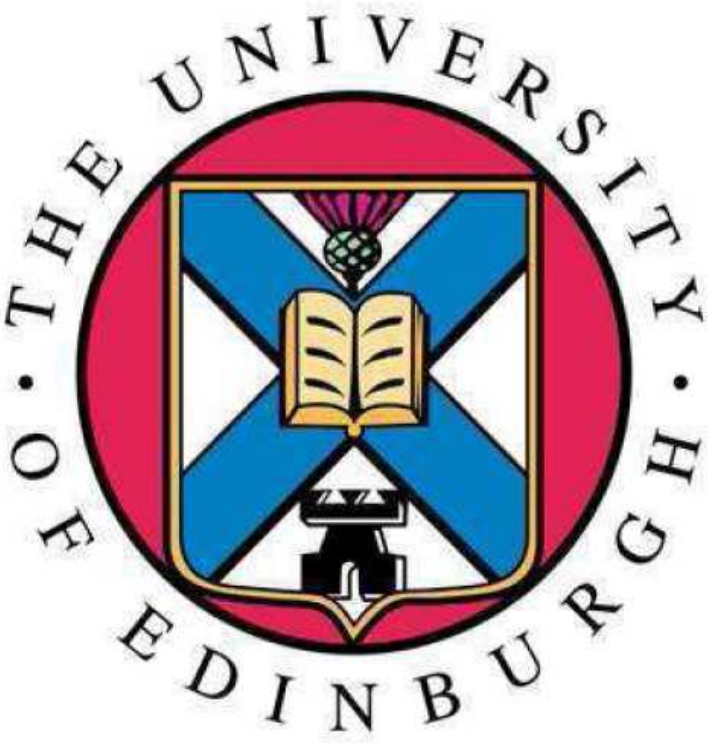
THE UNIVERSITY *of* EDINBURGH

This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e.g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

- This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
- A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
- This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
- The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
- When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.

Role of Insertion Sequences in the control of antibiotic resistance in *Acinetobacter baumannii*

Bruno S. Lopes



Thesis presented for the degree of
Doctor of Philosophy

University of Edinburgh
2011.

i. Abstract

Acinetobacter baumannii is an emerging multiresistant pathogen increasingly known to cause infections in the immuno-compromised patients. Carbapenems and colistin are considered to be the last resorts in treatment of infections involving multidrug resistant strains of *A. baumannii*. Resistance to carbapenems is well known due to the presence of intrinsic carbapenemase gene $bla_{\text{OXA-51-like,}}$ which may be governed by insertion elements, or by acquired carbapenemases like $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-58-like}}$ or $bla_{\text{OXA-40-like}}$ genes, most of which are frequently associated with insertion elements.

The acquired carbapenemases can be integrated with the host chromosome making the bacterium strongly resistant to a range of antibiotics. Recent reports also suggest that the ubiquitous and intrinsic enzymes encoded by the $bla_{\text{OXA-51-like}}$ gene can be mobilized on a plasmid.

In this thesis, the prevalence of antibiotic resistance was examined for 96 strains isolated from various parts of the world. The resistances to aminoglycosides, fluoroquinolones and cephalosporins were studied with a major focus on resistance to carbapenems.

Section 1 shows the transposition of IS*Aba1* and its varied influence in controlling the $bla_{\text{OXA-51-like}}$ gene and the bla_{ADC} gene. It explains how IS*Aba1* being a strong factor in influencing antibiotic resistance genes contributes to the plasticity of the organism

Section 2 is related with a novel insertion element IS*Aba125* controlling the *bla*$_{\text{ADC}}$ gene and as an element providing high resistance to ceftazidime in comparison to IS*Aba1*.

Section 3 analyses the multi-drug resistant profile of strains isolated from Cochabamba, Bolivia. Besides the classification of carbapenem resistance for the clinical strains, the aminoglycoside resistance and ciprofloxacin mechanisms are examined in this project

Section 4 relates with the pattern of resistance in strains isolated from the Aberdeen Royal Infirmary. It describes two novel variants of the *bla*$_{\text{OXA-51-like}}$ gene, namely *bla*$_{\text{OXA-216}}$ and *bla*$_{\text{OXA-217}}$ and also the acquisition of the *bla*$_{\text{OXA-23-like}}$ gene in two isolates from different years and deemed identical by their PFGE pattern.

Section 5 describes the influence of IS*Aba825* in controlling the *bla*$_{\text{OXA-51-like}}$ gene and the *bla*$_{\text{OXA-58-like}}$ from clinical isolates

Section 6 is related with the insertional inactivation of the *bla*$_{\text{OXA-132}}$ gene and the carbapenem resistance caused by the activation of the *bla*$_{\text{OXA-58}}$ gene in isolate Ab244

Section 7 describes the influence of insertion elements in strains having high ciprofloxacin resistance. This project is concerned with the role of efflux pump control system *adeRS* and how they influence the *adeABC* operon causing increased and decreased expression of the genes.

Section 8 describes the multi drug resistant pattern of 36 strains each isolated from Europe and the United States

In conclusion, there are various factors that influence the resistance profile of multi-drug resistant *A. baumannii* isolates with insertion sequences such as IS*Aba1*, IS*Aba2*, IS*Aba3*, IS*Aba825*, IS*1008*, IS*Aba125*, IS*Aba16* governing the expression or providing alternate mechanisms of resistance for the better fitness of the bacterium. Mutations in the genes identified in this study also have a crucial role in imparting resistance to this bacterium.

ii. Declaration.

The experiments and composition of this thesis are the work of the author unless otherwise stated.

Bruno S. Lopes

iii. Dedication

This thesis is dedicated to

My family who is the source of wisdom, love, support, strength and perseverance

And to

Catharine McAuley

Some great thing which He designs to accomplish would have been too much without a little bitter in the cup……..Catharine McAuley

iv. Acknowledgements.

Firstly I would like to acknowledge my supervisor Professor Sebastian Amyes whose advice and support has been invaluable for the completion of this study. I would like to thank him for not only his advice but also for the freedom he gave me in designing my own experiments which led me to think independently and because of which I had many fruitful and surprising outcomes.

I would like to thank Dr A. Hamouda for making me familiar with the technical skills involved in Molecular Microbiology which helped me to achieve my objectives.

Thanks to Prof. Lucía Gallego who worked with me on her summer project with her strains from Cochabamaba, Bolivia leading to a fruitful outcome. I would like to thank Dr Ian Gould for providing me with strains from Aberdeen Royal Infirmary. I would like to thank Dr Kevin Towner for providing me with all the European *Acinetobacter baumannii* isolates used in this study.

I would also like to thank Andres Opazo for having fruitful discussions on various laboratory techniques related with *A. baumannii*. Special thanks to Juachi, Maher, Leena, Monem and Norya who always wished the best for me and encouraged me to achieve my goals. Thanks to Jacqueline Finday, Ben Evans and Paul Higgins for their expertise. Thanks to Malcolm Baldock for the processing of orders and adding to the stock of chemical and reagents.

A very special thanks to Dr Cathy Doherty for allowing me to use all the various reagents and machines required for microbiological and molecular methods without which my work would not have been completed on time.

And last but not the least I would like to thank Prof Stephen Hillier and Dr Dorothy Watson for their help in providing me with the University of Edinburgh Overseas Research Scholarship and the College of Medicine and Veterinary Medicine bursary.

v. Permission to reproduce papers.

Published manuscripts have been reproduced here in accordance with the copyright guidelines of the publishers involved.

vi. Publications and presentations.

Lopes, B. S., A. Hamouda, J. Findlay, and S. G. B. Amyes. 2011. Effect of frame-shift mutagen acriflavine on control of resistance genes in *Acinetobacter baumannii*. *J. Med Microbiol*. **60:** 211-215.

Lopes, B. S., B. A. Evans and S .G.B Amyes. 2011. The disruption of the *bla*$_{\text{OXA-51-like}}$ gene by IS*Aba16* and the activation of the *bla*$_{\text{OXA-58}}$ gene leading to carbapenem resistance in *Acinetobacter baumannii* Ab244. *J Antimicrob Chemother*. **67**: 59-63. (Epub ahead of print on 5 October 2011)

Lopes, B. S., I. M. Gould, A.F. Opazo, S. G. B Amyes. 2011. The resistance profile of *Acinetobacter baumannii* strains isolated from the Aberdeen Royal Infirmary (Accepted in *Int J Antimicrob Agents*).

Lopes, B. S., and S.G.B Amyes. Role of IS*Aba825* in the expression of the *bla*$_{\text{OXA-51-like}}$ gene in clinical strains of *Acinetobacter baumannii* (Oral, ICAAC, 2011 Chicago)

Lopes, B. S., and S.G.B Amyes. The role of Insertion Elements in control of antibiotic resistance in *Acinetobacter baumannii* isolated from sources throughout the world (Oral, ICAAC, 2011 Chicago).

Opazo, A. F., B. S. Lopes, A. Sonnevend, T. Pal, A. Ghazawi, and S.G.B. Amyes. Ceftazidime resistance in *Acinetobacter baumannii* from the United Arab Emirates (Oral, ICAAC, 2011 Chicago).

Lopes, B., A. Hamouda, S. Amyes. The role of IS*30* in the expression of the *bla*$_{\text{ADC}}$ gene in *Acinetobacter baumannii* (Poster, ECCMID 2011, Milan, Italy)

Lopes, B., A. Hamouda, S. Amyes. The utilisation of gluconic acid in certain strains of *Acinetobacter baumannii* (Abstract, ECCMID 2011, Milan, Italy)

Lopes B. S., and S. G. B Amyes. 2011. First report of IS*Aba825* governing the expression of *bla*$_{\text{OXA-51-like}}$ gene in clinically relevant strains (Submitted to CMI).

Lopes, B. S., and S. G. B Amyes. 2011. The prevalence of IS*Aba1* and IS*Aba125*, governing the expression of the *bla*$_{\text{ADC}}$ in clinically relevant *Acinetobacter baumannii* strains resistant to cephalosporins (Submitted to JMM).

Lopes, B. S, L. Gallego and S. G. B Amyes. 2011. Multi drug resistance profiles and the genetic features that influences the resilient adaptation in *Acinetobacter baumannii* isolates from Bolivia (Submitted to JAC).

Opazo, A., A. Sonnevend, B. Lopes, A. Hamouda, A. Ghazawi, T. Pál, and S. G.B. Amyes.2011. Plasmid-encoded PER-7 beta-lactamase responsible for ceftazidime resistance in *Acinetobacter baumannii* isolated in the United Arab Emirates. (Accepted in JAC)

vii. Abbreviations

ADC	*Acinetobacter*-derived cephalosporinases
ARDRA	Amplified ribosomal DNA restriction analysis
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
BSAC	British Society for Antimicrobial Chemotherapy
CLSI	Clinical and Laboratory Standards Institute
cm	Centimetres
CN	Gentamicin
DNA	Deoxyribonucleic acid
EC	European clone
ESAC	Extended-spectrum AmpC
ESBL	Extended-spectrum β-lactamase
ICU	Intensive care unit
IST	Isosensitest
kb	Kilo-base pairs
kDa	Kilo-Dalton
L	Litre
M	Molar
Mg	Milligram
MIC	Minimum inhibitory concentration
Min	Minute
mL	Milli-litre
mM	Millimolar
NCBI	National Center of Biotechnology Information
NCTC	National Collection of Type Cultures
OMP	Outer-membrane protein
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	potential Hydrogen, measure of acidity or basicity of a solution
pmol	Pico-moles
RNA	Ribonucleic acid
RND	Resistance-nodulation-division
Rpm	Revolutions per minute
rRNA	Ribosomal-ribonucleic acid
s	Seconds
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis.
TAE	Tris/acetate/ ethylenediaminetetraacetic acid
TBE	Tris/borate/ethylenediaminetetraacetic acid
U	Units
V	Volts
w/v	Weight by volume
µL	Microlitre
µM	Micro-molar

Contents.

1.0 INTRODUCTION

1.1 The genus *Acinetobacter*

1.1.1 An historical perspective

The genus *Acinetobacter* has a complex history, and only recently it has become possible to distinguish various species within the genus. Phylogenetic classification places the genus *Acinetobacter* in the group of *Gammaproteobacteria* of the *Proteobacteria* (Juni, 2005). The history of the genus *Acinetobacter* begins in 1911 when the Dutch microbiologist Beijerinck isolated a micro-organism from soil by enrichment in calcium acetate containing minimal medium (Beijerinck, 1911). This organism was named as *Micrococcus calco-aceticus.* Over the following decades similar organisms were described and assigned to different genera and species, e.g; *Achromobacter anitratus* (Brisou, 1953), *Achromobacter mucosus* (Mannheim and Stenzel, 1962), *Alcaligenes haemolysans* (Henriksen, 1973), *Bacterium anitratum* (Schaub and Hauber, 1948) , *Diplococcus mucosus* (von Lingelsheim, 1908), *Herellea vaginicola* (DeBord, 1942), *Mima polymorpha* (DeBord, 1939), *Moraxella lwoffi* (Audureau, 1940), *Neisseria winogradskyi* (Lemoigne *et al*., 1952). The genus *Acinetobacter* (greek- akinetos, i.e nonmotile) was proposed by Brisou and Prévot in 1954 to separate the non-motile micro-organisms from the motile ones within the genus *Achromobacter*. Baumann *et al* (1968) published a comprehensive survey and concluded that the species listed above belonged to the proposed *Acinetobacter* genus. They also concluded that further phenotypic classification of various species was not possible (Baumann *et al*., 1968). By the end of 1980 the names *A. calcoaceticus* for strains producing acids from sugars and *A. lwoffii* for non acid- producing strains were approved (Skerman *et al*., 1980)

1.1.2 Taxonomic classification of *Acinetobacter* species

The genus *Acinetobacter* has been revised extensively since 1986. It comprises of 33 different species of which 22 are named (Table 1) (Dijkshoorn *et al*., 2007, Nemec *et al*., 2009, Nemec *et al*., 2011) and further 28 groups have been identified. The genus *Acinetobacter*, as currently defined, comprises of Gram-negative, strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive, oxidase-negative bacteria with DNA G+C content of 39% to 47% (reviewed by Peleg *et al*., 2008). It was proposed in 1991 that the members of the genus *Acinetobacter* should be classified in the new family *Moraxellaceae* within the order *Gammaproteobacteria*, which includes the genera *Moraxella, Acinetobacter, Psychrobacter* and other related organisms (Rossau *et al*., 1991). Twelve *Acinetobacter* genospecies were delineated on the basis of DNA-DNA hybridization (Bouvet and Grimont, 1986). Further work done by Bouvet and Jeanjean, Tjernberg and Ursing, and Nishimura *et al* added to the description of further *Acinetobacter* genomic species (Bouvet and Jeanjean., 1989, Tjernberg and Ursing, 1989, and Nishimura *et al*., 1988). *A. calcoaceticus*, *A. baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter* genomic species 13 TU are very closely related, it is very difficult to distinguish them from each other by phenotypic characteristics and these species are referred to as the *A. calcoaceticus- A. baumannii* complex (Grener-Smidt, 1992, Grener-Smidt *et al*., 1991). Further studies are essential to assess the relatedness of species clusters in more detail.

