Journal of Hospital Infection,* vol. 42, no. 1, pp. 27-35.

Charlier, D., Piette, J. & Glansdorff, N. 1982, "IS*3* can function as a mobile promoter in *E. coli*", *Nucleic Acids Research,* vol. 10, no. 19, pp. 5935-5948.

Charpentier, X., Kay, E., Schneider, D. & Shuman, H.A. 2011, "Antibiotics and UV radiation induce competence for natural transformation in *Legionella pneumophila*", *Journal of Bacteriology,* vol. 193, no. 5, pp. 1114-1121.

Charpentier, X., Polard, P. & Claverys, J.P. 2012, "Induction of competence for genetic transformation by antibiotics: convergent evolution of stress responses in distant bacterial species lacking SOS?", *Current Opinion in Microbiology,* vol. 15, no. 15, pp. 570-576.

Chen, C., Lin, Y., Sheng, W., Chen, Y., Chang, S., Hsia, K., Liao, M. & Li, S. 2011a, "Genome sequence of a dominant, multidrug-resistant *Acinetobacter baumannii* strain, TCDC-AB0715", *Journal of Bacteriology,* vol. 193, no. 9, pp. 2361-2362.

Chen, Y., Zhou, Z., Jiang, Y. & Yu, Y. 2011b, "Emergence of NDM-1-producing *Acinetobacter baumannii* in China", *Journal of Antimicrobial Chemotherapy,* vol. 66, no. 6, pp. 1255-1259.

Chen, Y.T., Shu, H.Y., Li, L.H., Liao, T.L., Wu, K.M., Shiau, Y.R., Yan, J.J., Su, I.J., Tsai, S.F. & Lauderdale, T.L. 2006, "Complete nucleotide sequence of pK245, a 98-kilobase plasmid conferring quinolone resistance and extended-spectrum-β-lactamase activity in a clinical *Klebsiella pneumoniae* isolate", *Antimicrobial Agents and Chemotherapy,* vol. 50, no. 11, pp. 3861-3866.

Choi, K.H. & Schweizer, H.P. 2006, "mini-Tn*7* insertion in bacteria with single *att*Tn*7* sites: example *Pseudomonas aeruginosa*", *Nature Protocols,* vol. 1, no. 1, pp. 153-161.

Choi, J.Y., Ko, G., Jheong, W., Huys, G., Seifert, H., Dijkshoorn, L. & Ko, K.S. 2013, "*Acinetobacter kookii* sp. nov., isolated from soil", *International Journal of Systematic and Evolutionary Microbiology,* vol. 63, no. 12, pp. 4402-4406.

Cirz, R.T., Chin, J.K., Andes, D.R., de Crécy-Lagard, V., Craig, W.A. & Romesberg, F.E. 2005, "Inhibition of mutation and combating the evolution of antibiotic resistance", *PLoS Biology,* vol. 3, no. 6, pp. e176.

Cisneros, J. & Rodriguez-Bano, J. 2002, "Nosocomial bacteremia due to *Acinetobacter baumannii*: epidemiology, clinical features and treatment", *Clinical Microbiology and Infection,* vol. 8, no. 11, pp. 687-693.

Cisneros, J.M., Reyes, M.J., Pachon, J., Becerril, B., Caballero, F.J., Garcia-Garmendia, J.L., Ortiz, C. & Cobacho, A.R. 1996, "Bacteremia due to *Acinetobacter baumannii*: epidemiology, clinical findings, and prognostic features", *Clinical Infectious Diseases,* vol. 22, no. 6, pp. 1026-1032.

Collis, C.M. & Hall, R.M. 1992a, "Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase", *Journal of Bacteriology,* vol. 174, no. 5, pp. 1574.

Collis, C.M. & Hall, R.M. 1992b, "Gene cassettes from the insert region of integrons are excised as covalently closed circles", *Molecular Microbiology,* vol. 6, no. 19, pp. 2875-2885.

Collis, C.M., Kim, M., Stokes, H. & Hall, R.M. 1998, "Binding of the purified integron DNA integrase IntI1 to integron- and cassette- associated recombination sites", *Molecular Microbiology,* vol. 29, no. 2, pp. 477-490.

Collis, C.M. & Hall, R.M. 1995, "Expression of antibiotic resistance genes in the integrated cassettes of integrons", *Antimicrobial Agents and Chemotherapy,* vol. 39, no. 1, pp. 155-162.

Corvec, S., Caroff, N., Espaze, E., Giraudeau, C., Drugeon, H. & Reynaud, A. 2003, "AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains", *Journal of Antimicrobial Chemotherapy,* vol. 52, no. 4, pp. 629-635.

Courcelle, J., Khodursky, A., Peter, B., Brown, P.O. & Hanawalt, P.C. 2001, "Comparative gene expression profiles following UV exposure in wild-type and SOS-deficient *Escherichia coli*", *Genetics,* vol. 158, no. 1, pp. 41-64.

Cox, M.M., Goodman, M.F., Kreuzer, K.N., Sherratt, D.J., Sandler, S.J. & Marians, K.J. 2000, "The importance of repairing stalled replication forks", *Nature,* vol. 404, no. 6773, pp. 37-41.

Damier-Piolle, L., Magnet, S., Bremont, S., Lambert, T. & Courvalin, P. 2008, "AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*", *Antimicrobial Agents and Chemotherapy,* vol. 52, no. 2, pp. 557-562.

Davis, M.W. 2013, *ApE - A plasmid Editor v. 2.0.46*. Available: http://biologylabs.utah.edu/jorgensen/wayned/ape/ [Accessed 2011]

Dawes, F.E., Kuzevski, A., Bettelheim, K.A., Hornitzky, M.A., Djordjevic, S.P. & Walker, M.J. 2010, "Distribution of class 1 integrons with IS*26*-mediated deletions in their 3′-conserved segments in *Escherichia coli* of human and animal origin", *PLoS One,* vol. 5, no. 9, pp. e12754.

Delihas, N. 2008, "Small mobile sequences in bacteria display diverse structure/function motifs", *Molecular Microbiology,* vol. 67, no. 3, pp. 475-481.

Di Cello, F., Pepi, M., Baldi, F. & Fani, R. 1997, "Molecular characterization of an *n*-alkane-degrading bacterial community and identification of a new species,*Acinetobacter venetianus*", *Research in Microbiology,* vol. 148, no. 3, pp. 237-249.

Diancourt, L., Passet, V., Nemec, A., Dijkshoorn, L. & Brisse, S. 2010, "The population structure of *Acinetobacter baumannii*: expanding multiresistant clones from an ancestral susceptible genetic pool", *PLoS One,* vol. 5, no. 4, pp. e10034.

Dijkshoorn, L., Nemec, A. & Seifert, H. 2007, "An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*", *Nature Reviews: Microbiology,* vol. 5, no. 12, pp. 939-951.

Dijkshoorn, L., Aucken, H., Gerner-Smidt, P., Janssen, P., Kaufmann, M.E., Garaizar, J., Ursing, J. & Pitt, T.L. 1996, "Comparison of outbreak and nonoutbreak *Acinetobacter baumannii* strains by genotypic and phenotypic methods", *Journal of Clinical Microbiology,* vol. 34, no. 6, pp. 1519-1525.

Doi, Y. & Arakawa, Y. 2007, "16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides", *Clinical Infectious Diseases,* vol. 45, no. 1, pp. 88-94.

Domingues, S., Harms, K., Fricke, W.F., Johnsen, P.J., da Silva, G.J. & Nielsen, K.M. 2012, "Natural Transformation Facilitates Transfer of Transposons, Integrons and Gene Cassettes between Bacterial Species", *PLoS Pathogens,* vol. 8, no. 8, pp. e1002837.

Domingues, S., Nielsen, K.M. & da Silva, G.J. 2011, "The*bla*$_{IMP-5}$-carrying integron in a clinical *Acinetobacter baumannii* strain is flanked by miniature inverted-repeat transposable elements (MITEs)", *Journal of Antimicrobial Chemotherapy,* vol. 66, no. 11, pp. 2667-2668.

Don, R., Cox, P., Wainwright, B., Baker, K. & Mattick, J. 1991, "'Touchdown' PCR to circumvent spurious priming during gene amplification", *Nucleic Acids Research,* vol. 19, no. 14, pp. 4008.

Dorer, M.S., Fero, J. & Salama, N.R. 2010, "DNA damage triggers genetic exchange in *Helicobacter pylori*", *PLoS Pathogens,* vol. 6, no. 7, pp. e1001026.

Edwards, J., Patel, G. & Wareham, D.W. 2007, "Low concentrations of commercial alcohol hand rubs facilitate growth of and secretion of extracellular proteins by multidrug-resistant strains of *Acinetobacter baumannii*", *Journal of Medical Microbiology,* vol. 56, no. Pt 12, pp. 1595-1599.

Edwards, J.S. & Palsson, B.O. 2000, "The *Escherichia coli* MG1655 in silico metabolic genotype: its definition, characteristics, and capabilities", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 97, no. 10, pp. 5528-5533.

Falagas, M.E., Bliziotis, I.A. & Siempos, I.I. 2006, "Attributable mortality of *Acinetobacter baumannii* infections in critically ill patients: a systematic review of matched cohort and case-control studies", *Critical Care,* vol. 10, no. 2, pp. R48.

Farrugia, D.N., Elbourne, L.D., Hassan, K.A., Eijkelkamp, B.A., Tetu, S.G., Brown, M.H., Shah, B.S., Peleg, A.Y., Mabbutt, B.C. & Paulsen, I.T. 2013, "The complete genome and phenome of a community-acquired *Acinetobacter baumannii*", *PloS One,* vol. 8, no. 3, pp. e58628.

Feng, G.D., Yang, S.Z., Wang, Y.H., Deng, M.R. & Zhu, H.H. 2014, "*Acinetobacter guangdongensis* sp. nov., isolated from abandoned lead-zinc ore", *International Journal of Systematic and Evolutionary Microbiology,* vol. 64, no. 10, pp. 3417-3421.

Fernández de Henestrosa, A., Ogi, T., Aoyagi, S., Chafin, D., Hayes, J.J., Ohmori, H. & Woodgate, R. 2000, "Identification of additional genes belonging to the LexA regulon in *Escherichia coli*", *Molecular Microbiology,* vol. 35, no. 6, pp. 1560-1572.

Feschotte, C., Osterlund, M.T., Peeler, R. & Wessler, S.R. 2005, "DNA-binding specificity of rice mariner-like transposases and interactions with stowaway MITEs", *Nucleic Acids Research,* vol. 33, no. 7, pp. 2153-2165.