Table 1. Classification of the genus *Acinetobacter* **(Dijkshoorn *et al*., 2007, Nemec *et al*., 2009, Nemec *et al*., 2010, Nemec *et al*., 2011)**		
Named species	**Genomic species**	**Source**
Acinetobacter calcoaceticus	1	Soil and humans (including clinical specimens)
Acinetobacter baumannii	2	Humans (including clinical specimens), soil, meat and vegetables
Acinetobacter haemolyticus	4	Humans (including clinical specimens)
Acinetobacter junii	5	Humans (including clinical specimens)
Acinetobacter johnsonii	7	Humans (including clinical specimens) and animals
Acinetobacter lwoffii	8,9	Humans (including clinical specimens) and animals
Acinetobacter radioresistens	12	Humans (including clinical specimens), soil and cotton
Acinetobacter ursingii		Humans (including clinical specimens)
Acinetobacter schindleri		Humans (including clinical specimens)
Acinetobacter parvus		Humans (including clinical specimens) and animals
Acinetobacter baylyi		Activated sludge and soil
Acinetobacter bouvetii		Activated sludge
Acinetobacter towneri		Activated sludge
Acinetobacter tandoii		Activated sludge
Acinetobacter grimontii		Activated sludge
Acinetobacter tjernbergiae		Activated sludge
Acinetobacter gerneri		Activated sludge
Acinetobacter venetianus		Sea water
Acinetobacter pittii	3	Humans (including clinical specimens), soil and vegetables
Acinetobacter nosocomialis	13TU	Humans (including clinical specimens)
	6	Humans (including clinical specimens)
Acinetobacter bereziniae	10	Humans (including clinical specimens), soil and vegetables
Acinetobacter guillouiae	11	Humans (including clinical specimens) and animals
	13BJ, 14TU	Humans (including clinical specimens)
	14BJ	Humans (including clinical specimens)
	15BJ	Humans (including clinical specimens)
	16	Humans (including clinical specimens) and vegetables
	17	Humans (including clinical specimens) and soil
	15TU	Humans (including clinical specimens) and animals
	‘Between 1 and 3’	Humans (clinical specimens)
	‘Close to 13TU’	Humans (clinical specimens)

1.1.3 Importance of *Acinetobacter* species

Acinetobacter baumannii has emerged as one of the most troublesome pathogens for health care institutions globally (reviewed by Peleg *et al*., 2008). It has a very high incidence in most hospitals and the misuse of broad-spectrum antibiotics has led to an increase in multi-drug resistance of this bacterium. Also the natural resistance of *Acinetobacter* spp. to several β-lactam antibiotics has caused an increase in its resistance profile (Amyes and Young, 1996). *A. baumannii* strains resistant to all known antibiotics have now been reported, signifying a sentinel event that should be acted on promptly by the international health care community (reviewed by Peleg *et al*., 2008). Acting in synergy with this emerging resistance profile is the uncanny ability of *A. baumannii* to survive for prolonged periods, thus potentiating its ability to spread. The organism mostly targets the vulnerable hospitalized patients who are critically ill with breaches in skin integrity and airway protection (reviewed by Peleg *et al*., 2008). The most common infection caused by this organism is hospital-acquired pneumonia (Glew *et al*., 1977). Although, recent reports suggest that it can also cause central nervous system and skin and soft tissue infections (reviewed by Peleg *et al*., 2008).

1.1.4 Identification and typing of *Acinetobacter* species

Acinetobacters may be identified presumptively as Gram-negative, catalase-positive, oxidase-negative, nonmotile and nonfermenting coccobacilli (reviewed by Peleg *et al*., 2008). The scheme proposed by Bouvet and Grimont in 1987 included growth at 37, 41 and 44°C; production of acid from glucose; gelatine hydrolysis; and assimilation of 14 different carbon sources (Bouvet and Grimont, 1987). This system correctly identified 136 *Acinetobacter* spp (95.6%) to the species level (Seifert *et al*.,

1997). Since this method of identification is laborious it is far from being suitable for routine laboratory microbiology testing in the labs. Species identification of acinetobacters for laboratory diagnostic purposes is currently done by systems such as API 20NE, VITEK 2, Phoenix, and MicroScan WalkAway. These phenotypic systems can be problematic. The four species belonging to *A. calcoaceticus- baumannii* complex can only be distinguished by molecular methods. Table 2 describes the genotypic methods available for *Acinetobacter* species identification (Seifert and Dijkshoorn, 2008)

Table 2. Genotypic methods for *Acinetobacter* species identification (Seifert and Dijkshoorn, 2008)

Method	**Target structure**
AFLP™ analysis	Whole genome
tRNA spacer fingerprinting	tRNA spacer
PCR-RFLP	16S rDNA sequence (in ADRA analysis)
	*rec*A
	16-23S spacer rDNA
Ribotyping	rDNA and adjacent regions
Hybridization with oligonucleotide probe	16-23S spacer rDNA
DNA sequence analysis	16S rDNA
	16-23S spacer rDNA
	gyrB
	recA
	rpoB
	efp

Amplified rDNA restriction analysis (ADRA) and amplified fragment length polymorphism (AFLP) are currently the most widely accepted and validated reference methods for species identification of acinetobacters with a large library of profiles available for both reference and clinical strains (reviewed by Peleg *et al*., 2008). ADRA is similar to the PCR-RFLP methods. The 16S rDNA sequence is amplified and separated by agarose electrophoresis after digestion with five different restriction

enzymes each. The combined patterns generated with the five enzymes are then compared to a library of profiles of strains of all known species (Seifert and Dijkshoorn, 2008). The ADRA combined with phenotypic characterization, i.e., consensus identification can compensate for the limitations of each of the two approaches (Nemec *et al*., 2000). AFLP analysis is a high resolution whole genomic fingerprinting method by which restriction fragments are selectively amplified (Vos *et al*., 1995). A simplified protocol as compared to the original procedure was set up at Leiden University Medical Center (LUMC). In this procedure, *Eco*RI and *Mse*I are used for restriction and the DNA is ligated to adapters in a single step, after which selective amplification is achieved with an Mse-C primer and a Cy-5 labeled Eco-A primer. Amplified fragments are separated electrophoretically on a sequencing machine and the banding patterns are analyzed by cluster analysis with dedicated software.

More recent developments include the identification of *A. baumannii* by detection of the $bla_{\text{OXA-51-like}}$ intrinsic to this species (Brown *et al*., 2005, Turton *et al*., 2006b).

1.1.5 Natural habitat of *Acinetobacter* species

Acinetobacter species are generally isolated from environmental samples such as soil and sludge, vegetables or from clinical human samples (Table 1).

Many members of the genus are generally considered to be environmental organisms as they are ubiquitously present in the environment (reviewed by Peleg *et al*., 2008). This holds true for the genus *Acinetobacter*, as they can be recovered from virtually all samples obtained from soil or surface water after enrichment (Baumann, 1968) but

not all species of the genus *Acinetobacter* have their natural habitat in the environment.

Acinetobacters are part of the human skin flora. In an epidemiological survey performed to investigate the colonization of human skin and mucous membranes with *Acinetobacter* species, up to 43% of non-hospitalized individuals tested positive (Seifert *et al*., 1997). The most frequently isolated species were *A. lwoffii* (58%), *A. johnsonii* (20%), *A. junii* (10%), and *A. pittii* (previosuly genomospecies 3) (6%). In a similar study, a carrier rate of 44% was found in healthy volunteers, with *A. lwoffii* (61%), *Acinetobacter* genomic species 15BJ (12%), *A. radioresistens* (8%), and *Acinetobacter pittii* (5%) being the most prevalent species (Berlau *et al*., 1999). In patients hospitalized on a regular ward, the carriage rate with these species was even higher, at 75% (Seifert *et al*., 1997). The faecal carriage of *Acinetobacter* was 25% among healthy individuals, with *A. johnsonii* and *Acinetobacter* genomic species 11 predominantly present (Dijkshoorn *et al*., 2005). A total of 226 samples were examined.

In a study of the microbial communities from the extreme environments of six Andean lakes, a total of five different acinetobacters were isolated, one of which was *A. johnsonii* (Ordonez *et al*., 2009). Members of the species *A. calcoaceticus* have been isolated from the soil surrounding plant roots and have been shown to promote plant growth *in vitro* (Peix *et al*., 2009). *A. baylyi*, another organism isolated from the soil, has been shown to be able to acquire plant DNA through horizontal gene transfer or by conjugation (Pontiroli *et al*., 2009). An *A. baumannii* isolate from petroleum-

contaminated desert soil in Kuwait has been shown to be able to degrade crude oil (Obuekwe *et al*., 2009).

A. baumannii, the most important nosocomial *Acinetobacter* spp, was found only rarely on human skin and in human faeces, and *Acinetobacter nosocomialis* could not be detected at all (Seifert *et al*., 1997; Berlau *et al*., 1999; Dijkshoorn *et al*., 2005). *A. baumannii* was occasionally found as aetiological agents in infected animals (Francey *et al*., 2000; Vaneechoutte *et al*., 2000). *A. baumannii* was recovered from 22% of body lice sampled from homeless people (La Scola and Raoult, 2004). It has been speculated that this finding might result from clinically silent bacteraemia in this group; the clinical implication of this observation, however, is not yet clear. In Hong Kong, 51% of local vegetables were found to be contaminated with acinetobacters, the majority of which were *A. pittii* (75%), but one sample grew *A. baumannii* (Houang *et al*., 2001). In particular, it has to be demonstrated that it is not human handling of vegetables that are the source of *A. pittii*. Acinetobacters were found in 22 of 60 soil samples collected in Hong Kong, and the most frequent species were *A. pittii* (27%) and *A. baumannii* (23%); only one sample yielded *A. calcoaceticus* (Houang *et al*., 2001).

Infected animals and surface contaminated vegetables and water can act as a source of transmission for *Acinetobacter* spp, allowing it to freely enter the human food chain. Furthermore silent carriers and fomites may also be responsible for the spread of this pathogenic bacterium.

A study of skin carriage among healthy individuals in UK showed an Acinetobacter carriage rate of 44% , with *A. lwoffii* accounting for 61% of isolates, *Acinetobacter* genospecies 15BJ accounting for 12.5%, and *A. radioresistens* accounting for 8% (Berlau *et al*., 1999). The percentage of Acinetobacters in a group of healthy people was 42.5%, mainly *A. lwoffii*, followed by *A. johnsonii*, and to a lesser extent of *A. junii* (Seifert *et al*., 1997). In a study performed in Hong Kong, it was found that 53% of medical students and new nurses colonized with acinetobacters in summer versus 32% in winter (Chu *et al*., 1999). A seasonal variation in the frequency of *Acinetobacter* infections was also observed in USA and attributed to increased humidity in the summer months (Retailliau *et al*., 1979; McDonald *et al*., 1999).

In conclusion, *A. baumannii*, *A. pittii* and *A. nosocomialis* may be found rarely in environmental samples, but they are emerging pathogens in the hospital environment and are frequently isolated from various clinical sources. More studies need to be performed to get a better understanding of the distribution of *Acinetobacter* species in the environment, in humans, and in animals. Systematic surveys in different geographical settings and under different climatic conditions should be carried out for detailed investigation purposes.

1.2 Clinical manifestations of infections caused by *A. baumannii.*

Acinetobacter baumannii is rapidly emerging as a multiresistant bacterium and today it can be considered as the paradigm of antibiotic resistant bacteria. The major problems confronting the clinicians in the ICUs are related to the severity of the clinical situation and multi-drug resistance (Bergogne- Bérézin, 2008). Acinetobacters are commonly found in the hospital environment, as colonizers of the patient's skin

and mucous membranes and they are responsible for the spread of nosocomial infections. The three major factors responsible for the strength of this bacterium are resistance to major antimicrobial agents, resistance to dessication and to disinfectants (Seifert and Dijkshoorn, 2008).

1.2.1 Urinary tract infections

A. baumannii rarely causes UTI with just 1.6% of ICU-acquired UTI, mainly catheter associated infection or colonization. Although, there has been a gradual increase in UTI caused by *A. baumannii* (Gaynes and Edwards, 2005). The use of endotracheal tubes, intravascular, ventricular, or urinary catheters often leads to opportunistic bacteria colonizing the site (Joly-Guillou, 2008). It is not unusual for this bacterium to cause uncomplicated UTI in healthy outpatients (reviewed by Peleg *et al*., 2008).

1.2.2 Meningitis

Acinetobacter is a rare cause of meningitis although post-neurosurgical *A. baumannii* meningitis is becoming an increasingly important entity with mortality rates as high as 64% been reported in patients with meningitis due to *A. baumannii* (Garcia-Garmendia *et al*., 2001). Sporadic cases have been reported following neurosurgical procedures (Chen *et al*., 2005). An outbreak of Acinetobacter meningitis was described in a group of children with leukaemia following administration of intrathecal methotrexate due to inappropriately sterilized needles which lead to death of three children. Risk factors for meningitis include the presence of a continuous connection between the ventricles and the external environment, a ventriculostomy, a CSF fistula, presence of an indwelling ventricular catheter for more than 5 days, and the use of antimicrobial agent. A wide prevalence of multiresistant Acinetobacter

causing meningitis in neurosurgical patients has been seen in Turkey (Metan *et al.*, 2007). The surveillance of local pathogens in neurosurgical wards should guide the selection of proper therapy with an effective infection control program (Joly-Guillou, 2008).

1.2.3 Suppurative infections

In recent times, acinetobacter infections involving skin and soft tissue have become very problematic. This may be because strains of *Acinetobacter* species are the only group of Gram-negative bacteria that may be present naturally on the human skin (Seifert *et al*., 1997). It was reported by Gaynes and Edwards (2005) that *Acinetobacter* was responsible for 2.1% of ICU-acquired skin/soft tissue infection. It is a very difficult pathogen to eradicate from a burns unit (Trottier *et al*., 2007). *A. baumannii* is commonly isolated from wounds of combat casualties from Iraq or Afghanistan (reviewed by Peleg *et al*., 2008). It was the most commonly isolated pathogen from patients with open tibial fractures, but due to its low pathogenicity at this site, it was completely eradicated (Johnson *et al*., 2007).

1.2.4 Bloodstream infections

A. baumannii was the 10th most common aetiologic agent, responsible for 1.3% of all nosocomial bloodstream infections in the United States (reviewed by Peleg *et al*., 2008). *A. baumannii* was a more common cause of ICU-acquired bloodstream infection than of non-ICU-ward infection (1.6% versus 0.9% of bloodstream infections, respectively, in those locations). Crude mortality overall from *A. baumannii* bloodstream infection was 34.0% to 43.4% in the ICU and 16.3% outside the ICU. *A. baumannii* bloodstream infection had the third highest crude mortality

rate in the ICU, exceeded only by infections caused by *P. aeruginosa* and *Candida* spp. *A. baumannii* infections were the latest of all bloodstream infections to occur during hospitalization, occurring at a mean of 26 days from the time of hospital admission (reviewed by Peleg *et al*., 2008). It is therefore not certain if the high crude mortality rate represents its occurrence in patients with ongoing underlying critical illness or whether the organism does have significant attributable mortality. It is notable that 102 patients had bloodstream infections at sites treating U.S. military members injured in Iraq or Afghanistan from 1 January 2002 and 31 August 2004 (reviewed by Peleg *et al*., 2008). Recent report suggests that *A. baumannii* is resistant to a range of antibiotics and, most importantly, *A. baumannii* bacteraemia has a worse prognosis than bacteraemia due to non-*A. baumannii* isolates (Lee *et al*., 2010).

1.2.5 Hospital acquired infections

A. baumannii infection is a pathogen of considerable importance associated with high mortality in patients with pneumonia (Fagon *et al*., 1989). It is one of the most important causes of nosocomial pneumonia especially after the use of a mechanical ventilator. The incidence of nosocomial pneumonia infections caused by *A. baumannii* between 1976 and 1990 increased from 0.64% to 6.4% (McDonald *et al*., 1999). In a review from the CDC, 7% of ICU-acquired pneumonias were due to Acinetobacter in 2003, compared to 4% in 1986 (Gaynes and Edwards, 2005). The majority of *A. baumannii* isolates were from the respiratory tracts of hospitalized patients. In many circumstances, it is very difficult to distinguish upper airway colonization from true pneumonia although there is no doubt that *A. baumannii* does cause ventilator-associated pneumonia (VAP). In large surveillance studies from the United States, between 5 and 10% of cases of ICU-acquired pneumonia were due to

A. baumannii (Gaynes and Edwards, 2005). The number of ICU-acquired pneumonia is very much a cause of concern today. Infections are associated with immunocompromised patients, with infection rates often being highest in intensive care units (ICUs) and surgical wards. It has been reported that 15% of all nosocomial infections were hospital-acquired pneumonia with the mortality rate exceeding 30% (Timsit *et al*., 1996). However, mechanical ventilation is a critical risk factor for developing hospital acquired pneumonia in patients who require prolonged mechanical ventilation (>48hrs); hospital acquired pneumonia occurs within 48hrs of incubation (Rello *et al*., 1999). *A. baumannii* is directly responsible for patient death, and therefore the risk factors for colonization between ventilation associated pneumonia due to *A. baumannii* infection could differ from other bacteria responsible for similar infection (Baraibar and Colleges, 1997). Patients with *A. baumannii* infections have had prolonged ICU stays, although in outbreak situations, earlier acquisition of infection may occur (reviewed by Peleg *et al*., 2008). Prolonged survival of *A. baumannii* in a clinical setting, i.e. on patients bed rails, has been found associated with an ongoing outbreak in an intensive care unit and illustrates that dry vectors can be secondary reservoirs where *A. baumannii* can survive (Catalano *et al*., 1999).