Fournier, P.E., Vallenet, D., Barbe, V., Audic, S., Ogata, H., Poirel, L., Richet, H., Robert, C., Mangenot, S. & Abergel, C. 2006, "Comparative genomics of multidrug resistance in *Acinetobacter baumannii*", *PLoS Genetics,* vol. 2, no. 1, pp. 62-72.

Friedberg, E.C., Walker, G.C. & Siede, W. 1995, *DNA repair and mutagenesis.* American Society for Microbiology (ASM).

Frost, L.S., Leplae, R., Summers, A.O. & Toussaint, A. 2005, "Mobile genetic elements: the agents of open source evolution", *Nature Reviews Microbiology,* vol. 3, no. 9, pp. 722-732.

Gaddy, J.A. & Actis, L.A. 2009, "Regulation of *Acinetobacter baumannii* biofilm formation", *Future Microbiology,* vol. 4, no. 3, pp. 273-278.

Gallego, L. & Towner, K.J. 2001, "Carriage of class 1 integrons and antibiotic resistance in clinical isolates of *Acinetobacter baumannii* from northern Spain", *Journal of Medical Microbiology,* vol. 50, no. 1, pp. 71-77.

Gasc, A., Sicard, N., Claverys, J. & Sicard, A. 1980, "Lack of SOS repair in *Streptococcus pneumoniae*", *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis,* vol. 70, no. 2, pp. 157-165.

Geospiza, I. 2006, , *FinchTV 1.4.0.* Available: http://www.geospiza.com/Products/finchtv.shtml [Accessed 2010].

Gerner-Smidt, P. 1992, "Ribotyping of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex", *Journal of Clinical Microbiology,* vol. 30, no. 10, pp. 2680-2685.

Gestal, A.M., Liew, E.F. & Coleman, N.V. 2011, "Natural transformation with synthetic gene cassettes: new tools for integron research and biotechnology", *Microbiology,* vol. 157, no. Pt 12, pp. 3349-3360.

Gillings, M.R. 2013, "Evolutionary consequences of antibiotic use for the resistome, mobilome and microbial pangenome", *Frontiers in Microbiology,* vol. 4, no. 4, pp. 1-10.

Gillings, M., Boucher, Y., Joss, M., Koenig, J., Doolittle, W., Polz, M., Stokes, H. & Labbate, M. 2009, "ACID: annotation of cassette and integron data", *BMC Bioinformatics,* vol. 10, no. 118, pp. 1-9.

Gregg-Jolly, L.A. & Ornston, L.N. 1994, "Properties of *Acinetobacter calcoaceticus recA* and its contribution to intracellular gene conversion", *Molecular Microbiology,* vol. 12, no. 6, pp. 985-992.

Gudas, L.J. & Pardee, A.B. 1975, "Model for regulation of *Escherichia coli* DNA repair functions", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 72, no. 6, pp. 2330-2334.

Guerin, E., Cambray, G., Sanchez-Alberola, N., Campoy, S., Erill, I., Da Re, S., Gonzalez-Zorn, B., Barbe, J., Ploy, M.C. & Mazel, D. 2009, "The SOS response controls integron recombination", *Science,* vol. 324, no. 5930, pp. 1034.

Guerin, E., Jové, T., Tabesse, A., Mazel, D. & Ploy, M.C. 2011, "High-level gene cassette transcription prevents integrase expression in class 1 integrons", *Journal of Bacteriology,* vol. 193, no. 20, pp. 5675-5682.

Gust, B., Challis, G.L., Fowler, K., Kieser, T. & Chater, K.F. 2003, "PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 100, no. 4, pp. 1541-1546.

Guzman, L.M., Belin, D., Carson, M.J. & Beckwith, J. 1995, "Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter", *Journal of Bacteriology,* vol. 177, no. 14, pp. 4121-4130.

Hanahan, D. 1983, "Studies on transformation of *Escherichia coli* with plasmids", *Journal of Molecular Biology,* vol. 166, no. 4, pp. 557-580.

Hare, J.M., Adhikari, S., Lambert, K.V., Hare, A.E. & Grice, A.N. 2012a, "The *Acinetobacter* regulatory $UmuD_{Ab}$ protein cleaves in response to DNA damage with chimeric LexA/UmuD characteristics", *FEMS Microbiology Letters,* vol. 334, pp. 57-65.

Hare, J.M., Bradley, J.A., Lin, C. & Elam, T.J. 2012b, "Diverse responses to UV light exposure in *Acinetobacter* include the capacity for DNA damage-induced mutagenesis in the opportunistic pathogens *Acinetobacter baumannii* and *Acinetobacter ursingii*", *Microbiology,* vol. 158, no. Pt 3, pp. 601-611.

Hare, J.M., Perkins, S.N. & Gregg-Jolly, L.A. 2006, "A constitutively expressed, truncated *umuDC* operon regulates the RecA-dependent DNA damage induction of a gene in *Acinetobacter baylyi* strain ADP1", *Applied and Environmental Microbiology,* vol. 72, no. 6, pp. 4036-4043.

Harmer, C.J., Moran, R.A. & Hall, R.M. 2014, "Movement of IS*26*-associated antibiotic resistance genes occurs via a translocatable unit that includes a single IS*26* and preferentially inserts adjacent to another IS*26*", *mBio,* vol. 5, no. 5, pp. e01801-14.

Harms, K., Starikova, I. & Johnsen, P.J. 2013, "Costly Class-1 integrons and the domestication of the functional integrase", *Mobile Genetic Elements,* vol. 3, no. 2, pp. e24774.

Harris, S.R., Cartwright, E.J., Török, M.E., Holden, M.T., Brown, N.M., Ogilvy-Stuart, A.L., Ellington, M.J., Quail, M.A., Bentley, S.D. & Parkhill, J. 2012, "Whole-genome sequencing for analysis of an outbreak of meticillin-resistant *Staphylococcus aureus*: a descriptive study", *The Lancet Infectious Diseases,* vol. 13, no. 2, pp. 130-136.

Heckman, K.L. & Pease, L.R. 2007, "Gene splicing and mutagenesis by PCR-driven overlap extension", *Nature protocols,* vol. 2, no. 4, pp. 924-932.

Henriksen, S.D. 1973, "*Moraxella*, *Acinetobacter*, and the *Mimeae*", *Bacteriological Reviews,* vol. 37, no. 4, pp. 522.

Héritier, C., Poirel, L., Lambert, T. & Nordmann, P. 2005, "Contribution of acquired carbapenem-hydrolyzing oxacillinases to carbapenem resistance in *Acinetobacter baumannii*", *Antimicrobial Agents and Chemotherapy,* vol. 49, no. 8, pp. 3198-3202.

Hoang, T.T., Karkhoff-Schweizer, R.R., Kutchma, A.J. & Schweizer, H.P. 1998, "A broad-host-range Flp-*FRT* recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa*mutants", *Gene,* vol. 212, no. 1, pp. 77-86.

Hocquet, D., Llanes, C., Thouverez, M., Kulasekara, H.D., Bertrand, X., Plésiat, P., Mazel, D. & Miller, S.I. 2012, "Evidence for induction of integron-based antibiotic resistance by the SOS response in a clinical setting", *PLoS Pathogens,* vol. 8, no. 6, pp. e1002778.

Howard, A., O'Donoghue, M., Feeney, A. & Sleator, R.D. 2012, "*Acinetobacter baumannii*: an emerging opportunistic pathogen", *Virulence,* vol. 3, no. 3, pp. 243-250.

Huang, H., Yang, Z., Wu, X., Wang, Y., Liu, Y., Luo, H., Lv, X., Gan, Y., Song, S. & Gao, F. 2012, "Complete genome sequence of *Acinetobacter baumannii* MDR-TJ and insights into its mechanism of antibiotic resistance", *Journal of Antimicrobial Chemotherapy,* vol. 67, no. 12, pp. 2825-2832.

Hunger, M., Schmucker, R., Kishan, V. & Hillen, W. 1990, "Analysis and nucleotide sequence of an origin of DNA replication in *Acinetobacter calcoaceticus* and its use for *Escherichia coli* shuttle plasmids", *Gene,* vol. 87, no. 1, pp. 45-51.

Iacono, M., Villa, L., Fortini, D., Bordoni, R., Imperi, F., Bonnal, R.J.P., Sicheritz-Ponten, T., De Bellis, G., Visca, P. & Cassone, A. 2008, "Whole-genome pyrosequencing of an epidemic multidrug-resistant *Acinetobacter baumannii* strain belonging to the European clone II group", *Antimicrobial Agents and Chemotherapy,* vol. 52, no. 7, pp. 2616-2625.

Janssen, P., Maquelin, K., Coopman, R., Tjernberg, I., Bouvet, P., Kersters, K. & Dijkshoorn, L. 1997, "Discrimination of *Acinetobacter* genomic species by AFLP fingerprinting", *International Journal of Systematic Bacteriology,* vol. 47, no. 4, pp. 1179-1187.

Jolley, K.A., Feil, E., Chan, M. & Maiden, M.C.J. 2001, "Sequence type analysis and recombinational tests (START)", *Bioinformatics,* vol. 17, no. 12, pp. 1230-1231.

Joly-Guillou, M. 2005, "Clinical impact and pathogenicity of *Acinetobacter*", *Clinical Microbiology and Infection,* vol. 11, no. 11, pp. 868-873.

Jové, T., Da Re, S., Denis, F., Mazel, D. & Ploy, M. 2010, "Inverse correlation between promoter strength and excision activity in class 1 integrons", *PLoS Genetics,* vol. 6, no. 1, pp. e1000793.

Kaase, M., Nordmann, P., Wichelhaus, T.A., Gatermann, S.G., Bonnin, R.A. & Poirel, L. 2011, "NDM-2 carbapenemase in *Acinetobacter baumannii* from Egypt", *Journal of Antimicrobial Chemotherapy,* vol. 66, no. 6, pp. 1260-1262.

Kang, Y., Jung, J., Jeon, C.O. & Park, W. 2011, "*Acinetobacter oleivorans* sp. nov. is capable of adhering to and growing on diesel-oil", *The Journal of Microbiology,* vol. 49, no. 1, pp. 29-34.

Karah, N., Giske, C.G., Sundsfjord, A. & Samuelsen, Ø 2011, "A diversity of OXA-carbapenemases and class 1 integrons among carbapenem-resistant *Acinetobacter baumannii* clinical isolates from Sweden belonging to different international clonal lineages", *Microbial Drug Resistance,* vol. 17, no. 4, pp. 545-549.

Kato, T. & Shinoura, Y. 1977, "Isolation and characterization of mutants of *Escherichia coli* deficient in induction of mutations by ultraviolet light", *Molecular and General Genetics,* vol. 156, no. 2, pp. 121-131.

Khairatkar-Joshi, N. & Ahmad, N.S. 1998, "Inverse polymerase chain reaction mediated chromosome walking within the human glutamic acid decarboxylase gene", *Journal of Biosciences,* vol. 23, no. 3, pp. 265-269.

Kibbe, W.A. 2007, "OligoCalc: an online oligonucleotide properties calculator", *Nucleic Acids Research,* vol. 35, no. suppl 2, pp. W43-W46.

Kim, D., Baik, K.S., Kim, M.S., Park, S.C., Kim, S.S., Rhee, M.S., Kwak, Y.S. & Seong, C.N. 2008, "*Acinetobacter soli* sp. nov., isolated from forest soil", *The Journal of Microbiology,* vol. 46, no. 4, pp. 396-401.

Kim, P.S., Shin, N., Kim, J.Y., Yun, J., Hyun, D. & Bae, J. 2014, "*Acinetobacter apis* sp. nov., isolated from the intestinal tract of a honey bee, *Apis mellifera*", *Journal of Microbiology,* vol. 52, no. 8, pp. 639-645.

Ko, K.S., Suh, J.Y., Kwon, K.T., Jung, S., Park, K., Kang, C.I., Chung, D.R., Peck, K.R. & Song, J. 2007, "High rates of resistance to colistin and polymyxin B in subgroups of *Acinetobacter baumannii* isolates from Korea", *Journal of Antimicrobial Chemotherapy,* vol. 60, no. 5, pp. 1163-1167.

Kochar, M., Crosatti, M., Harrison, E.M., Rieck, B., Chan, J., Constantinidou, C., Pallen, M., Ou, H.Y. & Rajakumar, K. 2012, "Deletion of Tn*AbaR23* results in both expected and unexpected antibiogram changes in a multidrug-resistant *Acinetobacter baumannii* strain", *Antimicrobial Agents and Chemotherapy,* vol. 56, no. 4, pp. 1845-1853.

Koeleman, J.G.M., Stoof, J., Van Der Bijl, M.W., Vandenbroucke-Grauls, C.M.J.E. & Savelkoul, P.H.M. 2001, "Identification of epidemic strains of *Acinetobacter baumannii* by integrase gene PCR", *Journal of Clinical Microbiology,* vol. 39, no. 1, pp. 8-13.

Krizova, L., Dijkshoorn, L. & Nemec, A. 2011, "Diversity and evolution of AbaR genomic resistance islands in *Acinetobacter baumannii* strains of European clone I", *Antimicrobial Agents and Chemotherapy,* vol. 55, no. 7, pp. 3201-3206.

Krizova, L. & Nemec, A. 2010, "A 63 kb genomic resistance island found in a multidrug-resistant *Acinetobacter baumannii* isolate of European clone I from 1977", *Journal of Antimicrobial Chemotherapy,* vol. 65, no. 9, pp. 1915-1918.

Kumar, A., Dalton, C., Cortez-Cordova, J. & Schweizer, H.P. 2010, "Mini-Tn*7*vectors as genetic tools for single copy gene cloning in *Acinetobacter baumannii*", *Journal of Microbiological Methods,* vol. 82, no. 3, pp. 296-300.

Kumarasamy, K.K., Toleman, M.A., Walsh, T.R., Bagaria, J., Butt, F., Balakrishnan, R., Chaudhary, U., Doumith, M., Giske, C.G. & Irfan, S. 2010, "Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study", *The Lancet Infectious Diseases,* vol. 10, no. 9, pp. 597-602.

La Scola, B., Gundi, V.A.K.B., Khamis, A. & Raoult, D. 2006, "Sequencing of the *rpoB* gene and flanking spacers for molecular identification of *Acinetobacter*species", *Journal of Clinical Microbiology,* vol. 44, no. 3, pp. 827-832.

Larkin, M., Blackshields, G., Brown, N., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A. & Lopez, R. 2007, "Clustal W and Clustal X version 2.0", *Bioinformatics,* vol. 23, no. 21, pp. 2947-2948.

Lee, A.M., Ross, C.T., Zeng, B. & Singleton, S.F. 2005, "A molecular target for suppression of the evolution of antibiotic resistance: inhibition of the *Escherichia coli* RecA protein by N^6-(1-Naphthyl)-ADP", *Journal of Medicinal Chemistry,* vol. 48, no. 17, pp. 5408-5411.

Lee, C., Kim, J., Shin, S.G. & Hwang, S. 2006, "Absolute and relative qPCR quantification of plasmid copy number in *Escherichia coli*", *Journal of Biotechnology,* vol. 123, no. 3, pp. 273-280.

Levesque, C., Piche, L., Larose, C. & Roy, P.H. 1995, "PCR mapping of integrons reveals several novel combinations of resistance genes", *Antimicrobial Agents and Chemotherapy,* vol. 39, no. 1, pp. 185.

Lévesque, C., Brassard, S., Lapointe, J. & Roy, P.H. 1994, "Diversity and relative strength of tandem promoters for the antibiotic-resistance genes of several integron", *Gene,* vol. 142, no. 1, pp. 49-54.

Lewis, L.K., Harlow, G.R., Gregg-Jolly, L.A. & Mount, D.W. 1994, "Identification of high affinity binding sites for LexA which define new DNA damage-inducible genes in *Escherichia coli*", *Journal of Molecular Biology,* vol. 241, no. 4, pp. 507-523.

Lewis, L.A., Cylin, E., Lee, H.K., Saby, R., Wong, W. & Grindley, N.D. 2004, "The left end of IS*2*: a compromise between transpositional activity and an essential promoter function that regulates the transposition pathway", *Journal of Bacteriology,* vol. 186, no. 3, pp. 858-865.

Li, W., Zhang, D., Huang, X. & Qin, W. 2014a, "*Acinetobacter harbinensis* sp. nov., isolated from the Songhua River in the northeast of China", *International Journal of Systematic and Evolutionary Microbiology,* vol. 64, no. 5, pp. 1507-1513.

Li, Y., He, W., Wang, T., Piao, C.G., Guo, L.M., Chang, J.P., Guo, M.W. & Xie, S.J. 2014b, "*Acinetobacter qingfengensis* sp. nov., isolated from canker bark of *Populus* × *euramericana*", *International Journal of Systematic and Evolutionary Microbiology,* vol. 64, no. Pt 3, pp. 1043-1050.

Li, Y., Piao, C.G., Ma, Y.C., He, W., Wang, H.M., Chang, J.P., Guo, L.M., Wang, X.Z., Xie, S.J. & Guo, M.W. 2013, "*Acinetobacter puyangensis* sp. nov., isolated from the healthy and diseased part of *Populus xeuramericana* canker bark", *International Journal of Systematic and Evolutionary Microbiology,* vol. 63, no. 8, pp. 2963-2969.

Lin, T.L., Lee, C.Z., Hsieh, P.F., Tsai, S.F. & Wang, J.T. 2008, "Characterization of integrative and conjugative element ICE*Kp1*-associated genomic heterogeneity in a *Klebsiella pneumoniae* strain isolated from a primary liver abscess", *Journal of Bacteriology,* vol. 190, no. 2, pp. 515-526.

Liou, M., Liu, C., Lu, C., Hsieh, M., Chang, K., Kuo, H., Lee, C., Chang, C., Yang, C. & Tang, C.Y. 2012, "Genome Sequence of *Acinetobacter baumannii* TYTH-1", *Journal of Bacteriology,* vol. 194, no. 24, pp. 6974-6974.

Little, J. 1991, "Mechanism of specific LexA cleavage: autodigestion and the role of RecA coprotease", *Biochimie,* vol. 73, no. 4, pp. 411-421.

Liu, C., Tang, C.Y., Chang, K., Kuo, H. & Liou, M. 2014, "A comparative study of class 1 integrons in *Acinetobacter baumannii*", *Gene,* vol. 544, no. 1, pp. 75-82.

Mahillon, J. & Chandler, M. 1998, "Insertion sequences", *Microbiology and Molecular Biology Reviews,* vol. 62, no. 3, pp. 725-774.

Malhotra, J., Anand, S., Jindal, S., Rajagopal, R. & Lal, R. 2012, "*Acinetobacter indicus* sp. nov., isolated from a hexachlorocyclohexane dump site", *International Journal of Systematic and Evolutionary Microbiology,* vol. 62, no. 12, pp. 2883-2890.

Mazel, D. 2006, "Integrons: agents of bacterial evolution", *Nature Reviews Microbiology,* vol. 4, no. 8, pp. 608-620.

McDonnell, G. & Russell, A.D. 1999, "Antiseptics and disinfectants: activity, action, and resistance", *Clinical Microbiology Reviews,* vol. 12, no. 1, pp. 147-179.

McEwen, S.A. & Fedorka-Cray, P.J. 2002, "Antimicrobial use and resistance in animals", *Clinical Infectious Diseases,* vol. 34, no. 3, pp. S93-S106.

Miriagou, V., Carattoli, A., Tzelepi, E., Villa, L. & Tzouvelekis, L.S. 2005, "IS*26*-associated In4-type integrons forming multiresistance loci in enterobacterial plasmids", *Antimicrobial Agents and Chemotherapy,* vol. 49, no. 8, pp. 3541-3543.

Moura, A., Soares, M., Pereira, C., Leitao, N., Henriques, I. & Correia, A. 2009, "INTEGRALL: a database and search engine for integrons, integrases and gene cassettes", *Bioinformatics,* vol. 25, no. 8, pp. 1096-1098.

Napolitano, R., Janel-Bintz, R., Wagner, J. & Fuchs, R. 2000, "All three SOS-inducible DNA polymerases (Pol II, Pol IV and Pol V) are involved in induced mutagenesis", *The EMBO journal,* vol. 19, no. 22, pp. 6259-6265.

Nemec, A., Dijkshoorn, L., Cleenwerck, I., De Baere, T., Janssens, D., van der Reijden, T.J.K., Ježek, P. & Vaneechoutte, M. 2003, "*Acinetobacter parvus* sp. nov., a small-colony-forming species isolated from human clinical specimens", *International Journal of Systematic and Evolutionary Microbiology,* vol. 53, no. 5, pp. 1563-1567.

Nemec, A., De Baere, T., Tjernberg, I., Vaneechoutte, M., Van Der Reijden, T. & Dijkshoorn, L. 2001, "*Acinetobacter ursingii* sp. nov. and *Acinetobacter schindleri* sp. nov., isolated from human clinical specimens.", *International Journal of Systematic and Evolutionary Microbiology,* vol. 51, no. 5, pp. 1891-1899.