The proportion of individuals with previous MDR *A. baumannii* isolation who remain carriers for prolonged periods was substantial in an Israeli hospital. Surveillance cultures involved six different body sites including nostrils, pharynx, skin, rectum, wounds, and endotracheal aspirates (Marchaim *et al*., 2007). It remains to be determined if long-term carriage of MDR *A. baumannii* in an individual patient may contribute to transmission of these organisms within and outside the hospital.

Epidemiological surveys involving acinetobacters have identified *A. baumannii* to be by far, the most frequent cause of infection, though there are reports of higher rates of isolation of *A. pittii* (Lim *et al*., 2007, Boo *et al*., 2009). While *A. pittii* and *A. nosocomialis* tend to cause infections sporadically they are usually relatively susceptible to antibiotics but the same cannot be said of *A. baumannii*. Mortality rates for *Acinetobacter* species other than *A. baumannii* have been found to be low, with values between 7% and 18% being reported (Seifert *et al*., 1994, Choi *et al*., 2006). However, crude mortality rates of between 8% and 43% have been reported for *A. baumannii* (Falagas *et al*., 2006). Additionally, the length of stay in hospital associated with *A. baumannii* infection was significantly longer than that for other *Acinetobacter* species (Choi *et al*., 2006).

1.2.6 Community acquired infections

Community-acquired pneumonia due to *A. baumannii* has been described for tropical regions of Australia and Asia (reviewed by Peleg *et al*., 2008). The source of infection may be throat carriage, which occurs in up to 10% of community residents with excessive alcohol consumption (Anstey *et al*., 2002).The infection is seen mainly in people with a history of alcohol abuse and may sometimes require admission to an ICU (Anstey *et al*., 2002). It is generally a fulminating infection or a secondary bloodstream infection, with the mortality rate as high as of 40 to 60% (Leung *et al*., 2006).

1.2.7 Other manifestations

Endocarditis due to *Acinetobacter* is a rare pathology with high mortality, reported mainly in hospitalized patients with predisposing risk factors (Valero *et al*, 1999). *Acinetobacter* spp. may cause endophthalmitis or keratitis, sometimes related to contact lens use or following eye surgery (Corrigan *et al*., 2001). *Acinetobacter* has been implicated in isolated infections following invasive ocular surgeries. Although ocular infections are rare, a recent study did associate *Acinetobacter* with adverse ocular responses in patients who wore extended-wear soft contact lenses (Corrigan *et al*., 2001). *A. baumannii* was also reported to cause preseptal and orbital cellulitis in a 39 year old woman (Miller, 2005). A case report also exists of a Shiga toxin-2 producing *A. haemolyticus* strain, which was associated with bloody diarrhoea in a 3-month-old infant in Pereira Rossell Pediatric Hospital, Uruguay (Grotiuz *et al*., 2006).

1.3 Molecular basis of *Acinetobacter* infections

A pathogen is a microorganism that is able to cause disease. Pathogenicity refers to the ability of microorganism to cause disease in a host organism. The pathogenicity of a microbe is expressed by its virulence, a term which refers to the degree of pathogenicity of the microbe. It has pathogenic determinants or virulence factors that enable it to infection the host. Virulence is the pathogenicity degree of a microorganism that can vary between the members of a same species of pathogens (Braun, 2008). The survival rates of Acinetobacters are very high with *A. radioresistens* surviving for an average of 157 days (Jawad *et al*., 1998) and *A. baumannii* beyond 30 days while *A. lwoffii* up to 21 days in the UK hospitals (Houang *et al*., 1998). This clearly shows that *Acinetobacter* spp can survive desiccation

persisting in the environment for many days. *Acinetobacter* also has been shown to survive better than other Gram-negative bacteria such as *E. coli* (Jawad *et al*., 1996).

1.3.1 *Acinetobacter* adhesins and fimbriae

The first step of infection is the ability of microorganism to penetrate the host and initiate the infection. It depends on the capacity of the microorganism to adhere and survive on the host mucosal surfaces. Bacterial adhesins are cell surface components that facilitate the adhesion of bacteria to inanimate surfaces; these include the fimbriae (pili), capsular polysaccharides, or cell wall components (Braun, 2008). The adherence of *A. baumannii* to the bladder tissue is a natural attribute of different strains, a comparable trait found in an uropathogenic strain of *Escherichia coli* (Sepulveda *et al*., 1998). Bacteria that express type 1 fimbriae are characterized by the ability to agglutinate erythrocytes in the absence of D-mannose. Fimbrial structures were observed in *A. baumannii* by transmission electron microscopy. The haemagglutining activity of the strains was not inhibited by either D-mannose or D-galactose (Sepulveda *et al*., 1998).The two types of adherence of *A. baumannii* to human bronchial epithelial cells were dispersed adherence of bacteria to the surface of the cell and adherence of clusters of bacteria at localized areas of the cell to form microcolonies. Scanning electron microscopy showed thin fimbrial-like extensions on the bacterial surface that were firmly anchored to the membrane surface of the cells. There were no significant quantitative differences in adherence between outbreak and non-outbreak strains (Lee *et al*., 2006b). The expression of a chaperone usher secretion system is required for fimbriae formation and the concomitant attachment to plastic surfaces and the ensuing formation of biofilms (Tomaras *et al.*, 2003). It has been hypothesized that there exists a correlation between the PER-1 gene that codes

for a beta-lactamase and Caco 2 cell adhesion in *Acinetobacter* strains (Sechi *et al*., 2004).

1.3.2 Toxins and enzymes

Many bacteria synthesize enzymes or toxins or have cellular constituents that play a role in toxic action or direct necrosis under inflammatory cells and other components of the host immune system (Braun, 2008). The presence of lipopolysaccharide (LPS) in a bacterium can influence the pathogenicity by causing macrophage activation, fever, and cardiovascular changes leading to shock and death (Peterson, 1996). The O polysaccharide of *Acinetobacter baumannii* and its antibodies have been investigated intensively (Haseley and Wilkinson, 1998). They are specific for a given serotype (Pantophlet *et al*., 2002). About the lipidic fraction, the chemical composition of lipid A of *A. calcoaceticus* exhibits unusual features, but its toxic and other biological properties are similar to those of enterobacterial endotoxin (Brade and Galanos, 1983). The endotoxins of both *A. baumannii* and *Acinetobacter* 'genomospecies 9' are potent stimulators of inflammatory signalling in human monocytic cells and that responses to these bacteria are dependent on both TLR2 and TLR4. An exaggerated innate immune response to the endotoxins of these organisms may contribute to the pathology of *Acinetobacter* infection. Hence *Acinetobacter baumannii* lipopolysaccharides are potent stimulators of human monocyte activation via Toll-like receptor 4 signalling (Erridge *et al*., 2007). *Acinetobacter baumannii* outer membrane protein A (AbOmpA) induced early-onset apoptosis and delayed-onset necrosis in dendritic cells. AbOmpA targeted the mitochondria and induced the production of reactive oxygen species (ROS). ROS are directly responsible for both apoptosis and necrosis of AbOmpA-treated dendritic cells. These results demonstrate that the

AbOmpA secreted by outer membrane vesicles from *A. baumannii* induces dendritic cell death, which may impair T cell biology to induce adaptive immune responses against *A. baumannii.* AbOmpA is a potential virulence factor that induces epithelial cell death (Lee *at al*., 2010). The ability of *A. baumannii* to produce esterase and urease may be responsible for damaging the lipid tissues and colonize the hypochlorhydric or achlorhydric walls of the stomach, thus inducing inflammation (Braun, 2008).

1.3.3 Surface and mitochondrial porins

Gram-negative bacteria are resistant to a large number of toxic agents as a result of the effective permeability barrier function of their outer membrane (OM) (reviewed by Vaara, 1992).

The OM is impermeable to macromolecules and allows only limited diffusion of hydrophobic substances through its LPS-covered surface (reviewed by Vaara, 1992). The small number and sizes of the different porins when coupled with efflux provide a significant barrier to the uptake of antibiotics in *A. baumannii* (Vila *et al*., 2007). Porins play a variety of roles depending on the bacterial species, including the maintenance of cellular structural integrity, bacterial conjugation and bacteriophage binding, antimicrobial resistance and pore formation to allow the penetration of small molecules (Braun, 2008). The outer membrane structures are integral part of the efflux assembly responsible for extrusion of toxic compounds from the cell. It is worth mentioning that even in the presence of a fully functional *acrAB* efflux pump, the loss of integrity of the outer membrane protein results in no resistance being seen in *E. coli* cells (Nikaido, 2001). This may not be entirely true for *Acinetobacter*

baumannii. Several studies state that a decrease in porin expression is associated with antimicrobial resistance in *A. baumannii* (Mussi *et al*., 2005; Siroy *et al*., 2006). It has been reported by Clark (1996) that imipenem resistance in *A. baumannii* is due to reduced expression of 33-36 kDa outer membrane protein. It has also been reported that a loss of 29 kDa outer membrane protein is associated with resistance to imipenem in strains having no carbapenemase activity (Limansky *et al*., 2002). The major band of outer membrane proteins (OMPs) of *A. baumannii* on SDS-polyacrylamide gel electrophoresis is a 38 kDa mitochondrial porin. Omp38 localizes to the mitochondria and induces a release of cytochrome c and apoptosis-inducing factor (AIF) into cytosol, which mediates caspase dependent and AIF-dependent apoptosis in epithelial cells. The cytotoxic effects of *A. baumannii* were investigated in human laryngeal HEp-2 cells, and the findings indicate that this bacterium induces apoptosis of HEp-2 *in vitro* through both caspase-dependent cascades, which are mediated by cell surface signalling and mitochondrial disintegration, and the AIF-dependent pathway. Purified Omp38 also induced apoptosis of human bronchial epithelial cells and human monocyte cells. Apoptosis of epithelial cells may disrupt the mucosal lining and allow the access of bacteria or bacterial products into the deep tissues (Choi *et al*., 2005). Mucins secreted by epithelial cells may offer a protective role and act as a barrier protecting against bacterial infections (Derrien *et al*., 2010).

1.3.4 The role of siderophores

Acinetobacter synthesizes siderophores which are relatively low-molecular weight agents, capable of converting polymeric ferric oxy-hydroxides to soluble iron chelates under low iron stress (Neilands, 1995). The ability of bacteria to assimilate iron is known to be related to invasiveness. The lung and systemic infections, where there is

iron restriction, suggest the presence of an iron uptake system in *Acinetobacter*. The presence of iron chelator 2, 3-dihydroxybenzoic acid (DHBA) and iron-repressible outer membrane proteins was detected in the culture supernatant of *A. calcoaceticus* (Smith *et al*., 1990). The *A. baumannii* clinical strains were able to grow under iron-deficient conditions, and some of them excreted an iron-regulated catechol siderophore into the culture supernatants (Echenique *et al*., 1992, Actis *et al*., 1993).

This siderophore was different from DHBA and other bacterial catechol-type siderophores (Braun, 2008). The structure of acinetobactin, a novel siderophore with both catechol and hydroxamate functional groups, isolated from *A. baumannii* ATCC 19606 was elucidated (Yamamoto *et al*., 1994). The same study proved that there exists strain-to-strain variation in the ability to produce acinetobactin (Yamamoto *et al*., 1994). The chemical structure of this siderophore is highly related to the iron chelator anguibactin produced by the fish pathogen *Vibrio anguillarum* (Dorsey *et al*., 2004).

Iron-regulated proteins are present in both inner and outer membranes of clinical strains of *A. baumannii*. The presence of two iron-regulated proteins localized in the inner membrane was reported (Echenique *et al*., 1992). The Fur protein responsible for iron uptake in *A. baumannii* was found to bear 63% similarity with that of *E. coli* (Daniel *et al*., 1999). Since Fur and Fur-like repressors are known to regulate some virulence determinant genes in other bacteria, the *A. baumannii* Fur-like repressor protein may also regulate a subset of genes with a role in pathogenesis (Braun, 2008).

1.3.5 Host- pathogen interactions

Many factors like strain virulence, host factors and bacterial load are responsible for the host-pathogen interactions. They play a crucial role in progression of disease in an infected host and can have detrimental effects on the host. A significant number of identified genes encoding resistance to antibiotics, heavy metals, and antiseptics likely originated in other highly pathogenic organisms (Fournier *et al*., 2006). A comparison between the whole genome of *A. baumannii* 17978 and the non pathogenic species *A. baylyi*, revealed 28 gene clusters unique to *A. baumannii*, with 16 having a potential role in virulence (Smith *et al*., 2007). The *csu* polycistronic operon involves five genes, some of which are homologous to genes that encode proteins related to chaperones and involved in pilus assembly in Gram-negative bacteria (Tomars *et al*., 2003). It has been shown that biofilm formation in *A. baumannii* is phenotypically associated with exopolysaccharide production and pilus formation (Tomars *et al*., 2003). This may explain the success of *A. baumannii* to form biofilms on inanimate objects in hospital environment. Adherence of *A. baumannii* to human bronchial epithelial cells and erythrocytes has also been demonstrated, with similar pilus-like structures (Gospodarek *et al*., 1998). Quorum sensing also has been shown to regulate a wide array of virulence mechanisms in many Gram-negative organisms. The quorum sensing molecules of the N-acyl homoserine lactones (AHSL) have been identified in *Acinetobacter*, indicating that this may be a central mechanism for auto-induction of various virulence factors (Gonzalez *et al*., 2001; Joly Guillou, 2005). The three molecules identified are 3-oxo substituted with acyl chain length from 4 to 12 carbons; 3- unsubstituted AHSL (with the exception of C4) and 3- hydroxyl AHSL (C6, C8 and C10) (Gonzalez *et al*., 2001).

Apart from biofilm formation, exopolysaccharide production is also thought to protect bacteria from host defenses (Joly Guillou, 2005). Recent studies have described the innate immune response to *A. baumannii* and the importance of Toll-like receptor (TLR) signaling (Knapp *et al*., 2006; Erridge *et al*., 2007). In a mouse pneumonia model, TLR4 gene-deficient mice showed increased bacterial counts, increased bacteraemia, impaired cytokine/chemokine responses, and delayed onset of lung inflammation compared to wild-type mice (Knapp *et al*., 2006). *A. baumannii* lipopolysaccharide (LPS) was also identified as the major stimulatory factor for triggering immune response (Knapp *et al*., 2006). This was further illustrated by the attenuated effects of *A. baumannii* on mice deficient in CD14, an important molecule that enables LPS binding to TLR4 (Knapp *et al*., 2006). These findings were confirmed using human cells but, in contrast to the mouse model, TLR2 was also identified as an important signaling pathway potent endotoxic potential of *A. baumannii* LPS, which stimulated the inflammatory cytokines interleukin-8 and tumour necrosis factor alpha equally to the stimulation by *E. coli* LPS at similar concentrations (Erridge *et al*., 2007). These studies suggest that *A. baumannii* endotoxin may incite a strong inflammatory response during infection.

Humoral immune responses have also been described for *Acinetobacter* infection, with antibodies being targeted toward iron-repressable OMPs and the O polysaccharide component of LPS (Smith and Alpar, 1991). Mouse-derived monoclonal antibodies directed at *A. baumannii* OMPs expressed in an iron-depleted environment have bactericidal and opsonizing effect, blocking the iron uptake (Goel and Kapil, 2001). Deliberately induced infections in well-defined animal models provide much useful information about disease processes. The results of experimental

infections should be interpreted carefully as they do provide information about disease processes in the natural context (Joly-Guillou and Wolff, 2008).