Nemec, A., Krizova, L., Maixnerova, M., van der Reijden, Tanny JK, Deschaght, P., Passet, V., Vaneechoutte, M., Brisse, S. & Dijkshoorn, L. 2011, "Genotypic and phenotypic characterization of the *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex with the proposal of *Acinetobacter pittii* sp. nov. (formerly *Acinetobacter* genomic species 3) and *Acinetobacter nosocomialis* sp. nov. (formerly *Acinetobacter* genomic species 13TU)", *Research in Microbiology,* vol. 162, no. 4, pp. 393-404.

Nemec, A., Musílek, M., Maixnerová, M., De Baere, T., van der Reijden, Tanny JK, Vaneechoutte, M. & Dijkshoorn, L. 2009, "*Acinetobacter beijerinckii* sp. nov. and *Acinetobacter gyllenbergii* sp. nov., haemolytic organisms isolated from humans", *International Journal of Systematic and Evolutionary Microbiology,* vol. 59, no. 1, pp. 118-124.

Nemec, A., Musílek, M., Šedo, O., De Baere, T., Maixnerová, M., van der Reijden, Tanny JK, Zdráhal, Z., Vaneechoutte, M. & Dijkshoorn, L. 2010, "*Acinetobacter bereziniae* sp. nov. and *Acinetobacter guillouiae* sp. nov., to accommodate *Acinetobacter* genomic species 10 and 11, respectively", *International Journal of Systematic and Evolutionary Microbiology,* vol. 60, no. 4, pp. 896-903.

Nemergut, D.R., Robeson, M.S., Kysela, R.F., Martin, A.P., Schmidt, S.K. & Knight, R. 2008, "Insights and inferences about integron evolution from genomic data", *BMC Genomics,* vol. 9, no. 261, pp. 1-12.

Nishimura, Y., Ino, T. & Iizuka, H. 1988, "*Acinetobacter radioresistens* sp. nov. isolated from cotton and soil", *International Journal of Systematic Bacteriology,* vol. 38, no. 2, pp. 209-211.

Niu, C., Clemmer, K.M., Bonomo, R.A. & Rather, P.N. 2008, "Isolation and characterization of an autoinducer synthase from *Acinetobacter baumannii*", *Journal of Bacteriology,* vol. 190, no. 9, pp. 3386-3392.

Nohmi, T., Battista, J.R., Dodson, L.A. & Walker, G.C. 1988, "RecA-mediated cleavage activates UmuD for mutagenesis: mechanistic relationship between transcriptional derepression and posttranslational activation", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 85, no. 6, pp. 1816-1820.

Norman, A., Hansen, L.H. & Sørensen, S.J. 2009, "Conjugative plasmids: vessels of the communal gene pool", *Philosophical Transactions of the Royal Society B: Biological Sciences,* vol. 364, no. 1527, pp. 2275-2289.

Norton, M.D., Spilkia, A.J. & Godoy, V.G. 2013, "Antibiotic resistance acquired through a DNA damage-inducible response in *Acinetobacter baumannii*", *Journal of Bacteriology,* vol. 195, no. 6, pp. 1335-1345.

Novick, R.P., Clowes, R.C., Cohen, S.N. & Curtiss, R. 1976, "Uniform nomenclature for bacterial plasmids: a proposal.", *Bacteriological Reviews,* vol. 40, no. 1, pp. 168-189.

Nunes-Duby, S.E., Kwon, H.J., Tirumalai, R.S., Ellenberger, T. & Landy, A. 1998, "Similarities and differences among 105 members of the Int family of site-specific recombinases", *Nucleic Acids Research,* vol. 26, no. 2, pp. 391-406.

Opazo, A., Sonnevend, A., Lopes, B., Hamouda, A., Ghazawi, A., Pal, T. & Amyes, S.G. 2012, "Plasmid-encoded PER-7 β-lactamase responsible for ceftazidime resistance in *Acinetobacter baumannii* isolated in the United Arab Emirates", *The Journal of Antimicrobial Chemotherapy,* vol. 67, no. 7, pp. 1619-1622.

Paetzel, M., Dalbey, R.E. & Strynadka, N.C. 2002, "Crystal structure of a bacterial signal peptidase apoenzyme: implications for signal peptide binding and the Ser-Lys dyad mechanism", *The Journal of biological chemistry,* vol. 277, no. 11, pp. 9512-9519.

Papagiannitsis, C.C., Tzouvelekis, L.S. & Miriagou, V. 2009, "Relative strengths of the class 1 integron promoter hybrid 2 and the combinations of strong and hybrid 1 with an active p2 promoter", *Antimicrobial Agents and Chemotherapy,* vol. 53, no. 1, pp. 277-280.

Park, J.Y., Kim, S., Kim, S., Cha, S.H., Lim, S. & Kim, J. 2011, "Complete genome sequence of multidrug-resistant *Acinetobacter baumannii* strain 1656-2, which forms sturdy biofilm", *Journal of Bacteriology,* vol. 193, no. 22, pp. 6393-6394.

Partridge, S.R., Tsafnat, G., Coiera, E. & Iredell, J.R. 2009, "Gene cassettes and cassette arrays in mobile resistance integrons", *FEMS Microbiology Reviews,* vol. 33, no. 4, pp. 757-784.

Peleg, A.Y., de Breij, A., Adams, M.D., Cerqueira, G.M., Mocali, S., Galardini, M., Nibbering, P.H., Earl, A.M., Ward, D.V. & Paterson, D.L. 2012, "The success of *Acinetobacter* species; genetic, metabolic and virulence attributes", *PloS One,* vol. 7, no. 10, pp. e46984.

Peleg, A.Y., Seifert, H. & Paterson, D.L. 2008, "*Acinetobacter baumannii*: emergence of a successful pathogen", *Clinical Microbiology Reviews,* vol. 21, no. 3, pp. 538-582.

Petrosino, J.F., Galhardo, R.S., Morales, L.D. & Rosenberg, S.M. 2009, "Stress-induced beta-lactam antibiotic resistance mutation and sequences of stationary-phase mutations in the *Escherichia coli* chromosome", *Journal of Bacteriology,* vol. 191, no. 19, pp. 5881-5889.

Pfaffl, M.W., Horgan, G.W. & Dempfle, L. 2002, "Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR", *Nucleic Acids Research,* vol. 30, no. 9, pp. e36-e36.

Pfeifer, Y., Wilharm, G., Zander, E., Wichelhaus, T.A., Göttig, S., Hunfeld, K., Seifert, H., Witte, W. & Higgins, P.G. 2011, "Molecular characterization of bla_{NDM-1} in an *Acinetobacter baumannii* strain isolated in Germany in 2007", *Journal of Antimicrobial Chemotherapy,* vol. 66, no. 9, pp. 1998-2001.

Philippe, N., Alcaraz, J., Coursange, E., Geiselmann, J. & Schneider, D. 2004, "Improvement of pCVD442, a suicide plasmid for gene allele exchange in bacteria", *Plasmid,* vol. 51, no. 3, pp. 246-255.

Piddock, L.J.V. 2006, "Multidrug-resistance efflux pumps? Not just for resistance", *Nature Reviews Microbiology,* vol. 4, no. 8, pp. 629-636.

Pilhofer, M., Bauer, A.P., Schrallhammer, M., Richter, L., Ludwig, W., Schleifer, K.H. & Petroni, G. 2007, "Characterization of bacterial operons consisting of two tubulins and a kinesin-like gene by the novel two-step gene walking method", *Nucleic Acids Research,* vol. 35, no. 20, pp. e135-e135.

Ploy, M.C., Denis, F., Courvalin, P. & Lambert, T. 2000, "Molecular characterization of integrons in *Acinetobacter baumannii*: description of a hybrid class 2 integron", *Antimicrobial Agents and Chemotherapy,* vol. 44, no. 10, pp. 2684-2688.

Poirel, L., Carrër, A., Pitout, J.D. & Nordmann, P. 2009, "Integron mobilization unit as a source of mobility of antibiotic resistance genes", *Antimicrobial Agents and Chemotherapy,* vol. 53, no. 6, pp. 2492-2498.

Post, V., White, P.A. & Hall, R.M. 2010, "Evolution of AbaR-type genomic resistance islands in multiply antibiotic-resistant *Acinetobacter baumannii*", *Journal of Antimicrobial Chemotherapy,* vol. 65, pp. 1162-1170.

Projan, S.J. 2003, "Why is big Pharma getting out of antibacterial drug discovery?", *Current Opinion in Microbiology,* vol. 6, no. 5, pp. 427-430.

Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B. & Claverys, J.P. 2006, "Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*", *Science Signalling,* vol. 313, no. 5783, pp. 89-92.

Quillardet, P., Rouffaud, M. & Bouige, P. 2003, "DNA array analysis of gene expression in response to UV irradiation in *Escherichia coli*", *Research in Microbiology,* vol. 154, no. 8, pp. 559-572.

Quinones, M., Kimsey, H.H. & Waldor, M.K. 2005, "LexA cleavage is required for CTX prophage induction", *Molecular Cell,* vol. 17, no. 2, pp. 291-300.

Radman, M. 1999, "Mutation: Enzymes of evolutionary change", *Nature,* vol. 401, no. 6756, pp. 866-869.

Radman, M. 1975, "SOS repair hypothesis: phenomenology of an inducible DNA repair which is accompanied by mutagenesis" in *Molecular Mechanisms for Repair of DNA*, eds. P.C. Hanawalt & R.B. Setlow, Springer, pp. 355-367.

Ramirez, M.S., Parenteau, T.R., Centron, D. & Tolmasky, M.E. 2008, "Functional characterization of Tn*1331* gene cassettes", *Journal of Antimicrobial Chemotherapy,* vol. 62, no. 4, pp. 669-673.

Ramirez, M.S., Don, M., Merkier, A.K., Bistue, A.J., Zorreguieta, A., Centron, D. & Tolmasky, M.E. 2010, "Naturally competent *Acinetobacter baumannii* clinical isolate as a convenient model for genetic studies", *Journal of Clinical Microbiology,* vol. 48, no. 4, pp. 1488-1490.

Ramirez, M.S., Quiroga, C. & Centron, D. 2005, "Novel rearrangement of a class 2 integron in two non-epidemiologically related isolates of *Acinetobacter baumannii*", *Antimicrobial Agents and Chemotherapy,* vol. 49, no. 12, pp. 5179-5181.

Rao, B.J. & Radding, C.M. 1995, "RecA protein mediates homologous recognition via non-Watson-Crick bonds in base triplets", *Philosophical Transactions of the Royal Society of London,* vol. 347, no. 1319, pp. 5-12.