1.4 Resistance mechanisms in *A. baumannii*

Acinetobacter spp. have an extraordinary ability to develop multiple resistance mechanisms against major antibiotic classes – they have become resistant to broad spectrum β-lactams; third generation cephalosporins, carboxypenicillins, and increasingly to carbapenems. They produce a wide range of aminoglycoside-inactivating enzymes and most strains are resistant to fluoroquinolones (Bergone-Bérézin, 2008). The rapid global emergence of *A. baumannii* strains resistant to all β-lactams, including carbapenems, illustrates the potential of this organism to respond swiftly to changes in selective environmental pressure (reviewed by Peleg *et al*., 2008).

There are various mechanisms of resistance that makes *A. baumannii* a successful pathogen of the 21st century. They include the class A β-lactamases which may also be extended spectrum β-lactamases (Nagano *et al*., 2004; Naiemi *et al*., 2005; Pasteran *et al*., 2006), class B β-lactamases which have metal ions governing the active site, hence they are also known as metallo β-lactamases (Chu *et al*., 2001; Lee *et al*.,2003; Lee *et al*., 2005); class C β-lactamases which are acinetobacter-derived cephalosporinases or the chromosomally-encoded AmpC enzymes (Bou and Martinez-Beltran, 2000), and class D β-lactamases which are mainly oxacillinases involved in resistance to carbapenems (Paton *et al*., 1993, reviewed by Peleg *et al*., 2008,).

Other enzymatic mechanisms of resistance include the aminoglycoside-modifying enzymes like acetyltransferases, nucleotidyltransferases and phosphotransferases (reviewed by Peleg *et al*., 2008). Besides all these resistance mechanisms efflux pumps like AdeABC (Magnet *et al*., 2001), AdeIJK (Damier-Piolle *et al*., 2008), AdeXYZ (Roca *et al*., 2011) AdeM (Su *et al*., 2005) and the tetracycline-specific efflux pumps Tet(A) and Tet(B) also play an important role in conferring resistance to various other antimicrobials (Guardabassi *et al*., 2000; Ribera *et al*., 2003a; Ribera *et al*., 2003b).

1.4.1 Antimicrobial agents

The modern era of chemotherapy began with the work of the German physician Paul Ehrlich. He coined the term 'chemotherapy' in the early 20th century in a search for compounds to act as ''magic bullets'' to treat bacterial infections (reviewed by Freter and Perry, 2008). According to the American Biochemist and Microbiologist Selman Waksman, 'It was the knowledge of the great abundance and wide distribution of actinomycetes, which dated back nearly three decades, and the recognition of the marked activity of this group of organisms against other organisms that led me in 1939 to undertake a systematic study of their ability to produce antibiotics.' He coined the term "antibiotic" in 1942 to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilution (Waksman, 1947). Antibiotics are chemicals produced by or derived from microoganisms that inhibit the growth of other microorganisms. The term antibiotic is now widely used to refer to all drugs that selectively target bacteria.

1.4.1.1 Penicillins

Alexander Fleming recognized the antibacterial activity of a substance secreted by *Penicillium notatum* on a contaminated culture plate in 1929 and thus discovered penicillin (Bush, 2010).

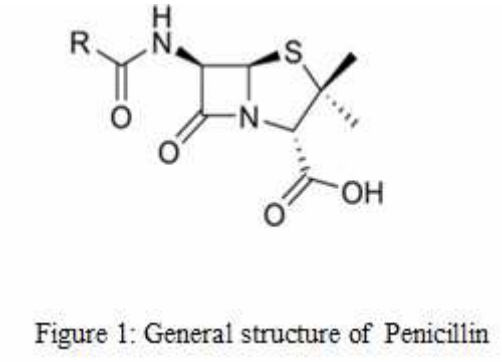


Figure 1: General structure of Penicillin

Penicillin (Figure 1) impairs cell wall synthesis by preventing cross-linking of the peptidoglycan polymers necessary for cell wall formation and does this by binding to the penicillin-binding proteins (PBPs) (carboxypeptidases, endopeptidases, and transpeptidases) that participate in cell wall synthesis (Tomaz, 1979). Allergy to any of the penicillins is the only absolute contraindication to the use of a penicillin agent. Allergic reactions occur in up to 10% of patients, with 0.001% dying from anaphylaxis (Lin, 1992).

1.4.1.2 Cephalosporins

The first cephalosporin (Figure 2) was discovered in 1948 by Guiseppe Brotzu, who observed that the fungus *Cephalosporium acremonium* produced a substance that inhibited the growth of *S. aureus* and other bacteria (reviewed by Griffith and Black, 1964). The cephamycins were created by adding a methoxy group on the β-lactam ring of the original compound, based on the structure of cefoxitin, produced by *Streptomyces lactamdurans*. By altering the chemical groups substituted on the basic

molecule, greater antimicrobial activity and longer half-lives have been obtained (Kees and Grobecker, 1995)

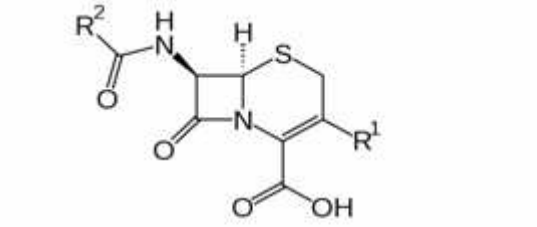


Figure 2: General structure of Cephalosporin

Cephalosporins are β-lactams that have the β-lactam ring fused with a dihydrothiazine ring. Cephalosporins inhibit bacterial cell wall synthesis by blocking the transpeptidases and other PBPs involved in the synthesis and cross-linking of peptidoglycan (Wise, 1990; Fontana *et al.*, 2000). Resistance to cephalosporins may result from mutations in the penicillin-binding proteins resulting in altered binding site or due to the production of extended-spectrum beta-lactamases responsible for the hydrolysis of the drug or both (Koch, 2000). Recently developed cephalosporins include ceftaroline and ceftobiprole which are regarded as 5th generation cephalosporins (Kollef, 2009).

1.4.1.3 Carbapenems

Carbapenems (Figure 3) which include meropenem, imipenem, biapenem, doripenem, razupenem and ertapenem are parenteral synthetic β-lactams derived from thienamycin, an antibiotic produced by *Streptomyces cattleya* (Kahan *et al*., 1979; Chen and Livermore, 1994; Livermore *et al*., 2009). Carbapenems have a lactam ring, like the penicillins and cephalosporins, but have a methylene moiety in the ring replacing sulphur in position 1 in the lactam ring hence the name carbapenem. (Kumagai *et al*., 2002).

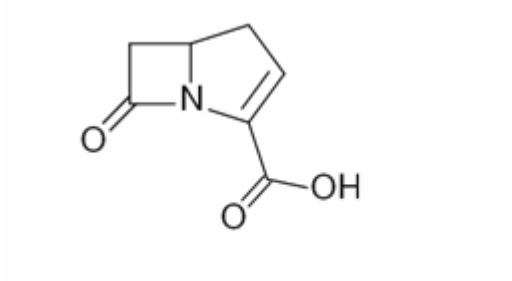


Figure 3: General structure of Carbapenem

Like other β-lactams, the carbapenems inhibit mucopeptide synthesis in the bacterial cell wall by binding to PBPs, leading to lysis and cell death. Carbapenems have the ability to retain activity against Gram-negative bacteria either with an extended spectrum of β-lactamases or those which hyperproduce the AmpC type cephalosporinase (reviewed by Livermore, 1995). Bacterial resistance may occur due to carbapenemases, which may be intrinsic or acquired and are responsible for the hydrolysis of carbapenems. Another source of resistance is the lack of the outer membrane porin, thus inhibiting the transport of the drug into the cell (Rybak, 2004).

1.4.1.4 Monobactams

Monobactams have a single β-lactam ring which is not fused with other ring, hence then name monobactam. (Ennis and Cobbs, 1995). The monobactam aztreonam was originally extracted from *Chromobacterium violaceum*.

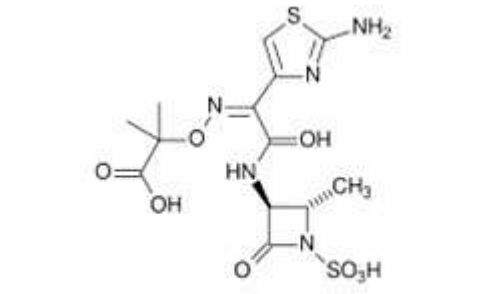


Figure 4: General structure of Aztreonam

It is now manufactured as a synthetic antibiotic (Durán *et al*., 2010). As with other β-lactams, aztreonam (Figure 4) inhibits mucopeptide synthesis in the bacterial cell wall by binding to the penicillin-binding protein (PBP) 3 of Gram-negative bacteria, leading to the lysis of the cell. It also has mild affinity for PBP-1a. It has been shown to have a synergistic effect with aminoglycosides (Georgopapadakou *et al*., 1982).

1.4.1.5 Aminoglycosides

The aminoglycosides were discovered during the 1940s during an extensive study of *Actinomycetes* for possible antimicrobial by-products. It was found that *Micromonospora purpurea* (suffix-micin) and *Streptomyces griseus* (suffix-mycin) produced inhibitory molecules Streptomycin (Figure 5) and Gentamicin (Figure 6) respectively. Streptomycin, discovered in the laboratory of Selman Walksman was the first aminoglycoside to be developed (Begg and Barclay, 1995).

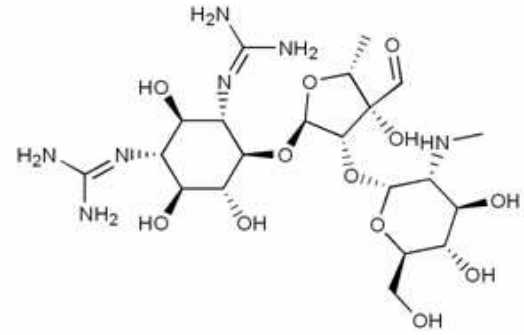


Figure 5: General structure of Streptomycin

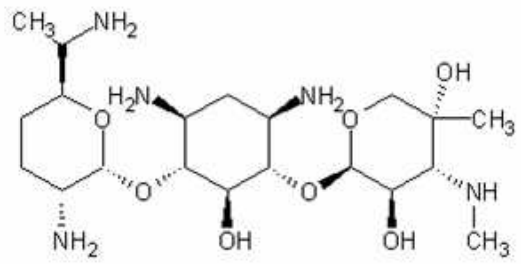


Figure 6: General structure of Gentamicin

An aminoglycoside is a molecule composed of amino-modified sugars. The aminoglycosides have two main effects on the bacterial cell that ultimately result in cell death. These agents bind to the negative charges in the outer phospholipid membrane, displacing the cations that link the phospholipids leading to disruption of the integral cell membrane. The aminoglycosides also inhibit protein synthesis by

binding to the 30S subunit of the ribosome, causing miscoding and termination (Moellering Jr, 1983).

1.4.1.6 Macrolides

The original macrolide (Figure 7), erythromycin, was discovered in 1952 by J.M. McGuire and marketed under the brand name Ilotycin (McGuire *et al*., 1952).
It is produced by *Saccharopolyspora erythraea* (formerly known as *Streptomyces erythreus*) (Labeda, 1987). Semisynthetic derivatives like clarithromycin and azithromycin have been produced from erythromycin, with modifications that improve acid stability, antibacterial spectrum, and tissue penetration (Kanatani and Guglielmo, 1994).

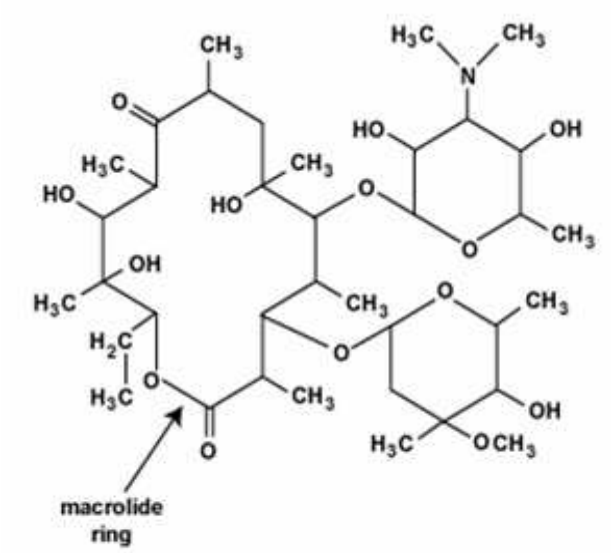


Figure 7: General structure of Macrolide

The macrolides are bacteriostatic, inhibiting protein synthesis by binding at the 50S ribosomal unit and by blocking transpeptidation and translocation. At high concentrations or with rapid bacterial growth, the effects may be bactericidal (Kanatani and Guglielmo, 1994).

1.4.1.7 Quinolones

The first quinolone, nalidixic acid (Figure 8) was formed as a result of chloroquine synthesis and introduced in 1962. As with other classes of synthetic and semisynthetic antimicrobials, alterations of side chains affect antimicrobial activity and pharmacokinetics (Andersson and MacGowan, 2003)

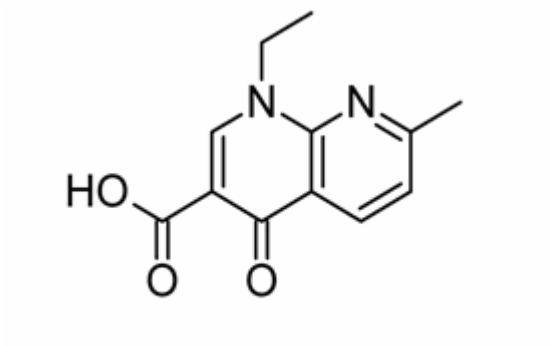


Figure 8: General structure of Nalidixic acid

Quinolones cause bacterial cell death by inhibiting DNA synthesis. They inhibit DNA gyrase and DNA topoisomerase, enzymes that mediate DNA supercoiling, transcription, and repair (Drlica and Zhao, 1997). Bacterial resistance develops as a result of spontaneous mutations that change the binding sites for quinolones on the DNA gyrase and the DNA topoisomerase (Willmott and Maxwell, 1993).

1.4.1.8 Sulphonamides

Sulfonamides are azo dyes derivatives which were discovered by Gerhard Domagk, who was awarded the Nobel Prize in the year 1939. The first agent was sulfachrysoidine, used in 1935, which released sulfanilamide *in vivo* (Woods, 1940). In bacteria, sulphonamides act as competitive inhibitors of the enzyme dihydropteroate synthetase, an enzyme involved in folate synthesis. Trimethoprim was discovered by George Hitchings during the 1940s and he was the Nobel laureate for medicine in the year 1988. Trimethoprim is a bacteriostatic antibiotic and acts by

interfering with the action of bacterial dihydrofolate reductase, inhibiting synthesis of tetrahydrofolic acid. Folic acid is an essential precursor in the de novo synthesis of the DNA nucleosides thymidine and deoxyuridine. Trimethoprim (Figure 9) and sulfamethoxazole (Figure 10) have a greater effect when given together; the reason is because they inhibit successive steps in the folate synthesis pathway (Burchall, 1973).

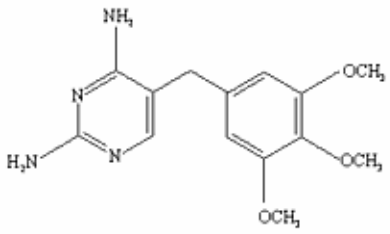


Figure 9: General structure of Trimethoprim

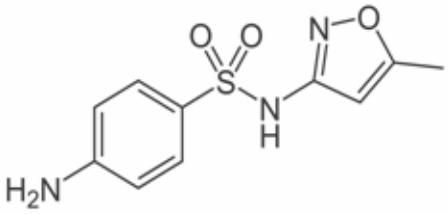


Figure 10: General structure of Sulfamethoxazole

The synergy between trimethoprim and sulfamethoxazole was first described in a series of *in vitro* and *in vivo* experiments published in the late 1960s (Bushby and Hitchings, 1968; Böhni, 1969).