Rauch, P.J.G., Palmen, R., Burds, A.A., Gregg-Jolly, L.A., van der Zee, J.R. & Hellingwerf, K.J. 1996, "The expression of the *Acinetobacter calcoaceticus recA* gene increases in response to DNA damage independently of RecA and of development of competence for natural transformation", *Microbiology,* vol. 142, no. 4, pp. 1025-1032.

Ravasi, P., Limansky, A.S., Rodriguez, R.E., Viale, A.M. & Mussi, M.A. 2011, "IS*Aba825*, a functional insertion sequence modulating genomic plasticity and bla_{OXA-58} expression in *Acinetobacter baumannii*", *Antimicrobial Agents and Chemotherapy,* vol. 55, no. 2, pp. 917-920.

Recchia, G.D., Stokes, H. & Hall, R.M. 1994, "Characterisation of specific and secondary recombination sites recognised by the integron DNA integrase", *Nucleic Acids Research,* vol. 22, no. 11, pp. 2071-2078.

Reif, H. & Saedler, H. 1975, "IS*1*is involved in deletion formation in the *gal* region of *E. coli*K12", *Molecular and General Genetics,* vol. 137, no. 1, pp. 17-28.

Reuven, N.B., Arad, G., Maor-Shoshani, A. & Livneh, Z. 1999, "The mutagenesis protein UmuC is a DNA polymerase activated by UmuD', RecA, and SSB and is specialized for translesion replication", *The Journal of Biological Chemistry,* vol. 274, no. 45, pp. 31763-31766.

Rice, L.B. 2008, "Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE", *Journal of Infectious Diseases,* vol. 197, no. 8, pp. 1079-1081.

Rieck, B., Tourigny, D.S., Crosatti, M., Schmid, R., Kochar, M., Harrison, E.M., Ou, H., Turton, J.F. & Rajakumar, K. 2012, "*Acinetobacter* insertion sequence IS*Aba11* belongs to a novel family that encodes transposases with a signature HHEK motif", *Applied and Environmental Microbiology,* vol. 78, no. 2, pp. 471-480.

Riley, R. & Nardell, E. 1989, "Clearing the air", *The American Review of Respiratory Disease,* vol. 139, pp. 1286-1294.

Roberts, A.P., Chandler, M., Courvalin, P., Guédon, G., Mullany, P., Pembroke, T., Rood, J.I., Jeffery Smith, C., Summers, A.O. & Tsuda, M. 2008, "Revised nomenclature for transposable genetic elements", *Plasmid,* vol. 60, no. 3, pp. 167-173.

Robinson, A., Brzoska, A.J., Turner, K.M., Withers, R., Harry, E.J., Lewis, P.J. & Dixon, N.E. 2010, "Essential biological processes of an emerging pathogen: DNA replication, transcription, and cell division in *Acinetobacter* spp", *Microbiology and Molecular Biology Reviews,* vol. 74, no. 2, pp. 273-297.

Rose, A. 2010, "Tn*AbaR1*: a novel Tn*7*-related transposon in *Acinetobacter baumannii* that contributes to the accumulation and dissemination of large repertoires of resistance genes", *Bioscience Horizons,* vol. 3, no. 1, pp. 40-48.

Rozen, S. & Skaletsky, H. 1999, "Primer3 on the WWW for general users and for biologist programmers" in *Bioinformatics Methods and Protocols*, eds. S. Misener & S.A. Krawetz, Springer, , pp. 365-386.

Safi, H., Barnes, P.F., Lakey, D.L., Shams, H., Samten, B., Vankayalapati, R. & Howard, S.T. 2004, "IS*6110* functions as a mobile, monocyte-activated promoter in *Mycobacterium tuberculosis*", *Molecular Microbiology,* vol. 52, no. 4, pp. 999-1012.

Sambrook, J. & Russell, D.W. 2006, "Purification of nucleic acids by extraction with phenol:chloroform" in *Cold Spring Harbor Protocols*, eds. J. Sambrook & D.W. Russel, 3rd edn, Cold Spring Harbor, United States.

Saroj, S.D., Clemmer, K.M., Bonomo, R.A. & Rather, P.N. 2012, "Novel mechanism for fluoroquinolone resistance in *Acinetobacter baumannii*", *Antimicrobial Agents and Chemotherapy,* vol. 56, no. 9, pp. 4955-4957.

Schneider, D. & Lenski, R.E. 2004, "Dynamics of insertion sequence elements during experimental evolution of bacteria", *Research in Microbiology,* vol. 155, no. 5, pp. 319-327.

Scott, P., Petersen, K., Fishbain, M., Craft, D., Ewell, A., Moran, K., Hack, D., Deye, G., Riddell, S. & Christopher, G. 2004, "*Acinetobacter baumannii* infections among patients at military medical facilities treating injured US service members, 2002-2004", *Morbidity and Mortality Weekly Report,* vol. 53, no. 45, pp. 1063-1066.

Segal, H., Garny, S. & Elisha, B.G. 2005, "Is IS*Aba1*customized for *Acinetobacter*?", *FEMS Microbiology Letters,* vol. 243, no. 2, pp. 425-429.

Seifert, H., Boullion, B., Schulze, A. & Pulverer, G. 1994, "Plasmid DNA profiles of *Acinetobacter baumannii*: clinical application in a complex endemic setting", *Infection Control and Hospital Epidemiology,* vol. 15, no. 8, pp. 520-528.

Seifert, H., Dijkshoorn, L., Gerner-Smidt, P., Pelzer, N., Tjernberg, I. & Vaneechoutte, M. 1997, "Distribution of *Acinetobacter* species on human skin: comparison of phenotypic and genotypic identification methods.", *Journal of Clinical Microbiology,* vol. 35, no. 11, pp. 2819-2825.

Sexton, J.Z., Wigle, T.J., He, Q., Hughes, M.A., Smith, G.R., Singleton, S.F., Williams, A.L. & Yeh, L.A. 2010, "Novel Inhibitors of *E. coli* RecA ATPase Activity", *Current Chemical Genomics,* vol. 4, pp. 34-42.

Shaikh, F., Spence, R.P., Levi, K., Ou, H.Y., Deng, Z., Towner, K.J. & Rajakumar, K. 2009, "ATPase genes of diverse multidrug-resistant *Acinetobacter baumannii* isolates frequently harbour integrated DNA", *Journal of Antimicrobial Chemotherapy,* vol. 63, no. 2, pp. 260-264.

Shaw, K.J., Rather, P.N., Hare, R.S. & Miller, G.H. 1993, "Molecular Genetics of Aminoglycoside Resistance Genes and Familial Relationships of the Aminoglycoside-Modifying Enzymes", *Microbiological Reviews,* vol. 57, no. 1, pp. 138-163.

Simon, R., Priefer, U. & Pühler, A. 1983, "A broad host range mobilization system for*in vivo*genetic engineering: transposon mutagenesis in Gram-negative bacteria", *Nature Biotechnology,* vol. 1, no. 9, pp. 784-791.

Skulj, M., Okrslar, V., Jalen, S., Jevsevar, S., Slanc, P., Strukelj, B. & Menart, V. 2008, "Improved determination of plasmid copy number using quantitative real-time PCR for monitoring fermentation processes", *Microbial cell factories,* vol. 7, no. 6, pp. 1-12.

Smet, A., Cools, P., Krizova, L., Maixnerova, M., Sedo, O., Haesebrouck, F., Kempf, M., Nemec, A. & Vaneechoutte, M. 2014, "*Acinetobacter gandensis* sp. nov. isolated from horse and cattle", *International Journal of Systematic and Evolutionary Microbiology,* vol. 64, no. 12, pp. 4007-4015.

Smillie, C., Garcillan-Barcia, M.P., Francia, M.V., Rocha, E.P. & de la Cruz, F. 2010, "Mobility of plasmids", *Microbiology and Molecular Biology Reviews,* vol. 74, no. 3, pp. 434-452.

Smith, M.G., Gianoulis, T.A., Pukatzki, S., Mekalanos, J.J., Ornston, L.N., Gerstein, M. & Snyder, M. 2007, "New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis", *Genes & Development,* vol. 21, no. 5, pp. 601-614.

Solomon, J.M. & Grossman, A.D. 1996, "Who's competent and when: regulation of natural genetic competence in bacteria", *Trends in Genetics,* vol. 12, no. 4, pp. 150-155.

Starikova, I., Harms, K., Haugen, P., Lunde, T.T.M., Primicerio, R., Samuelsen, Ø, Nielsen, K.M. & Johnsen, P.J. 2012, "A trade-off between the fitness cost of functional integrases and long-term stability of integrons", *PLoS Pathogens,* vol. 8, no. 11, pp. e1003043.

Steiniger-White, M., Rayment, I. & Reznikoff, W.S. 2004, "Structure/function insights into Tn*5* transposition", *Current Opinion in Structural Biology,* vol. 14, no. 1, pp. 50-57.

Stokes, H.W. & Gillings, M.R. 2011, "Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens", *FEMS Microbiology Reviews,* vol. 35, no. 5, pp. 790-819.

Stokes, H. & Hall, R.M. 1989, "A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons", *Molecular Microbiology,* vol. 3, no. 12, pp. 1669-1683.

Tang, M., Pham, P., Shen, X., Taylor, J., O'Donnell, M., Woodgate, R. & Goodman, M.F. 2000, "Roles of *E. coli* DNA polymerases IV and V in lesion-targeted and untargeted SOS mutagenesis", *Nature,* vol. 404, no. 6781, pp. 1014-1018.

Tang, M., Bruck, I., Eritja, R., Turner, J., Frank, E.G., Woodgate, R., O'Donnell, M. & Goodman, M.F. 1998, "Biochemical basis of SOS-induced mutagenesis in *Escherichia coli*: reconstitution of in vitro lesion bypass dependent on the UmuD'2C mutagenic complex and RecA protein", *Proceedings of the National Academy of Sciences of the United States of America,* vol. 95, no. 17, pp. 9755-9760.

Tennstedt, T., Szczepanowski, R., Braun, S., Pühler, A. & Schlüter, A. 2003, "Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant", *FEMS Microbiology Ecology,* vol. 45, no. 3, pp. 239-252.

Touchon, M., Hoede, C., Tenaillon, O., Barbe, V., Baeriswyl, S., Bidet, P., Bingen, E., Bonacorsi, S., Bouchier, C. & Bouvet, O. 2009, "Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths", *PLoS Genetics,* vol. 5, no. 1, pp. e1000344.

Turton, J.F., Kaufmann, M.E., Glover, J., Coelho, J.M., Warner, M., Pike, R. & Pitt, T.L. 2005, "Detection and typing of integrons in epidemic strains of *Acinetobacter baumannii* found in the United Kingdom", *Journal of Clinical Microbiology,* vol. 43, no. 7, pp. 3074.