1.4.1.9 Tetracyclines

Chlortetracycline or Aureomycin, the first tetracycline (Figure 11), was developed in 1948 from *Streptomyces aureofaciens* by Benjamin Duggar (Duggar, 1948).

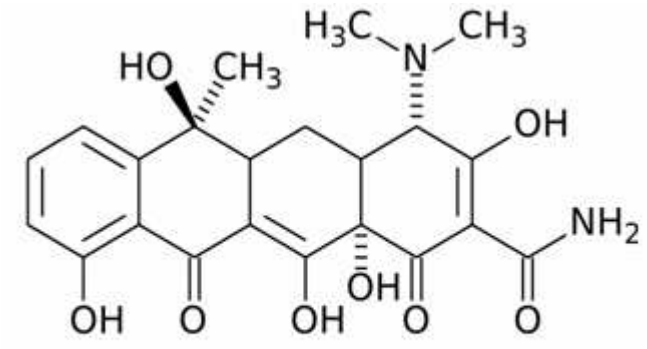


Figure 11: General structure of Tetracycline

Doxycycline and minocycline are semisynthetic derivatives of tetracycline (Smith and Leyden, 2005). Tetracyclines bind to the 30S ribosomal subunit, blocking the binding of aminoacyl t-RNA resulting in the inhibition of protein synthesis. It has a bacteriostatic effect (Craven *et al*., 1969).

1.4.1.10 Polymyxins

Polymyxins are antibiotics produced by *Bacillus polymyxa* which act by disrupting the lipopolysaccharide bilayer (Dixon and Chopra, 1986).

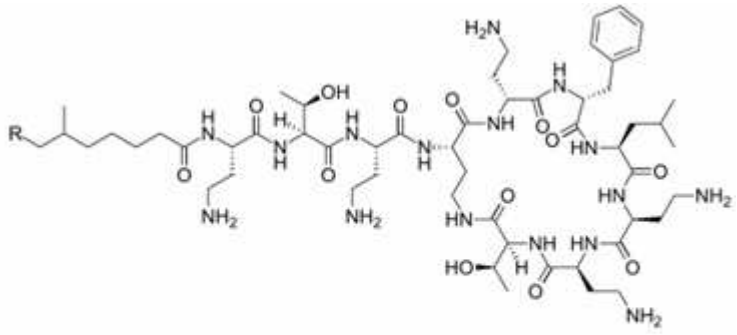


Figure 12: General structure of Polymyxin B

Polymyxin B (Figure 12) has been extensively used for topical applications (Kohonen and Tarkanen, 1969). Colistin or Polymyxin E (Figure 13) is a multicomponent polypeptide antibiotic, comprised mainly of colistin A and B that became available for clinical use in the 1960s, but was replaced in the 1970s by antibiotics considered less toxic (Li *et al*., 2006b).

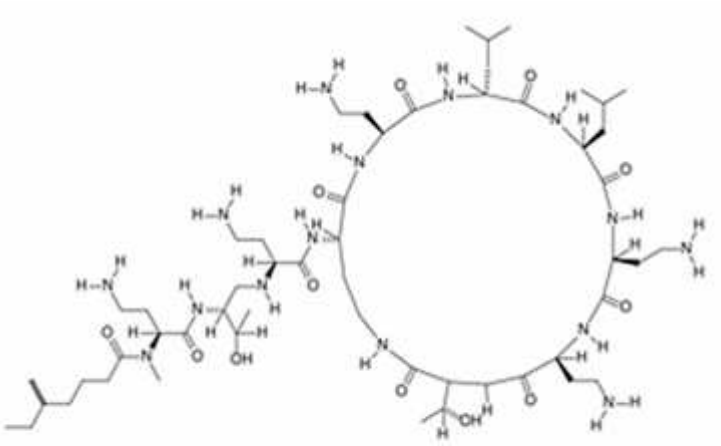


Figure 13: General structure of Polymyxin E

Colistin is produced by *Bacillus polymyxa* var. *colistinus,* and is now considered to be the last resort for serious infections involving multi-drug resistant isolates of *Pseudomonas aeruginosa, Acinetobacter baumannii* or *Klebsiella pneumoniae* (Falagas *et al*., 2008).

1.4.1.11 Tigecycline

Tigecycline is the first glycylcycline antibiotic and was discovered by Francis Tally (Projan, 2010). It was developed in order to overcome bacterial mechanisms of tetracycline resistance such as ribosomal protection and efflux pumps.

The primary backbone consists of minocycline with an *N*-alkyl-glycylamido group substituted at position nine and this gives this drug its broad spectrum of activity and protection from resistance mechanisms. Tigecycline is generally bacteriostatic, but some in vitro studies have reported bactericidal activity against *Streptococcus pneumoniae, Haemophilus influenzae,* and *Neisseria gonorrhoeae* (Meagher *et al*., 2005; Noskin, 2005).

This drug binds to the 30S ribosomal subunit of bacteria and blocks entry of amino-acyl t-RNA into the A site of the ribosome (Noskin, 2005). Tigecycline was efficacious both against NDM-1-producing *K. pneumoniae* and *E. coli* (Docobo-Pérez *et al*., 2012).

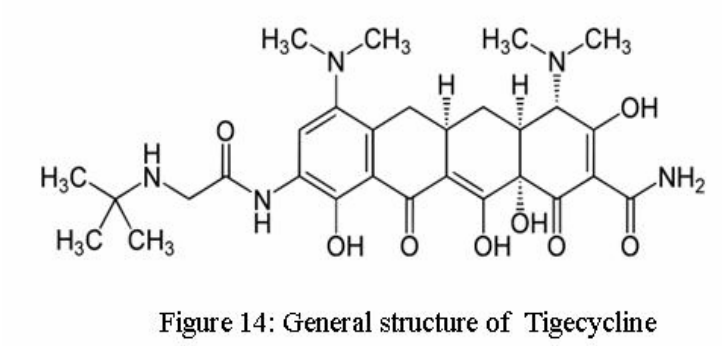


Figure 14: General structure of Tigecycline

1.4.2 Mechanisms of resistance to antibiotics in *A. baumannii*

1.4.2.1 Resistance to β-Lactams

1.4.2.1.1 β-Lactamases

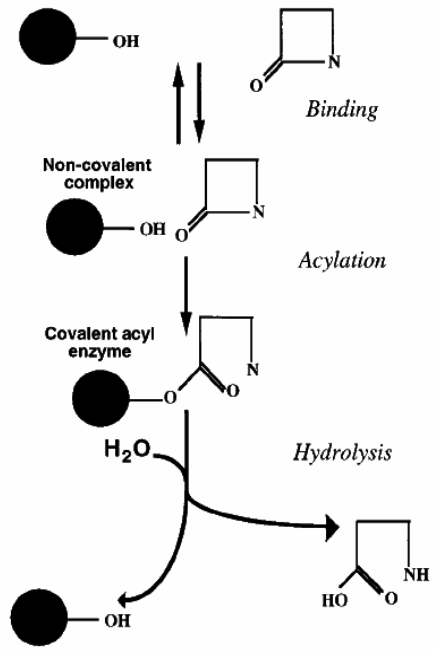


Figure 15: General mechanism of hydrolysis by serine β-lactamase.

β-lactamases (EC 3.5.2.6), are enzymes produced by bacteria, which destroy β-lactam ring of antibiotics. The β-lactam agent becomes changed in its chemical structure and is no longer recognized by the enzymes responsible for making the peptidoglycan or the murein layer of the bacterial cell wall (Frere, 1995). Enzymes of class A, C, and D have at their active site a serine residue but class B enzymes utilize a Zn^{2+} ion to attack the β-lactam ring. The mechanism of hydrolysis by a serine β-lactamase is depicted in figure 15 (reviewed by Livermore, 1995). Table 3 gives a systematic classification of β-lactamases and their functional groups according to Ambler and Bush scheme (reviewed by Livermore, 1995). Figure 16 gives the mode of action of five main antibacterial target sites.

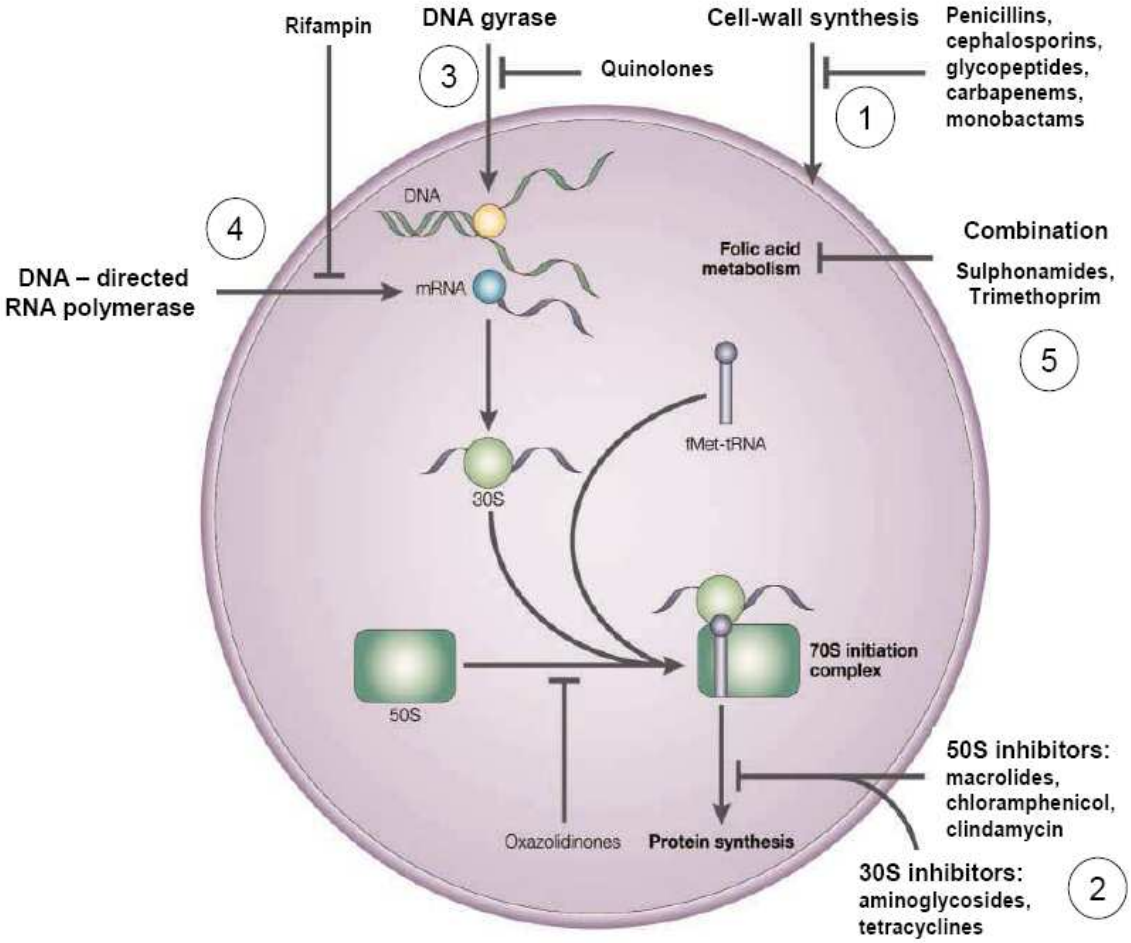


Figure 16: The five main antibacterial drug targets (Coates *et al*., 2002).

Table 3: Classification of β-lactamases and their functional groups (reviewed by Livermore, 1995).

Structural class (Ambler)	Functional group (Bush)	Activity[b]							Inhibited by clavulanate
		Penicillin	Carbenicillin	Oxacillin	Cephaloridine	Cefotaxime	Aztreonam	Imipenem	
Serine-β lactamases									
A	2a	+++	+	-	+/-	-	-	-	++
	2b	+++	+	+	++	-	-	-	++
	2be	+++	+	+	++	++	++	-	++
	2br	+++	+	+	+	-	-	-	-
	2c	++	+++	+	+	-	-	-	+
	2e	++	++	-	++	++	++	-	++
	2f	++	+	?	+	+	++	++	+
C	1	++	+/-	Inhibitor	+++	+	Inhibitor	-	-
D	2d	++	+	+++	+	-	-	-	+/-
Undetermined[c]	4[c]	++	++	++	V	V	-	-	-
Zinc-β lactamases									
B	3	++	++	++	++	++	-	++	-

b +++, preferred substrate (highest Vmax); ++, good substrate; +, hydrolyzed; +/-, barely hydrolyzed; -, stable; V, variable within group; ?, uncertain.
c None of Bush's group 4 enzymes has yet been sequenced; they are assumed to be serine types because they lack carbapenemase activity

1.4.2.1.1.1 Class A β-Lactamases and ESBLs

The class A enzymes have the ability to hydrolyze a broad spectrum of antibiotics including 3rd generation cephalosporins, penicillins, carbapenems and monobactams. They are inhibited by β-lactamase inhibitors such as clavulanic acid and tazobactam (Gin *et al*., 2007; Thomson, 2010). This group of enzymes includes the majority of the extended-spectrum β-lactamases (ESBLs) as it encompasses the KPC, GES, NMC, SME, IMI, TEM, SHV, PER, VEB, CTX-M ESBL families (Poirel and Nordman, 2008; Thomson, 2010). The gene encoding the enzyme PER-1 has been found in many isolates of *A. baumannii* in the USA, Korea and Turkey (Poirel *et al*., 2005a; Hujer *et al*., 2006, Kim *et al*., 2008), and a significant number of isolates from Belgium and France encode the gene for the VEB-1 enzyme (Naas *et al*., 2006a, Naas *et al*., 2006b). The emerging group of ESBLs in Enterobacteriaceae, which are of the CTX-M- type, have been identified rarely in *A. baumannii*. A strain producing CTX-M-2 was identified in Japan, which is a country where the first CTX-M-type enzymes were identified more than 15 years ago (Nagano *et al*., 2004). TEM, SHV type enzymes have also been described in *Acinetobacter* (Hujer *et al*., 2006; Naas *et al*., 2007). The expression of ESBLs in *A. baumannii* may contribute significantly to its resistance to expanded-spectrum β-lactams and to the increasingly observed multidrug resistance profile in this species. Due to generally widespread high-levels of resistance in *A. baumannii* to the penicillins and cephalosporins conferred by the intrinsic class C β-lactamases, these drugs are not usually considered appropriate for treatment of *A. baumannii* infections. Clinical detection of ESBL producers may be difficult since combined mechanisms of resistance are often associated in those multidrug-resistant *A. baumannii* isolates. A combined over-expression of the naturally occurring cephalosporinase may be antagonized by addition of cloxacillin,

oxacillin or boronic acid that inhibits the cephalosporinase activity making such ESBL detection easier (Coudron, 2005; Poirel and Nordmann, 2008). The prevalence of the class A β-lactamases in *A. baumannii* may not be a very major problem in comparison to other carbapenemases in this species; however the potential of this species to act as a reservoir for mobilisable resistance genes in the hospital may be alarming.

1.4.2.1.1.2 Class B β-Lactamases (metallo-β-lactamases)

The first classification of molecular class B enzymes was by Ambler in 1980 distinguishing them from serine β-lactamases. He also proposed that β-lactamases have a polyphyletic origin (Ambler, 1980); since then an alternative categorization was suggested by Bush in 1989, placing them into separate groups according to their functional enzymes (Bush *et al*., 1995). The molecular class B enzymes are metallo-β-lactamases (MBL), which require two divalent cations, usually zinc as metal cofactors for the enzyme activity. They have the ability to hydrolyse penicillin, cephalosporins and carbapenems, and are not inhibited by β-lactamase inhibitors and monobactams (Walsh *et al*., 2005). They are inhibited by EDTA or mercaptopropionic acid (Arakawa *et al*., 2000; Yong *et al*., 2002). It is interesting to note that they are not hydrolysed by aztreonam particularly well; however in animal pneumonia model studies it was observed that infection caused by *P. aeruginosa* producing VIM-2, could not be eradicated with aztreonam even when the animals were given high drug doses (Bellais *et al*., 2002). To date, nine families of acquired MBLs enzymes have been identified – IMP (Imipenemase first discovered in Japan), VIM (Verona MBL), SIM (Seoul MBL), SPM (São Paulo imipenemase), GIM (German MBL), DIM (Dutch imipenemase) (Poirel *et al*., 2010a), KHM (Kyorin

university hospital imipenemase, Japan) (Poirel *et al*., 2010a), AIM (Australian imipenemase) (Poirel *et al*., 2010a) and the most recent NDM (New Delhi MBL) (Poirel and Nordmann, 2008, Yong *et al*., 2009).