Turton, J., Gabriel, S., Valderrey, C., Kaufmann, M. & Pitt, T. 2007, "Use of sequence-based typing and multiplex PCR to identify clonal lineages of outbreak strains of *Acinetobacter baumannii*", *Clinical Microbiology and Infection,* vol. 13, no. 8, pp. 807-815.

Utnes, A., Sørum, V., Hülter, N., Primicerio, R, Hegstad, J., Kloos, J., Nielsen, K.M. & Johnsen, P.J. 2015, "Growth phase-specific evolutionary benefits of natural transformation in *Acintetobacter baylyi*", *The International Society for Microbial Ecology Journal*, vol. 15, pp 1751-7362.

van Aartsen, J.J. & Rajakumar, K. 2011, "An optimized method for suicide vector-based allelic exchange in *Klebsiella pneumoniae*", *Journal of Microbiological Methods,* vol. 86, no. 3, pp. 313-319.

Vaneechoutte, M., Young, D.M., Ornston, L.N., De Baere, T., Nemec, A., Van Der Reijden, T., Carr, E., Tjernberg, I. & Dijkshoorn, L. 2006, "Naturally transformable *Acinetobacter* sp. strain ADP1 belongs to the newly described species *Acinetobacter baylyi*", *Applied and Environmental Microbiology,* vol. 72, no. 1, pp. 932-936.

Vaz-Moreira, I., Novo, A., Hantsis-Zacharov, E., Lopes, A.R., Gomila, M., Nunes, O.C., Manaia, C.M. & Halpern, M. 2011, "*Acinetobacter rudis* sp. nov., isolated from raw milk and raw wastewater", *International Journal of Systematic and Evolutionary Microbiology,* vol. 61, no. 12, pp. 2837-2843.

Vila, J., Marti, S. & Sanchez-Cespedes, J. 2007, "Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*", *Journal of Antimicrobial Chemotherapy,* vol. 59, pp. 1210-1215.

Villa, L. & Carattoli, A. 2005, "Integrons and transposons on the *Salmonella enterica* serovar *typhimurium* virulence plasmid", *Antimicrobial Agents and Chemotherapy,* vol. 49, no. 3, pp. 1194-1197.

Wagner, J., Gruz, P., Kim, S., Yamada, M., Matsui, K., Fuchs, R.P. & Nohmi, T. 1999, "The *dinB* gene encodes a novel *E. coli* DNA polymerase, DNA pol IV, involved in mutagenesis", *Molecular Cell,* vol. 4, no. 2, pp. 281-286.

Wang, M., Tran, J.H., Jacoby, G.A., Zhang, Y., Wang, F. & Hooper, D.C. 2003, "Plasmid-mediated quinolone resistance in clinical isolates of *Escherichia coli* from Shanghai, China", *Antimicrobial Agents and Chemotherapy,* vol. 47, no. 7, pp. 2242-2248.

Wang, X., Zhang, Z., Hao, Q., Wu, J., Xiao, J. & Jing, H. 2014, "Complete Genome Sequence of *Acinetobacter baumannii* ZW85-1", *Genome Announcements,* vol. 2, no. 1, pp. 1-2.

Wigle, T.J., Sexton, J.Z., Gromova, A.V., Hadimani, M.B., Hughes, M.A., Smith, G.R., Yeh, L.A. & Singleton, S.F. 2009, "Inhibitors of RecA activity discovered by high-throughput screening: cell-permeable small molecules attenuate the SOS response in *Escherichia coli*", *Journal of Biomolecular Screening,* vol. 14, no. 9, pp. 1092-1101.

Wilharm, G., Piesker, J., Laue, M. & Skiebe, E. 2013, "DNA uptake by the nosocomial pathogen *Acinetobacter baumannii* occurs during movement along wet surfaces", *Journal of Bacteriology,* vol. 195, no. 18, pp. 4146-4153.

Winokur, P.L., Vonstein, D.L., Hoffman, L.J., Uhlenhopp, E.K. & Doern, G.V. 2001, "Evidence for transfer of CMY-2 AmpC β-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans", *Antimicrobial Agents and Chemotherapy,* vol. 45, no. 10, pp. 2716-2722.

Yang, Z., Jiang, X., Wei, Q., Chen, N. & Lu, Y. 2009, "A novel and rapid method for determining integration frequency catalyzed by integron integrase *intI1*", *Journal of Microbiological Methods,* vol. 76, no. 1, pp. 97-100.

Young, D.M., Parke, D. & Ornston, L.N. 2005, "Opportunities for genetic investigation afforded by *Acinetobacter baylyi*, a nutritionally versatile bacterial species that is highly competent for natural transformation", *Annual Review of Microbiology,* vol. 59, pp. 519-551.

Zhou, H., Zhang, T., Yu, D., Pi, B., Yang, Q., Zhou, J., Hu, S. & Yu, Y. 2011, "Genomic analysis of the multidrug-resistant *Acinetobacter baumannii* strain MDR-ZJ06 widely spread in China", *Antimicrobial Agents and Chemotherapy,* vol. 55, no. 10, pp. 4506-4512.

Zhu, L., Yan, Z., Zhang, Z., Zhou, Q., Zhou, J., Wakeland, E.K., Fang, X., Xuan, Z., Shen, D. & Li, Q. 2013, "Complete genome analysis of three *Acinetobacter*

baumannii clinical isolates in China for insight into the diversification of drug resistance elements", *PloS One,* vol. 8, no. 6, pp. 1-13.

Appendices

Appendix 1. Culture media

Brain heart infusion (BHI) broth plus 30 % glycerol

USE: Storage of bacterial stocks at -20 and -80ºC

47 g of brain heart infusion broth powder (Oxoid) was dissolved in a final volume of 1 l of dH_2O containing 30 % (v/v) glycerol and was autoclaved before use.

Lysogeny broth (LB) and agar (LA)

USE: Standard liquid (LB) and solid growth (LA) medium for bacterial cultures

Lysogeny broth (LB) was prepared by dissolving 4 g of tryptone, 2 g of yeast extract and 2 g of NaCl in dH_2O to a final volume of 400 ml. LB agar (LA) was prepared as for LB with 1.5 % w/v of agar. Both media types were autoclaved before use.

LAS

USE: LAS was used as counterselection media for allelic exchange

LB agar with 10 % sucrose (LAS) was prepared by dissolving 4 g of tryptone, 2 g of yeast extract, 6 g of agar powder and 40 g of sucrose in dH_2O to a final volume of 400 ml.

Simmon's Citrate Agar

USE: Selection for A. baumannii during conjugation experiments

9.2 g of Simmon's citrate agar powder (Oxoid) was dissolved in a final volume of 400 ml dH_2O. Media was autoclaved before use.

SOC (Super Optimal broth with Catabolite repression)

USE: Broth for non-selective outgrowth of bacteria post-transformation

SOC was prepared by dissolving 5 g of tryptone, 2.5 g of yeast extract and 5 g of NaCl into 200 ml of dH_2O. After autoclaving, 50 µl of 2 M $MgCl_2$ (filter sterilized) and 200 µl of 1M glucose (filter sterilized) were added to 1 ml of medium.

Appendix 2. Reagents

Tris-EDTA (TE) buffer

10 mM Tris-HCl, pH 8.0

1 mM EDTA, pH 8.0

5x SDS sample loading buffer

10 % (w/v) SDS

5 % (v/v) β-mercaptoethanol

20 % (v/v) glycerol

0.2 M Tris-HCl pH 6.8

0.05 % (w/v) bromophenolblue

50x Tris-acetate-EDTA (TAE) buffer

2 M Tris-HCl

2 M Acetic acid

50 mM EDTA

20 × SSC (1 L)

175.3 g of NaCl

88.2 g of sodium citrate

800 mL dH_2O. pH to 7.0 with

10 M NaOH

Add dH_2O to 1 L

Sterilised by autoclaving

10 x Washing Buffer

1 M Maleic acid

1. 5 M NaCl, pH 7.5 (20°C)

3 % (v/v) Tween 20

10 x Maleic acid buffer

1 M Maleic acid

1. 5 M NaCl

adjust with NaOH (solid) to pH 7.5 (20°C)

10 x Detection buffer

1 M Tris-HCl

1 M NaCl, pH 9.5 (20°C)

Appendix 3. *E. coli* and *A. baumannii* wildtype strains

Strain designation	KR number[a]	Relevant characteristics	Reference
***E. coli* wildtype strains**			
DH5α	1231	F- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(rK-mK+) *phoA supE*44 λ- *thi*-1 *gyrA96 relA1*	(Hanahan 1983)
S17-1λpir	145	F- *thi pro hsdR- recA*::RP4-2-Tc::Mu Km::Tn7 λ*pir*	Kanamycin (Simon, Priefer & Pühler 1983)
MG1655	2577	F- λ- *ilvG- rfb*-50 *rph*-1	(Edwards, Palsson 2000)
***A. baumannii* wildtype strains**			
A1	105	Isolate obtained from the private collection of Dr Kevin Towner, QMC, originally from the UK	
A13	106	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A14	107	Isolate obtained from the private collection of Dr Kevin Towner, QMC, originally from the Netherlands	
A20	108	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A92	109	Isolate obtained from the private collection of Dr Kevin Towner, QMC, originally from Spain	
A97	111	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A63	112	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A332	113	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A52	114	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
1237 (A473)	1237	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A472	261	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A387	262	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A474	263	Isolate obtained from the private collection of Dr Kevin Towner, QMC	

Strain designation	KR number[a]	Relevant characteristics	Reference
A401	265	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A369	266	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A384	267	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A397	268	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A380	269	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A392	270	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A457	272	Isolate obtained from the private collection of Dr Kevin Towner, QMC, originally from Estonia	
A377	273	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A443	274	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A388	275	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A442	276	Isolate obtained from the private collection of Dr Kevin Towner, QMC, originally from Bulgaria	
A371	277	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A418	278	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A424	279	Isolate obtained from the private collection of Dr Kevin Towner, QMC, originally from Croatia	
A390	280	Isolate obtained from the private collection of Dr Kevin Towner, QMC, originally from Bulgaria	
A297	281	Isolate obtained from the private collection of Dr Kevin Towner, QMC, originally from the Netherlands	
A473	301	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A25	331	Isolate obtained from the private collection of Dr Kevin Towner, QMC, originally from the UK	
A37	332	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A167	335	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A186	336	Isolate obtained from the private collection of Dr Kevin Towner, QMC	