The IMP, VIM and SIM have been identified in *A. baumannii* with several variants of the IMP and VIM families found in Hong Kong, Canada, Taiwan, Japan and in Italy France, Greece, Korea, Spain, Portugal, Poland and other countries (Walsh *et al*., 2005). The SPM enzyme was first discovered in *P. aeruginosa* in Brazil (Toleman *et al*., 2002). The GIM enzyme was also first identified in *P. aeruginosa* (Castanheira *et al*., 2004). Interspecies spread of SPM and GIM enzyme has not yet been reported. Since their initial discoveries, SPM, GIM, and SIM metallo-β-lactamases have not spread beyond their countries of origin. Genetic analysis of the surrounding genes identified in *A. baumannii* shows similar structures since the gene families of VIM, IMP and SIM have been included in the class-1 integron structure. The dissemination of these genes among the strains of *A. baumannii* and *P. aeruginosa* in specific areas has been clarified by the plasmid location of MBL genes (Livermore and Woodford, 2006). The NDM enzyme, first found in *K. pneumoniae* is now also prevalent in *A. baumannii* in India (Yong *et al*., 2009; Karthikeyan *et al*., 2010). To date, 33 variants of IMP, 33 variants of VIM and 6 variants of NDM have been reported (http://www.lahey.org/Studies/other.asp, Last accessed on 12/03/12). It is clear that VIM and IMP family are growing at an equal rate but it is also a matter of concern that the $bla_{\text{NDM-1}}$ gene first identified on plasmid can be integrated on the chromosome in certain *E. coli* strains as the surrounding genetic structures may facilitate its integration (Pfeifer *et al*., 2011). Recent reports by Walsh *et al*. (2011) suggest the integration of $bla_{\text{NDM-1}}$ in the chromosomes of *Aeromonas caviae* and *Vibrio cholerae*.

1.4.2.1.1.3 Class C β-Lactamases (ampC's)

AmpC-type β -lactamases constitute molecular class C in Ambler's classification scheme and are commonly found encoded on the chromosome in Gram-negative organisms. As well as these intrinsic enzymes, there are several families of plasmid-borne *ampC* genes that are spreading particularly within the *Enterobacteriaceae* (Jacoby, 2009). In *Acinetobacter* species these genes are known as *Acinetobacter*-derived cephalosporinases (ADC) (Hujer *et al*., 2005). As this name implies, these enzymes are able to hydrolyse penicillins and the narrow-spectrum cephalosporins. When over-expressed, the enzymes can confer resistance to the extended-spectrum cephalosporins. AmpC enzymes were considered as exclusively chromosomal until 1989, when MIR-1, the first plasmid-encoded AmpC enzyme, was discovered (Papanicolaou *et al*., 1990). In other Gram-negative bacteria the expression of the cephalosporinase gene is inducible but, in *A. baumannii*, the *ampC* is expressed at a basal level but the expression is enhanced when the insertion sequence IS*Aba1*or IS*Aba125* inserts upstream of the gene providing a better promoter for gene expression (Corvec *et al*.,2003; Lopes *et al*., 2011. P601, ECCMID). The widespread nature of the bla_{ADC} genes is the major reason for high levels of resistance in *A. baumannii* to the penicillins and cephalosporins and as such these drugs are generally not effective for treatment of this organism.

1.4.2.1.1.4 Class D β-Lactamases (oxacillinases)

Oxacillinases are peculiar β-lactamases that are grouped in a heterogeneous class of enzymes either regarding their structural or their biochemical properties (Poirel and Nordman, 2008). The OXA group mainly occurs in *Acinetobacter* and *Pseudomonas* species with naturally occuring variants in many Gram-negative non fermentors. The OXA β-lactamases hydrolyze amoxicillin, methicillin, cephaloridine and, to some extent, cefalotin. They usually hydrolyze oxacillin more efficiently than benzylpenicillin (Poirel and Nordmann 2008, Girlich *et al*., 2004). The OXA group enzymes are generally not regarded as ESBLs, because they do not hydrolyse the extended-spectrum cephalosporins, although certain exceptions like OXA-37 from *A. baumannii* and OXA-20 from *P. aeruginosa* can be seen (Naas *et al*., 1998; Navia *et al*., 2002; Girlich *et al*., 2004). Currently, over 232 different variants of OXA enzymes have been identified on the protein level (http://www.lahey.org/Studies/other.asp, last accessed 12/03/12). The class D oxacillinases have been found both on plasmids and chromosome (Heritier *et al*., 2005b; Corvec *et al*., 2007). The naturally occurring class-D oxacillinase OXA-50 is expressed constitutively in *P. aeruginosa* (Girlich *et al*., 2004). The genes $bla_{\text{OXA-51}}$-like, $bla_{\text{OXA-23}}$-like and $bla_{\text{OXA-134}}$ for instance, are present intrinsically in *A. baumannii*, *A. radioresistens* and *A. lwoffii*, respectively (Brown *et al*., 2005; Poirel *et al*., 2008; Figueiredo *et al*., 2010).

1.4.2.1.1.4.1 OXA-23-like β-Lactamases

OXA-23, originally named ARI-1 for "*Acinetobacter* resistant to imipenem" was identified in Scotland and then renamed OXA-23 based on its genetic characterization. It was isolated from the plasmid of a strain of *A. baumannii* isolated

in 1985 which possessed an MIC of imipenem of 16 mg/L (Paton *et al*., 1993). The OXA-23-like enzyme OXA-27, isolated from Singapore, shares 99% identity with OXA-23 (Afzal-Shah *et al*., 2004). Another variant, OXA-49, was isolated in China from a carbapenem resistant *A. baumannii* strains (Brown *et al*., 2005). The OXA-23 enzymes have been identified in *A. baumannii* in various parts of the world like, Brazil (Dalla-Costa *et al*., 2003), China (Yu *et al*., 2004), London (Coelho *et al*., 2004) and Singapore (Koh *et al*., 2007). They can be present on both plasmid and chromosome (Paton *et al*., 1993; Bonnet *et al*., 2002). *In vivo* the OXA-23 β-lactamase contributes towards resistance to amoxicillin, ticarcillin, meropenem and imipenem. When, a naturally-occurring plasmid containing the $bla_{\text{OXA-23}}$ gene is electro-transformed into the same recipient strains, moderate to high MICs are achieved of 16 to >32 mg/L of carbapenems, with the highest values again found in the recipient over-expressing the AdeABC efflux pump, indicating the involvement of other genetic factors associated with the $bla_{\text{OXA-23}}$ gene in conferring resistance (Héritier *et al*., 2005c). Other variants of OXA-23 enzymes are OXA-27, OXA-102, OXA-103, OXA-105, and OXA-133. NCBI blast search reveals that OXA-146 (FJ194494), OXA-165 (HM488986), OXA-165 (HM488987), OXA-166(HM488988), OXA-167 (HM488989), OXA-168 (HM488990), OXA-169 (HM488991), OXA-170 (HM488992), OXA-171 (HM488992) and OXA-225 (JN638887) are also OXA-23-like carbapenemases. OXA-133 has been found in *A. radioresistens* (Mendes *et al*., 2009; reviewed by Poirel *et al*., 2010b). OXA-134 is found to be present intrinsically in *A. lwoffii* and it could act as a source of dissemination of OXA-23-like enzymes (Figueiredo *et al*., 2010). Chomosomally encoded OXA-23 enzyme has also been shown to be present in *Proteus mirabilis* (Bonnet *et al*., 2002). OXA-73, an acquired OXA-23- like carbapenemase, has also

been found in *K. peumoniae* (reviewed by Poirel *et al*., 2010b). Recent reports point to the increase in carbapenem-resistant *A. baumannii* isolates encoding OXA-23 from South America and South-East Asia (Villegas *et al*., 2007, Fu *et al*., 2010). A study of *A. baumannii* isolates from Colombian institutions found an average of 33.6% of isolates resistant to a carbapenem, and in a subset of 66 of these resistant isolates, 65 were positive by PCR for $bla_{\text{OXA-23}}$. It was observed that one clone was present in two hospitals within one city, while another had spread between two hospitals in different cities (Villegas *et al*., 2007). In a Chinese teaching hospital, the rates of carbapenem resistance have increased from 5% to 50% from 1999 to 2005 in isolates from ICU wards and 20% in non-ICU isolates in 2004; and a subsequent study of 221 resistant isolates from 11 institutions, including 117 isolates from the same teaching hospital, found 97.7% were positive for $bla_{\text{OXA-23}}$ gene (Wang *et al*., 2007). Recent report identifies Tn*2008* having IS*Aba1* upstream and *ATPase* gene downstream of the $bla_{\text{OXA-23}}$, as a major vehicle carrying $bla_{\text{OXA-23}}$ in *Acinetobacter baumannii* in China (Wang *et al*., 2011).

1.4.2.1.1.4.2 OXA-40-like β-Lactamases

The OXA-40 β-lactamase has a mainly narrow-spectrum hydrolytic profile including ceftazidime and imipenem. Its activity is resistant to inhibition by clavulanic acid, tazobactam, sulbactam. Like most of the other carbapenem-hydrolyzing oxacillinases, the OXA-40 enzyme is not inhibited by NaCl (Héritier *et al*., 2003).The OXA-40 enzyme, originally named OXA-24, was identified in Spain in a highly carbapenem resistant *A. baumannii* strain and it shares 63% and 60% amino acid identity with OXA-51/69 and OXA-23 respectively (Bou *et al*., 2000a). This group also includes the variants OXA-24, OXA-25, OXA-26, OXA-33 (AY008291), OXA-72 OXA- 139

(AM991978) and OXA-160 (Tian *et al*., 2011). The variants OXA-25, OXA- 26 were identified in strains from Spain and Belgium subsequently (Afzal-Shah *et al*., 2001), while OXA-72 was identified in *A. baumannii* strains were identified from Thailand (Walther-Rasmussen and Høiby, 2006). The *bla*$_{\text{OXA-33}}$ has also been found to be co-present with the carbapenemase gene *bla*$_{\text{OXA-62}}$ on the integron of *Pandoraea pnomenusa* (Schneider *et al*., 2006).

Kinetic studies carried out on OXA-40-like enzymes demonstrate that these enzymes are capable of hydrolysing penicillins, have weak activity against carbapenems, and very weak activity against some cephalosporins (Bou *et al*., 2000a; Afzal-Shah *et al*., 2001; Heritier *et al*., 2003). It is observed that *in vivo* the OXA-40 enzyme confers resistance to carbapenems as well as increased MICs of penicillins and cephalosporins (Héritier *et al*., 2005c). Despite having only weak hydrolytic activity against the carbapenems of the purified enzyme, isolates encoding an OXA-40-like β-lactamase had an imipenem MIC of >128mg/L (Ruiz *et al*., 2007). When transformed into susceptible reference *A. baumannii* strains, the enzyme only confers intermediate to low level resistance, with the higher of these values associated with an isolate over-expressing the AdeABC efflux pump, suggesting that other resistance mechanisms are required to achieve the high levels of resistance seen in clinical isolates (Héritier *et al*., 2005c).

The *bla*$_{\text{OXA-40-like}}$ genes have been found in other *Acinetobacter* spp as well as in *Pseudomonas aeruginosa*, and to be both chromosomally and plasmid encoded (Quinteira *et al*., 2007; Lee *et al*., 2009a; Sevillano *et al*., 2009). Isolates encoding the enzymes have been reported in Spain and in Asia (Ruiz *et al*., 2007, Wang *et al*.,

2007, Lee *et al*., 2009a), but Spain and Portugal are the main countries where it has been widely disseminated, and this is because there was evidence of its plasmid location in several isolates (Lopez-Otsoa *et al*., 2002; Quinteira *et al*., 2007; Ruiz *et al*., 2007). High prevalence of $bla_{\text{OXA-40-like}}$ has been seen on the Iberian Peninsula and it appears to be the hot spot for the dissemination of these genes (Da Silva *et al*., 2004). A recent study of 83 imipenem resistant *A. baumannii* isolates from 12 hospitals in Spain found 42% of isolates were positive for a $bla_{\text{OXA-40-like}}$ gene (Ruiz *et al*., 2007). A second recent study from a hospital in Portugal found that of 222 imipenem-resistant *A. baumannii* collected over the period of January 2001 to October 2004, 36.6% carried a $bla_{\text{OXA-40-like}}$ gene, the majority of which were associated with plasmid DNA, which was also found in *Acinetobacter haemolyticus* isolates in the same institution (Quinteira *et al*., 2007). The identification of a high percentage of $bla_{\text{OXA-40-like}}$ genes presents a worrying possibility of these genes becoming more globally established thereby hampering treatment options in compromised patients.

Recently two new carbapenemases, OXA-143 and OXA-182 have been identified. The OXA-182 carbapenemase has 93% amino acid identity with OXA-143 and 89% with OXA-40 (Kim *et al*., 2010). The OXA-143 carbapenemase has 88% amino acid identity with OXA-40, 63% with OXA-23 and 52% with OXA-58 (Higgins *et al*., 2009)

1.4.2.1.1.4.3 OXA-51-like β-Lactamases

The OXA-51-like β-lactamases are an enzyme group that are intrinsic and ubiquitous in *A. baumannii* (Brown *et al*., 2005; Merkier and Centron, 2006; Turton *et al*., 2006). The first report of OXA-51, was in 2005 in isolates obtained from Argentina in 1996 (Brown *et al*., 2005), and a large number of variants of enzymes have since been discovered. OXA-51 shares < 63% amino acid identity with subgroups 1 (OXA-23-like enzymes), subgroup 2 (OXA-40-like enzymes) and <50% with subgroup 4 (OXA-58-like enzymes). They are less efficiently inhibited by clavulanate or tazobactam and their activity is inhibited by NaCl (Héritier *et al.* 2005b; Poirel and Nordmann, 2008).The OXA-51-like enzymes currently represent one of the largest groups of β-lactamases, with 81 enzymes currently identified (http://www.lahey.org/Studies/ Last accessed on 12/03/12).

The $bla_{\text{OXA-51-like}}$ genes have generally been found to be chromosomally encoded and non-transferable. However, recent reports and PCR analysis for $bla_{\text{OXA-51-like}}$ genes indicate plasmid as well as chromosomal location of the $bla_{\text{OXA-51-like}}$ genes (Chen *et al*., 2010). A second report mentions that the investigators were able to transfer a $bla_{\text{OXA-87}}$, a $bla_{\text{OXA-51-like}}$ gene, to *E. coli* DH5α, indicating that $bla_{\text{OXA-51-like}}$ variants might jump to plasmids as well (Vahaboglu *et al*., 2006).