Strain designation	KR number[a]	Relevant characteristics	Reference
A187	337	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A230	339	Isolate obtained from the private collection of Dr Kevin Towner, QMC, originally from the UK	
A329	340	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A335	341	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A343	342	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A365	343	Isolate obtained from the private collection of Dr Kevin Towner, QMC, originally from the UK	
A479	345	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A480	346	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A481	347	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
A482	348	Isolate obtained from the private collection of Dr Kevin Towner, QMC	
1376	2339	Sputum isolate obtained from UHL-LRI 22/11/2007	
1377	2340	Sputum isolate obtained from UHL-LRI 26/11/2007	
1378	2341	Sputum isolate obtained from UHL-LRI 25/11/2007	
1379	2342	Extra-tracheal aspirate isolate obtained from UHL-LRI 20/10/2007	
1380	2343	Broncho-tracheal lavage isolate obtained from UHL-LRI 10/11/2007	
1381	2344	Sputum isolate obtained from UHL-LRI 06/11/2008	
1401	2345	Wound swab of trachea isolate obtained from UHL-LRI 31/10/2008	
1402	2346	Sputum isolate obtained from UHL-LRI 08/12/2008	
1403	2347	Urine isolate obtained from UHL-LRI 30/01/2009	
1404	2348	Peripheral blood culture isolate obtained from UHL-LRI 18/10/2007	
UHL-Crete	3116	Isolate from road traffic accident patient transferred from Crete to UHL-LRI. Resulted in closure of LRI-ICU	
ACICU	1306	Clone of an outbreak from Rome; sequenced	(Iacono et al. 2008)
AYE	1153	Epidemic strain in France; sequenced	(Fournier et al. 2006)

Strain designation	KR number[a]	Relevant characteristics	Reference
AB0057	1935	Bloodstream isolate from Walter Reed Army Medical Center; sequenced	(Adams et al. 2008)
ATCC 19606	623	Type strain available from ATCC; sequenced	(Peleg et al. 2012)
Other strains			
MGH78578	KR640	*K. pneumoniae* sputum isolate; sequenced	Washington University

[a] KR number corresponds to a physical catalogue in Dr Kumar Rajakumar's laboratory. [b] UHL-LRI, University Hospitals Leicester-Leicester Royal Infirmary

Appendix 4. *A. baumannii* mutant strains constructed in this study

Strain designation	KR number[a]	Relevant characteristics	Resistance[b]
AB0057 *umuD*$_{Ab}$::pJ-hyg-umuD$_{Ab}$	KR3867	*A. baumannii* AB0057 with pJ-hyg-umuD$_{Ab}$ inserted downstream of *umuD*$_{Ab}$	Hyg, Cml
AYE *umuD*$_{Ab}$::pJ-hyg-umuD$_{Ab}$	KR3868	*A. baumannii* AYE with pJ-hyg-umuD$_{Ab}$ inserted downstream of *umuD*$_{Ab}$	Hyg, Cml
AB0057 Δ*umuD*$_{Ab}$	KR3981	*A. baumannii* AB0057 with an insertion/deletion mutation of *umuD*$_{Ab}$;Δ*umuD*$_{Ab}$::*hph* constructed by allelic exchange	Hyg
AYE Δ*umuD*$_{Ab}$	KR4001	*A. baumannii* AYE with an insertion/deletion mutation of *umuD*$_{Ab}$;Δ*umuD*$_{Ab}$::*hph* constructed by allelic exchange	Hyg

[a] KR number corresponds to a physical catalogue in Dr Kumar Rajakumar's laboratory. [b] Hyg, hygromycin; Cml, chloramphenicol.

Appendix 5. Plasmids used in this study

Plasmid name	Alternative designation	pKR number[a]	Relevant characteristics	Resistance[b]	Reference
Commercial and published plasmids					
pQE-80L	-	pKR313	Expression vector with phage T5 promoter and *lacIQ*	Amp	Qiagen
pGEM-T Easy	-	-	TA cloning vector for PCR products	Amp	Promega
pJTOOL-3	-	pKR35	Lambda pir-based suicide vector with added MCS	Cml	(van Aartsen, Rajakumar 2011)
pWH1266	-	pKR646	*E. coli – A. baumannii* shuttle vector	Amp, Tet	ATCC
pIJ773	-	-	Contains RP4 *oriT* and FRT-flanked *aacA4* resistance cassette	Amp, Apr	(Gust et al. 2003)
pFLP2	-	pKR19	FLP recombinase-expressing plasmid	Amp	(Hoang et al. 1998)
Plasmids constructed in this study					
pQE-bla	pQE-int-bla	pKR477	pQE-80L-based vector expressing *intI1* under a P_{lac} promoter, repressed with $LacI^q$. Also contains a single gene cassette integron (bla_{IMP-5}).	Amp	This work
pQE-WH-bla	-	pKR736	As for pQE-bla, but containing the pWH1277 origin of replication amplified from pWH1266	Amp	This work
pQE-BHR2A	pQE-bla-BHR2A	pKR563	As for pQE-bla, but containing an broad host-range origin of replication from pRO1600 in orientation 1	Amp	This work
pG-AYEint		pKR468	pGEM-T vector containing an integron from AYE amplified using primers PR1562 and PR1377	Amp	This work
pJ-hyg-$umuD_{Ab}$	pJ-umu_{Ab}-hyg	pKR648	contains SOE product for creating knock out of $umuD_{Ab}$ in *A. baumannii* using allelic exchange, replacing $umuD_{Ab}$ with hygromycin gene	Cml, Hyg	This work

Plasmid name	Alternative designation	pKR number[a]	Relevant characteristics	Resistance[b]	Reference
pWHA-umuD$_{Ab}$	pWH-apra-umuD	pKR738	1378 bp BamHI-SalI fragment excised from pIJ773, containing *aac3-IV* and *oriT*; 1019 bp product (PR3409/PR3474) from AYE; *umuD$_{Ab}$* gene added by PvuI and BamHI; ligated into pWH1266	Amp, Apr	This work

[a] KR number corresponds to a physical catalogue in Dr Kumar Rajakumar's laboratory. [b] Amp, ampicillin; Apr, apramycin; Cml, chloramphenicol; Hyg, hygromycin; Tet, tetracycline;

Appendix 6. Primers used in this study

Primer name	Catalogue number[a]	Sequence[b]	Special features	Reference
5'-CS	PR121	GGCATCCAAGCAGCAAG		
3'-CS	PR122	AAGCAGACTTGACCTGA		
GmF	PR351	CGAATTAGCTTCAAAAGCGCTCTGA		
qacEDelta1F	PR978	GCGAAGTAATCGCAACATCC		
sul1D	PR1213	CCGACTTCAGCTTTTGAAGG		
intI1L	PR1241	ACATGTGATGGCGACGCACGA		
intI1R	PR1242	ATTTCTGTCCTGGCTGGCGA		
intI2L	PR1243	CACGGATATGCGACAAAAAGGT		
intI2R	PR1244	GTAGCAAACGAGTGACGAAATG		
intI3L	PR1245	GCCTCCGGCAGCGACTTTCAG		
intI3R	PR1246	ACGGATCTGCCAAACCTGACT		
AbaR1_Med_Int-Rev	PR1377	ATA**GAATTC**ATTGCGAATCGTGATGAACC	5' EcoRI restriction site for directional cloning	
AbaR1_Med_Internal-For	PR1562	AGT**CTCGAG**CGTGATCGAAATCCAGATCC	5' XhoI restriction site for directional cloning	
Aba Int R	PR1569	ATA**AAGCTT**GCTGCACTGAACGTCAGAAG	5' HindIII restriction site for directional cloning	
blaIMP-5_cF	PR1679	TTGGAGACGCATCACTCTTG		

Primer name	Catalogue number[a]	Sequence[b]	Special features	Reference
blaIMP-5_cR	PR1680	AACCACCAAGCCGTGTTTAG		
intI1-F3	PR1719	TAC**GAATTC**AATAAAGAGGAGAAATTAACTATGAAAACCGC CACTGCGC	5' EcoRI restriction site for directional cloning	
16S 338F	PR1734	ACTCCTACGGGNGGCNGCA		
16S 515R	PR1735	GTATTACCGCNNCTGCTGGCAC		
sul1Cinv	PR1751	GTCTAAGAGCGGCGCAATAC		
aacCc-R	PR1953	CGACCGAAAAGATCAAGAGC		
aacCc-F	PR1954	TACGTGCAAGCAGATTACGG		
aadA1c-R	PR1955	AGCCGAAGTTTCCAAAAGGT		
aadA1c-F	PR1956	GGCGATGAGCGAAATGTAGT		
orfXc-R	PR1957	CGTCATAGTGAGACGCTCCA		
orfXc-F	PR1958	CAGGTGTTTCGCTCTTCACA		
blaVEB cF	PR2020	GACATTGCAAAAATAACGTGGAA		
blaVEB cR	PR2021	AAGTTGTCAGTTTGAGCATTTGAA		
Aba Int-F4	PR2022	AGGGCAGATCCGTGCACA		
hp-cF	PR2023	TCATACTGCTCGGGCCTATT		
hp-cR	PR2024	CACCACGTCACTACCGTCAA		
Citrato R12	PR2026	GCAGAGATACCAGCAGAGATACACG		(Bartual et al. 2005)

Primer name	Catalogue number[a]	Sequence[b]	Special features	Reference
RA2	PR2034	GTTTCTGGGCTGCCAAACATTAC		(Bartual et al. 2005)
drfI cF	PR2044	TTACCCAAGACTTCGCCTCT		
drfI cR	PR2045	CCCGATAACTCCATTCTTCG		
aac6-Ib cF	PR2233	GAGGCAAGGTACCGTAACCA		
aac6-Ib cR	PR2234	CATAGAGCATCGCAAGGTCA		
aadB cF	PR2257	GGAAAAGGTTGAGGTCTTGC		
Aba Int R2	PR2259	CAGCGAAGTCGAGGCATT		
AbRecAF	PR2260	CAAAATGGCTCCTCCGTTTA		
AbRecAR	PR2261	GGCCAATTTTATTGCCTTGA		
aadB cR2	PR2320	AATGTGACCTGCGTTGTGTC		
catB8 cR	PR2436	TCAGAAAGAAGTTCCCCTTTGA		
catB8 cF	PR2437	TCGTCAAATATTTTTGGTCTGC		
gyrB_Ab_F	PR2439	CACCCTGAAGAAGGGGTTTC		
gyrB_Ab_R	PR2440	AACACCTACGCCGTGTAAGC		
oriT-F2-NcoI	PR2510	CTC**CCATGG**GCTCACTCAAAGGCGGTAAT	5' NcoI restriction site	
oriT-R2-NcoI	PR2511	CTC**CCATGG**CTGGCCTTTTGCTCACATAA	5' NcoI restriction site	
qacED1 R3	PR2523	GGATGTTGCGATTACTTCGC		
IS6 tnpF	PR2524	TTGCAAATAGTCGGTGGTGA		

Primer name	Catalogue number[a]	Sequence[b]	Special features	Reference
3'CSBinv	PR2525	GCTCACAGCCAAACTATCAGG		
BHRoriR-F-BamHI	PR2546	CG**GGATCC**CGGCATCAGAGCAGATTGTA	5' BamHI restriction site	
ColE1-F	PR2602	GGCGCTTTCTCATAGCTCAC		
ColE1-R	PR2603	AGTCGTGTCTTACCGGGTTG		
BHRoriR-R2-BamHI	PR2614	CG**GGATCC**ATCGCCCTTCCCAACAGT	5' BamHI restriction site	
gdhB2 M13 R	PR2717	CAGGAAACAGCTATGACCTATGGGGGCCAGATAATCAA	5’ M13 R primer tag	
gdhB M13 F	PR2718	TGTAAAACGACGGCCAGTTGAGTCGGAGTATGCTGTGC	5’ M13 F primer tag	
Gm-R2	PR2773	AATTGGGGATCTTGAAGTTCCT		
Group1ompAF306	PR2938	GATGGCGTAAATCGTGGTA		(Turton et al. 2007)
Group1and2ompAR660	PR2939	CAACTTTAGCGATTTCTGG		(Turton et al. 2007)
Group1csuEF	PR2940	CTTTAGCAAACATGACCTACC		(Turton et al. 2007)
Group1csuER	PR2941	TACACCCGGGTTAATCGT		(Turton et al. 2007)
Gp1OXA66F89	PR2942	GCGCTTCAAAATCTGATGTA		(Turton et al. 2007)

Primer name	Catalogue number[a]	Sequence[b]	Special features	Reference
Gp1OXA66R647	PR2943	GCGTATATTTTGTTTCCATTC		(Turton et al. 2007)
Group2ompAF378	PR2944	GACCTTTCTTATCACAACGA		(Turton et al. 2007)
Group2csuEF	PR2945	GGCGAACATGACCTATTT		(Turton et al. 2007)
Group2csuER	PR2946	CTTCATGGCTCGTTGGTT		(Turton et al. 2007)
Gp2OXA69F169	PR2947	CATCAAGGTCAAACTCAA		(Turton et al. 2007)
Gp2OXA69R330	PR2948	TAGCCTTTTTTCCCCATC		(Turton et al. 2007)
umuD-UF-F	PR3002	AGC**CTGCAG**CTTTCGGCTGCTTCACTTTC	5' PstI restriction site	
umuD-UF-R	PR3003	**AGGAACTTCAAGATCCCCAATT**CATATCGCCTCCATTTCACC	5’ SOE PCR overlapping region with Gm-R2	
umuD-DF-F	PR3004	**TCAGAGCGCTTTTGAAGCTAATTCG**GGGGCAAGAACTGGTGATTT	5’ SOE PCR overlapping region with GmF	
umuD-DF-R	PR3005	CGG**CTGCAG**TGAAAAGGCTCAGTCATGGTT	5' PstI restriction site	
UmuD SF	PR3075	TGAGTGGGTAAGGGGATGTAA		

Primer name	Catalogue number[a]	Sequence[b]	Special features	Reference
UmuD SR	PR3076	TTTTATTGGTAGCGGCTGGT		
pWHoriR-R-BamHI	PR3136	CG**GGATCC**CGTTCGGGTCTTTCATGTCT	5' BamHI restriction site	
pWHoriR-F-BamHI	PR3137	CG**GGATCC**AATGCAGGCAAGTTGATTCC	5' BamHI restriction site	
Citrato F1 M13 F	PR3253	**TGTAAAACGACGGCCAGT**AAT TTACAGTGGCACATTAGGTCC C	5’ M13 F primer tag	(Bartual et al. 2005)
gyrB_F M13 F	PR3254	**TGTAAAACGACGGCCAGT**TGAAGGCGGCTTATCTGAGT	5’ M13 F primer tag	(Bartual et al. 2005)
gyrB_R	PR3255	GCTGGGTCTTTTTCCTGACA		(Bartual et al. 2005)
RA1 M13 F	PR3256	**TGTAAAACGACGGCCAGT**CCTGAATCTTCYGGTAAAAC	5’ M13 F primer tag	(Bartual et al. 2005)
cpn60_F M13 F	PR3257	**TGTAAAACGACGGCCAGT**GGTGCTCAACTTGTTCGTGA	5’ M13 F primer tag	(Bartual et al. 2005)
cpn60_R	PR3258	CACCGAAACCAGGAGCTTTA		(Bartual et al. 2005)
rpoD-F M13 F	PR3259	**TGTAAAACGACGGCCAGT**ACCCGTGAAGGTGAAATCAG	5’ M13 F primer tag	(Bartual et al. 2005)
rpoD-R	PR3260	TTCAGCTGGAGCTTTAGCAAT		(Bartual et al. 2005)

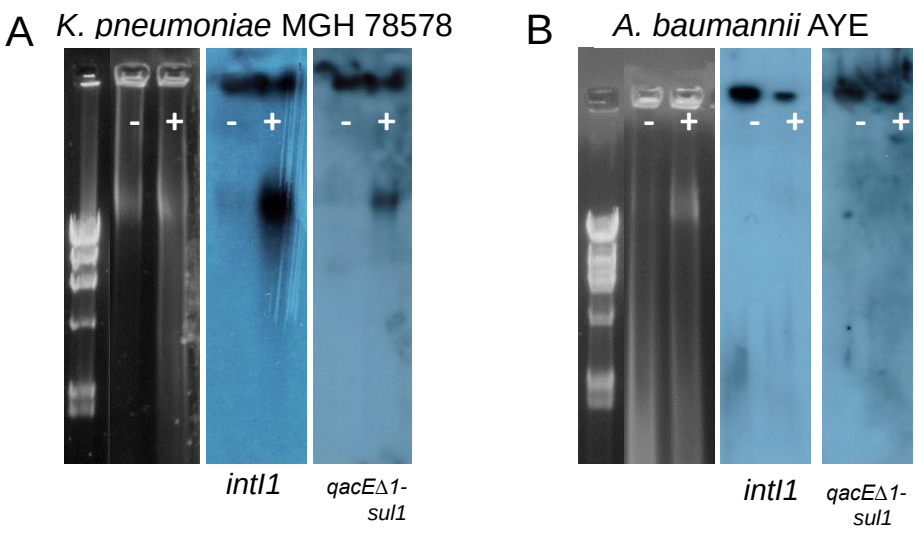
Primer name	Catalogue number[a]	Sequence[b]	Special features	Reference
hyg-SR	PR3313	CTCGCCGAACAGCTTGATC		
hyg-SF	PR3314	CAACTGCATCTCAACGCCTT		
aac nest F	PR3323	GCTCATCAATCTCCTCAAGCA		
aac nest R	PR3324	CCGGCTGATGTTGGGAGTA		
umuD-F-PvuI	PR3409	AT**CGATCG**CATTTCGCATGTCTCCATACCA	5' PvuI restriction site	
umuD-R-BamHI	PR3474	CGC**GGATCC**GGTGGCTTGATGGTCGTTAT	5' BamHI restriction site	
GroEL F	PR3507	GATCACGCTCGGTTCTTCAC		
ISAba1R	PR3508	GAGCTAATCCTCTGTACACGAC		
gyrBEc-F	PR3509	GCTGCGGAATGTTGTTGGTA		
gyrBEc-R	PR3510	AGAACAAAACGCCGATCCAC		
intI1-seq-F	PR3546	GTTACCACCGCTGCGTTC		
intI1-seq-R	PR3547	CCGCCAACTTTCAGCACAT		

[a] Catalogue number corresponds to a physical primer catalogue in Dr Kumar Rajakumar's laboratory. [b] Primer sequence (5' to 3' orientation) with special features highlighted in bold and underlined text.

Appendix 7. S1 nuclease digestion method

A further method by which integrons or other genetic elements may be simply located to the plasmid or chromosome is through S1 nuclease digestion of plasmids, an enzyme isolated from *Aspergillus oryzae*. In a study by Barton, et al. (1995), it was shown that through its nicking action, S1 nuclease can convert supercoiled plasmids into linear fragments, allowing the plasmid to travel through a gel in a manner consistent with its linearized size, thus facilitating a greater accuracy in plasmid sizing. This method is useful in this instance, because due to their large size, many supercoiled mega plasmids may become entangled with the chromosomal DNA, which prevents their travel through conventional gel separation methods. In addition, S1 nuclease is useful in such instances, as in this case, where the samples were clinical isolates containing unknown plasmids. Since the S1 nuclease functions by nicking and does not have a specific restriction site, it is not necessary to know restriction maps of the plasmids before use.

Figure S7.1 Detection of integrons on plasmids using S1 nuclease method



Southern blot analysis with Si nuclease treated DNA from cell lysate. *K. pneumoniae* MGH 78578 (A) is known to contain an integron on a megaplasmid; *A. baumannii* AYE (B) contains integrons on the chromosome only. Samples were run without and with S1 treatment, indicated by a – and a + at the top of each lane, respectively.

It can be seen from Figure S7.1 that without S1 nuclease treatment, insufficient amount of plasmid is able to travel from the well in order to give a

clear band to which the probe can bind. With the use of the S1 enzyme, a strong band is seen in the positive control strain *K. pneumoniae* MGH 78578, which is known to contain a plasmid-borne integron. Conversely, under the same experimental conditions, negative control strain *A. baumannii* AYE (known to contain only chromosomal integrons) does not show binding to any band, with or without S1 nuclease treatment. Both cases show some binding in the gel well region, which may be due to retardation of the chromosome and very large plasmids in the gel. The plasmid size for pKPN3, the plasmid known to contain an integron is 175,879 in size.

The strong bands in the top of the well show that the probe has bound to the chromosomal DNA, which has too large molecular weight in order to move through the 0.8 % agarose. Strong binding was also observed in the well of the S1 nuclease-treated *K. pneumoniae* strain, suggesting that the nicking of the plasmid was incomplete, possibly due to some plasmid remaining inaccessible to the enzyme because of intercalation with the chromosomal DNA.

Although this method gives a useful and quick way of determining whether a specific genetic target is carried on the chromosome or on the plasmid, it might give ambiguous results for genes that are carried on both genetic structures. In addition, this method may not be able to illuminate whether a strain is carrying multiple integrons on the same structure, since the plasmids are only linearised, and not broken down into smaller segments. Due to this, it was decided that the AleI digestion method was to be used for surveying the integron-positive strains, as the results from this method gave a more enriched view of integron carriage in isolates.