The $bla_{\text{OXA-51-like}}$ genes have been identified in *A. baumannii*, except $bla_{\text{OXA-138}}$, which has been identified in *A. nosocomialis* from Taiwan, further suggesting that there is the potential for these genes to be mobilised within the *A. baumannii- calcoaceticus* complex (Lee *et al*., 2009b). The G+C content of the $bla_{\text{OXA-51-like}}$ gene $bla_{\text{OXA-69}}$ was found to be 39.3%, which compares with the G+C content of the *A. baumannii*

genome (38.8%) lending further weight to the suggestion that these genes are native to *A. baumannii* (Héritier *et al.* 2005b). Kinetic analysis has only been performed on OXA-51 and OXA-69 enzymes (Brown *et al*., 2005, Heritier *et al*., 2005b) and they both demonstrate poor hydrolysis of oxacillin and cloxacillin, a feature that has been observed in carbapenem-hydrolysing oxacillinases (Bou *et al*., 2000a ; Afzal-Shah *et al*., 2001).The contribution of the enzymes to carbapenem resistance is difficult to determine. It has been suggested that the $bla_{\text{OXA-51-like}}$ genes are generally poorly expressed and that they may only confer resistance when they have high levels of expression with the insertion of an element such as IS*Aba1* upstream of the gene, providing a better promoter (Turton *et al*., 2006a). The large group of enzymes clustered around the OXA-66 enzymes are found in isolates belonging to an *A. baumannii* lineage including the prevalent European clone 2, while those clustered around the OXA-69 enzyme are found in another lineage encompassing European clone 1. The OXA-71 enzyme is associated with European clone 3. The most commonly identified enzymes are those of the OXA-66 cluster, which are particularly highly represented in South America and Asia (Merkier and Centron, 2006; Koh *et al*., 2007; Wang *et al*., 2007; Evans *et al*., 2008). Enzymes of the OXA-69 cluster are also common, particularly in Eastern Europe and of OXA-71, associated with European clone 3 have been widely identified in isolates from the Iberian Peninsula (Evans *et al*., 2008). To date, the OXA-51-like group comprises of OXA-51, OXA-64 to OXA-71, OXA-75 to OXA-80, OXA-82 to OXA-84, OXA-86 to OXA-95, OXA-98 to OXA-100 (AM231720), OXA-104, OXA-106 to OXA-113, OXA-115 to OXA-117, OXA-120 to OXA-127, OXA-138, OXA-144 (FJ872530), OXA-148 to OXA-150 (GQ853679 to GQ853681), OXA-172 to OXA-180 (HM113558 to HM570036) OXA-194 to OXA-197 (HQ425492 to HQ425495), OXA-200 to OXA-202

(HQ734811 to HQ734813), OXA-208 (FR853176), OXA-216 (FR865168), OXA-217 (JN603240), OXA-219 (JN215211), OXA-223 (JN248564). These enzymes have been identified according to the data cited by Brown & Amyes, 2005; Evans *et al*, 2007; reviewed by Poirel *et al*., 2010b; Lee *et al*., 2009b and by personal communication with B. A. Evans.

1.4.2.1.1.4.4 OXA-58-like β-Lactamases

The *bla*$_{\text{OXA-58}}$ gene was first identified in France from a strain isolated in Toulouse in 2003 (Poirel *et al*., 2005b). It hydrolyses penicillins, oxacillin, and imipenem but not expanded-spectrum cephalosporins. The *bla*$_{\text{OXA-58}}$ gene was located on a 30-kb non-self-transferable plasmid. It was electrotransformed in the *A. baumannii* reference strain and was shown to conferred reduced susceptibility to carbapenems (Poirel *et al*., 2005b). Kinetic analysis of OXA-58 has shown that it has similar properties to the other OXA-type carbapenemases of *A. baumannii*; however, it hydrolyses imipenem twice as efficiently as OXA-40. The purified enzyme has weak activity against penicillins and imipenem, very weak activity against meropenem, and some activity against cephalothin and cefpirome, but not against ceftazidime or cefotaxime (Poirel *et al*., 2005b). The *bla*$_{\text{OXA-58}}$ gene, which has been identified only in *Acinetobacter* spp. isolates, has been associated with a variety of different genetic structures. It has been shown to confer high level of carbapenem resistance only when insertion sequences provide a strong promoter in the expression of the gene along with synergistic over-expression of the AdeABC efflux pump (reviewed by Peleg *et al*., 2008). OXA-58 has a low percentage of amino acid identity compared with the other oxacillinases (Brown and Amyes, 2006). Genes encoding OXA-58-like enzymes have been found in a range of *Acinetobacter* species and are usually plasmid borne, though

a chromosomal location has also been described (Bogaerts *et al*., 2006, Poirel *et al*., 2005b; Poirel and Nordmann, 2006a). Isolates carrying $bla_{\text{OXA-58-like}}$ genes are most frequently reported from Europe, though the genes have been reported in isolates from South America, North America, Anatolia, Asia and Australia (Marqué *et al*., 2005; Bogaerts *et al*., 2006; Coelho *et al*., 2006; reviewed by Peleg *et al*., 2006; Koh *et al*., 2007; Wang *et al*., 2007; Gur *et al*., 2008; Merkier *et al*., 2008; Zarrilli *et al*., 2008; Lee *et al*., 2009a; Mendes *et al*., 2009). In addition to being dependent on IS elements, the level of expression of the $bla_{\text{OXA-58}}$ gene may be related to the gene copy number, as exemplified in one Italian clone exhibiting various levels of resistance to imipenem as a consequence of the $bla_{\text{OXA-58}}$ copy number (Bertini *et al*., 2007). It has been shown recently that the $bla_{\text{OXA-58}}$ has been duplicated in one isolate of *A. pittii* conferring intermediate level of resistance to carbapenems (Evans *et al*., 2010). Other variants of OXA-58 found in *Acinetobacter* spp are OXA-96, OXA-97 and OXA-164 (Gene bank accession number, GU831575) (reviewed by Poirel *et al*., 2010b)

1.4.2.1.2 Outer membrane proteins

A tough outer membrane, low numbers of protein-porins, and several other constitutive characteristics play a role in behaviour of *Acinetobacter* with regards to the capacity of resistance and survival in the environment. The small number and sizes of the different porins when coupled with efflux provide a significant barrier to the uptake of antibiotics in *A. baumannii* (Hooper, 2005; Vila *et al*., 2007). Several studies state that a decrease in porin expression is associated with antimicrobial resistance in *A. baumannii* (Bou *et al*., 2000b; Fernandez-Cuenca *et al*., 2003; Mussi *et al*., 2005; Siroy *et al*., 2006).

The major outer membrane protein in *Acinetobacter* spp is HMP-AB, a member of the OmpA-like family and is a heat-modifiable protein (Dupont *et al*., 2005; Walzer *et al*., 2006). It shares sequence identity and structural similarities with OprF in *P. aeruginosa* and with OmpA in *E. coli* and is sized at 37 kDa, but heating shifts this to 45 kDa (Gribun *et al*., 2003). The HMP-AB protein has been shown to allow the penetration of β-lactams and saccharides up to 800 Da, but has a significantly lower efficiency than the OmpF protein in E. coli (Gribun *et al*., 2003). The OmpA-like protein contributes poorly to membrane permeability and it is possible that it may work in association with one of the clinically relevant efflux pumps such as AdeAB and AbeM although this link has not been established experimentally. Recent report shows that OmpA of *A. baumannii* 19606 plays a partial role in the development of robust biofilms on plastic and it is essential for bacterial attachment to *Candida albicans* filaments and A549 human alveolar epithelial cells. The interaction with biotic surfaces is independent of the CsuA/BABCDE-mediated pili and the interaction of *A. baumannii* 19606 with fungal and epithelial cells results in their apoptotic death. Furthermore, the bacterial adhesion phenotype correlates with the ability of bacteria to invade A549 epithelial cells. The same study also proves that the killing activity of cell-free culture supernatants is protease and temperature sensitive, suggesting that its cytotoxic activity is due to secreted proteins, some of which are different from OmpA (Gaddy *et al*., 2009).

The 33-36 kDa outer membrane protein has been implicated in several studies involving carbapenem resistance in clinical strains (Clark, 1996; del Mar Tomas *et al*., 2005). The levels of the 33-36 kDa outer membrane protein were decreased in a carbapenem-resistant isolate of *A. baumannii* and cloning and over-expression of the

gene encoding this outer membrane protein restored a β-lactam sensitive phenotype to the previously resistant isolate. N-terminal sequencing of this protein shows that it shares homology with outer membrane proteins from *Ralstonia* spp. and OmpF from *Serratia marcescens* (del Mar Tomas *et al*., 2005).

The CarO protein, another heat-modifiable protein, co-migrates on SDS-PAGE gels with the HMP-AB protein. When heated, the size of the protein changes from 25 to 29kDa (Mussi *et al*., 2005). The mature protein comprises 228 residues is found to be approximately 24 kDa. Amino-terminal sequencing analysis shows that it shares a 100% similarity with the CarO protein in the Swiss-Prot database. Blast analysis indicates that this protein is highly similar to ACIAD2598 (70% identity to outer membrane protein CarO precursor) and Omp25 in *A. baumannii* ATCC19606 (Accession No: CAI 79415) (Schneiders *et al*., 2008). The loss or disruption of this protein results in carbapenem resistance as indicated by other studied but no binding site for imipenem has been identified suggesting that it might function as a nonspecific monomeric channel (Siroy *et al*., 2005).

1.4.2.1.3 Efflux mechanisms

In *A. baumannii*, pumps of the resistance nodulation cell-division (RND), multidrug and toxic compound extrusion (MATE), and major facilitator superfamily (MFS) have been associated with antimicrobial resistance (Magnet *et al*., 2001; Marchand *et al*., 2004; Su *et al*., 2005). Many studies have shown that efflux pumps play a crucial role in bacterial pathogenesis and in the survival of the pathogen in the host where the deletion of the transporter genes has resulted in reduced survival and virulence potential of the organism (Hirakata *et al*., 2002; Su *et al*., 2005; Piddock, 2006).

Studies on *P. aeruginosa* lacking efflux pumps show that the bacteria were unable to invade canine-kidney epithelial cells but the invasion potential was restored upon complementation with the *mexAB-oprM* genes (Hirakata *et al*., 2002).

It is likely that the RND-type efflux pumps, AdeABC, AdeDE, AdeIJK, and AdeXYZ, contribute to the virulence and fitness of *Acinetobacter* spp. Although described as independent pumps, AdeIJK and AdeXYZ share 93% identity at the nucleotide level and 99% similarity at the protein level. AdeIJK and AdeXYZ represent the same pump belonging to *Acinetobacter* genomic DNA groups 2, 3, and 13TU. AdeABC pump has been described and characterized in a non-*Acinetobacter baumannii* strain and all three pumps can coexist (Roca *et al*., 2011). The retrospective analysis of the active efflux systems show that the AdeAB efflux pump was specific to *A. baumannii* and both AdeE and AdeY were only found in *Acinetobacter* genomospecies 3 (Chau *et al*., 2004; Chu *et al*., 2006).

It has been shown that AdeAB is controlled by a two component regulatory system AdeR (regulator) and AdeS (sensor kinase). AdeA is the membrane fusion protein, AdeB is the multidrug transporter and AdeC is the outer membrane protein. The lack of the AdeC gene in *A. baumannii* ATCC17978 in contrast to BM4454 suggests that AdeAB efflux pump might use alternative outer membrane proteins to export substrates, however when AdeB gene was disrupted in BM4454 the bacterium became susceptible to a vast range of antibiotics (Magnet *et al*., 2001; Schneiders *et al*., 2008). This is consistent with the observation that not all structural components of the AdeABC pump are required to be encoded by members of the same genomic

DNA group and that AdeC does not contribute significantly to the multidrug resistance phenotype (Marchand *et al*., 2004).

AbeM is an H^+-dependent drug antiporter and member of the MATE family. It is shown to confer resistance to gentamicin and hydrophilic quinolones (Chau *et al*., 2004). The *tet*(A) or *tet*(B) genes encode for membrane-associated efflux proteins (Marti *et al*., 2006). These proteins belong to the MF superfamily and exchange a proton for the tetracycline cation complex (Roberts, 1996). When expressed, the *tet* (A) gene confers resistance to tetracycline and the *tet* (B) gene confers resistance to both tetracycline and minocycline (Roberts, 1996).

The *cmlA* gene encodes an efflux pump that confers resistance to chloramphenicol and is a part of the gene cluster that harbours *tet*(R) and *tet*(A). It belongs to the MF superfamily and was found to be located within the pathogenicity island present in the epidemic multidrug resistant *A. baumannii* AYE strain. The AYE strain also harboured the *qacE*Δ1 gene responsible for resistance to quarternary ammonium compounds (Fournier *et al*., 2006).

1.4.2.2 Antibiotic resistance

1.4.2.2.1 Aminoglycosides

All of the major classes of enzymes conferring resistance to amino-glycosides have been described; these include acetyltransferases, nucleotidyltransferases, and phosphotransferases (Noppe-Leclercq *et al*., 1999; Nemec *et al*., 2004; Hujer *et al*., 2006). One of the most important mechanisms of aminoglycoside resistance is post-transcriptional rRNA methylation by 16S rRNA methylases. 16S rRNA methylation

by the *armA* gene has described for *A. baumannii* strains from Japan, Korea, and North America (Yamane *et al*., 2005; Lee *et al*., 2006a; Doi *et al*., 2007). The genetic surroundings of *armA* appear very similar across Gram-negative organisms, as it is plasmid-borne and within a transposon (Tn*1548*) (Doi *et al*., 2007). Other 16S rRNA methylase genes like *rmtA*, *rmtB*, *rmtC* and *rmtD* have also been described in the Enterobacteriaceae family (Yokoyama *et al*., 2003; Yamane *et al*., 2005). They are the cause of high-levels of resistance to all clinically useful aminoglycosides, including gentamicin, tobramycin, and amikacin.

1.4.2.2.2 Fluoroquinolones

The two target proteins of the quinolones are the DNA gyrase (topoisomerase II) and topoisomerase IV. DNA gyrase is a tetrameric enzyme with two subunits A and two subunits B, encoded in the *gyrA* and *gyrB* genes, respectively. Topoisomerase IV is also a tetrameric enzyme, which also has two A and two B subunits, encoded in the *parC* and *parE* genes, respectively (Vila and Pachón, 2008). Modifications of the DNA gyrase and topoisomerase IV, due to the mutations in the *gyrA* and *parC* genes, have been well described for *A. baumannii* and they interfere with the binding of the target site (Hamouda and Amyes, 2004; Higgins *et al*., 2004., reviewed by Peleg *et al*., 2008).

Both aminoglycosides, and quinolones have been shown as the substrates for multidrug efflux pumps (Ribera *et al*., 2002), including the AdeABC and AbeM (Magnet *et al*., 2001; Higgins *et al*., 2004; Su *et al*., 2005). The plasmid genes *qnrA*, *qnrB* and *qnrS* conferring quinolone resistance are not widespread with only a single report on identification of the *qnrA* gene in *A. baumannii* strain recovered in Algeria

(Touati *et al*., 2008). QepA plasmid mediated quinolone resistance by fluoroquinolone efflux pump has been reported in *E.coli* but yet not in *A. baumannii* (Yamane *et al*., 2007).

1.4.2.2.3 Polymyxins

According to the recent reports in vitro resistance and heteroresistance to the polymyxins in *A. baumannii* is increasing with the underlying mechanism still unclear (Li *et al*., 2006a). Mutations in PmrAB system have shown to confer colistin resistance (Adams *et al*., 2009). It has previously been shown that reduced binding to the lipopolysaccharide (LPS) target site can lead to resistance in *E. coli*, *Salmonell*a spp., and *P. aeruginosa* (Peterson *et al*., 1987; Conrad and Galanos, 1989). Changes in outer membrane proteins have shown to cause reduced susceptibility to polymyxins in *P. aeruginosa* (Nicas *et al*., 1980). These factors may have implications in the contribution of colistin resistance in *A. baumannii* strains.

1.4.2.2.4 Trimethoprim-sulfamethoxazole

The prevalence of trimethoprim-sulfamethoxazole resistance in *A. baumannii* is high in China and Europe (Van Looveren *et al*., 2004; Gu *et al*., 2007). Integrons are very common in multidrug resistant *A. baumannii* strains. The 3'-conserved region of an integron mostly contains a *qac* gene fused to a *sul* gene, conferring resistance to antiseptics and sulphonamides, respectively (reviewed by Peleg *et al*., 2008). Consequently, sulphonamide resistance has been shown to be highly predictive of integron-carrying strains of *A. baumannii* (Gu *et al*., 2007). Similarly *dhfr* genes coding for trimethoprim resistance and *cat* genes coding for chloramphenicol

resistance have also been reported within integron structures in *A. baumannii* (Gu *et al*., 2007; reviewed by Peleg *et al*., 2008).

1.4.2.2.5 Tigecycline

Tigecycline is an extended-spectrum antibiotic with activity against *Acinetobacter* spp, an increasingly common cause of nosocomial pneumonia. It is one of the last resorts for the treatmeant of multi-resistant *Acinetobacter baumannii* infections (Koomanachai *et al*., 2009). High resistance rates to tigecycline, in multiple clones of MDR *A. baumannii* have been reported in Tel Aviv medical centre, Israel (Navon-Venezia *et al*., 2007). These findings are worrying.

Other mechanisms like alteration of drug targets (penicillin-binding proteins), permeability problems, resistance to tetracyclines and carbapenems have also been cited (Amyes and Young, 1996; Poirel and Nordmann, 2008).

1.4.3 Integrons

Integrons are assembly platforms that incorporate exogenous open reading frames (ORFs) by site-specific recombination and convert them to functional genes (Figure 17). All integrons characterized to date are composed of three main elements necessary for the capture of exogenous genes: a gene *intI* encoding an integrase belonging to the tyrosine-recombinase family; a primary recombination site *attI*; and an outward- orientated promoter (Pc) that directs transcription of the captured genes (Mazel, 2006). At present, five classes of integrons are known to have a role in the dissemination of antibiotic resistance genes. These classes have been defined based on the sequence of the encoded integrases, which show 40–58% identity (Mazel, 2006). All five classes are physically linked to mobile DNA elements, such as insertion sequences, transposons and conjugative plasmids, all of which can serve as vehicles for the intraspecies and interspecies transmission of genetic material (Mazel, 2006). Three classes of integrons are 'historical' classes that are involved in the multiple-antibiotic-resistance phenotype. Class 1 integrons are associated with functional and non-functional transposons derived from transposon Tn*402* that can be embedded in larger transposons, such as Tn*21* (Rådström *et al*., 1994; Brown *et al*., 1996; Mazel, 2006). Class 2 integrons are exclusively associated with Tn*7* derivatives (Sundström *et al*., 1991; Rådström *et al*., 1994). The class 3 integrons are thought to be located in a transposon inserted in as-yet-uncharacterized plasmids (Collis *et al*., 2002; Mazel, 2006). The other two classes of mobile integrons, class 4 and class 5 have been described in *Vibrio cholerae* and *Vibrio salmonicida* respectively (Mazel, 2006).

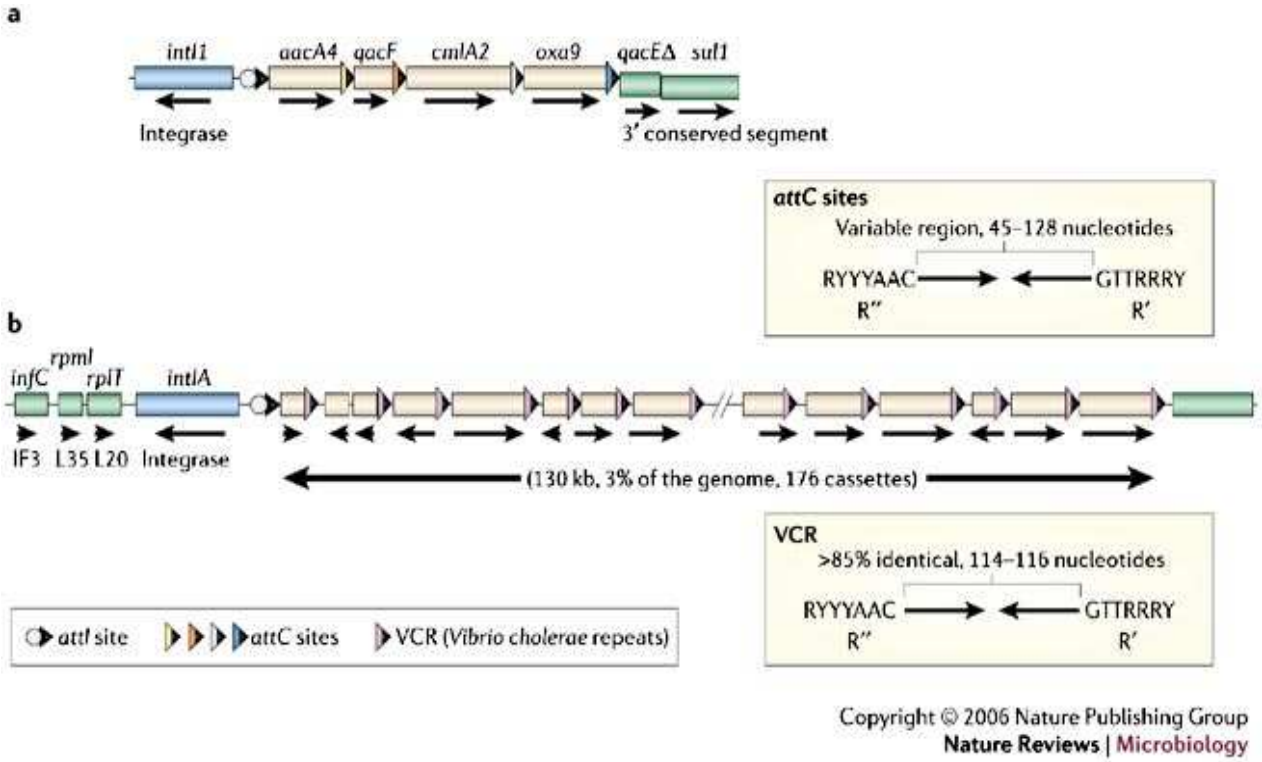


Figure 17: Structural comparison of a 'classical' mobile integron and a superintegron from *Vibrio cholerae* strain N16961.

a) A schematic representation of the class 1 integron In40. The various resistance-gene cassettes carry different attC sites. The following antibiotic-resistance cassettes confer resistance to the following compounds: *aacA4*, aminoglycosides; *cmlA2*, chloramphenicol; *oxa-9*, β-lactams; *qacF* and *qacE*, quarternary ammonium compounds. The *sul* gene, which confers resistance to sulphonamides, is not a gene cassette.

b) A schematic representation of the chromosomal superintegron in *V. cholerae*; the open reading frames are separated by highly homologous sequences, the *V. cholerae* repeats (VCRs). *infC*, encodes translation initiation factor IF3; *rpmI* and *rplT* encode ribosomal proteins L35 and L20, respectively (Mazel, 2006).

To date only class 1 and class 2 integrons have been reported in *A. baumannii,* class 1 being the most common (Seward and Towner, 1999) and can confer resistance to the aminoglycosides, sulphonamides, chloramphenicol, trimethoprim and β-lactams (Seward and Towner, 1999; Ploy *et al*., 2000; Koeleman *et al*., 2001; Nemec., 2004; Gu *et al* 2007). Genes encoding metallo-β-lactamase such as IMP and VIM have also been reported *A. baumannii* from isolates in Italy and Korea (Riccio *et al*., 2000; Yum *et al*., 2002). Horizontal transfer of the resistance genes can be achieved when an integron containing one or more such genes is incorporated into a broad-host-range plasmid. Likewise, single cassettes integrated at secondary sites in a broad-host-range plasmid can also move across species boundaries (Hall and Collis, 1998).

1.4.2.4 Insertion Sequences

The IS finder database (www-is.biotoul.fr/is.html, last accessed 12/3/2012) reveals the presence of 35 insertion sequences, 23 of which have been associated with *A. baumannii* (Table 4).

Table 4: Insertion elements found in various *Acinetobacter spp*

N^0	Name	Family	Origin	Accession number
1	IS*1007*	IS*6*	*Acinetobacter* sp. LS56-7	AJ250860
2	IS*18*	IS*30*	*Acinetobacter* sp. 13 BM2716	AF043676
3	IS*1236*	IS*3*	*Acinetobacter calcoaceticus*	U03772
4	IS*Aca1*	IS*3*	*Acinetobacter calcoaceticus*	AF121266
5	IS*1008*	IS*6*	*Acinetobacter calcoaceticus*	AJ251307
6	IS*17*	IS*5*	*Acinetobacter haemolyticus*	U95013
7	IS*Aha1*	IS*5*	*Acinetobacter haemolyticus*	
8	IS*Aha2*	IS*5*	*Acinetobacter haemolyticus*	
9	IS*Alw1*	IS*5*	*Acinetobacter lwoffii*	
10	IS*1006*	IS*6*	*Acinetobacter junii*	NC_004361
11	IS*Aba1*	IS*4*	*Acinetobacter baumannii*	AY758396
12	IS*Aba2*	IS*3*	*Acinetobacter baumannii*	AY665723
13	IS*Aba3*	IS*1*	*Acinetobacter baumannii*	AY665723
14	IS*Aba4*	IS*982*	*Acinetobacter baumannii*	EF059914
15	IS*Aba5*	IS*5*	*Acinetobacter baumannii*	CU459140
16	IS*Aba6*	IS*982*	*Acinetobacter baumannii*	CU468230
17	IS*Aba7*	IS*5*	*Acinetobacter baumannii*	CU468230
18	IS*Aba8*	IS*21*	*Acinetobacter baumannii*	EF102240
19	IS*Aba9*	IS*982*	*Acinetobacter baumannii*	EU850412
20	IS*Aba10*	IS*5*	*Acinetobacter baumannii*	GQ379223
21	IS*Aba11*	IS*701*	*Acinetobacter baumannii*	CP000521
22	IS*Aba12*	IS*5*	*Acinetobacter baumannii*	NC_009085
23	IS*18*	IS*30*	*Acinetobacter baumannii*	EU294228
24	IS*Aba125*	IS*30*	*Acinetobacter baumannii*	AY751533
25	IS*Aba825*	IS*982*	*Acinetobacter baumannii*	AY751532
26	IS*Aba13*	IS*5*	*Acinetobacter baumannii*	CP001182
27	IS*Aba14*	IS*3*	*Acinetobacter baumannii*	CP001921
28	IS*Aba15*	IS*5*	*Acinetobacter baumannii*	JN187417
29	IS*Aba16*	IS*66*	*Acinetobacter baumannii*	JN415682
30	IS*Aba17*	IS*66*	*Acinetobacter baumannii*	
31	IS*Aba18*	IS*3*	*Acinetobacter baumannii*	CP000521
32	IS*Aba19*	IS*3*	*Acinetobacter baumannii*	
33	IS*Aba20*	IS*3*	*Acinetobacter baumannii*	
34	IS*Acsp1*	Tn*3*	*Acinetobacter species EB104*	
35	IS*Acsp2*	IS*982*	*Acinetobacter genomospecies 15TU*	

Barbara McClintock's discovery of jumping genes early in her career earned her the Nobel Prize in 1983. She records her discovery of transposable elements which have either a "copy and paste" or "cut and paste" mechanism (McClintock, 1950). An insertion sequence (Figure 18) is a short DNA sequence that acts as a simple transposable element. The two major characteristics of insertion elements are: they are smaller in length (upto 2500bp) relative to other transposable elements and only code for proteins implicated in the transposition activity (Campbell and Reece, 2002). These proteins are usually the transposase which catalyses the enzymatic reaction allowing the IS to move, and also one regulatory protein which either stimulates or inhibits the transposition activity (Campbell and Reece, 2002). The coding region in an insertion sequence is usually flanked by inverted repeats (Figure 18). For example, the well-known IS*911* is flanked by two 36 bp inverted repeats and the coding region have two partially overlapping genes *orfA* and *orfAB*, coding for the transposase (OrfAB) and a regulatory protein (OrfA)(Campbell and Reece, 2002).

Insertion sequences have a very specific role to play with respect to the expression of oxacillinases in *A. baumannii*. IS*Aba1* has been shown to cause the over-expression of the naturally occurring $bla_{\text{OXA-51-like}}$ gene leading to carbapenem resistance in *A. baumannii* (Turton *et al*., 2006a). IS*Aba825* has been found upstream of the $bla_{\text{OXA-51-like}}$ and is responsible for the expression of the $bla_{\text{OXA-51-like}}$ gene (Lopes and Amyes, unpublished observations). It has also been shown that IS*Aba1* controls the expression of the bla_{ADC} (Corvec *et al*., 2003). Recent observations also show the role of IS*Aba125* in providing a better promoter than IS*Aba1* in the over-expression of the bla_{ADC} gene (Lopes *et al*., 2011. P601, ECCMID). It has been reported that insertion sequences IS*Aba1*, IS*Aba2*, IS*Aba3* and IS*18* are associated with the $bla_{\text{OXA-58}}$ gene

expression (Poirel and Nordmann, 2006b). The same study also identified a composite transposon-like structure formed by IS*Aba3* and it concluded that the acquisition of the *bla*$_{OXA-58}$ gene was not the result of a transposition process but due to homologous recombination (Poirel and Nordmann, 2006b). IS*Aba1* and IS*Aba4* have been associated with expression of the *bla*$_{OXA-23}$ gene (Corvec *et al*., 2007; Bogaerts *et al*., 2008). Insertion sequences in *A. baumannii* have also been involved in altering the copy number of the *bla*$_{OXA-58}$ gene. The best known example is of the duplication of the *bla*$_{OXA-58}$ gene, mediated by the IS*26* element. The study reports three clonally-related strains, resistant to imipenem having one, two and three copies of the *bla*$_{OXA-58}$ gene (Bertini *et al*., 2007). This study shows that IS*26* element is able to duplicate the entire IS*Aba2*/IS*Aba3*- *bla*$_{OXA-58}$-IS*Aba3* region by imperfect duplicative transposition, starting with the cointegrate fusion and duplication of the element but lacking the resolution of the cointegrate providing better promoters for the expression of *bla*$_{OXA-58}$ resulting in resistance to carbapenems (Bertini *et al*., 2007).

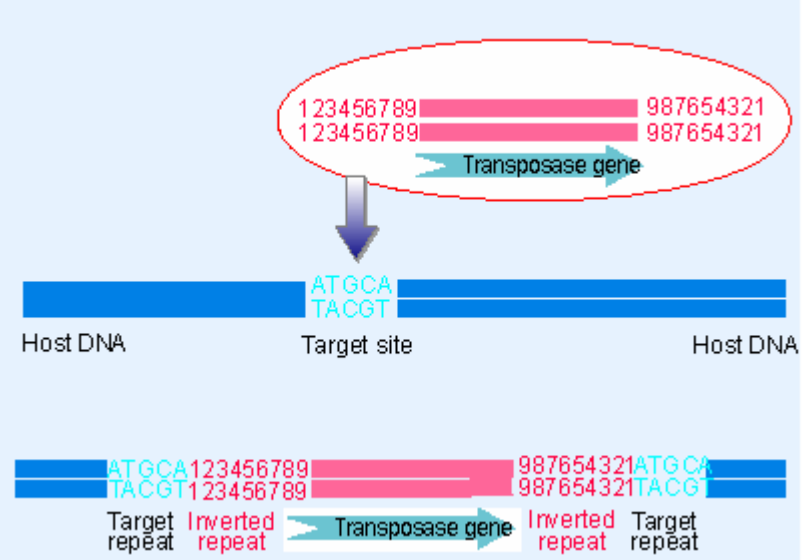


Figure 18: General structure of an insertion element having the transposase gene

Recent studies also demonstrate the acquisition of plasmid-located IS*Aba1*-*bla*$_{\text{OXA-51-like}}$ genes as a result of one ended transposition (Chen *et al*., 2010). It is clear by these studies that the ‘‘intrinsic’’ genes can confer high levels of resistance and can be freely mobilised interspecifically in *Acinetobacter* spp. The acquisition of the *bla*$_{\text{OXA-138}}$ (a gene of the *bla*$_{\text{OXA-51-like}}$ cluster) in *A. nosocomialis* may have occurred by a similar process. Insertion sequences such as IS*Aba125* and IS*Aba825* have also been involved in the disruption of the *carO* gene leading to a multi-drug resistant phenotype in *A. baumannii* (Mussi *et al*., 2005). IS*Aba825*-IS*Aba3*-like hybrid promoter has also been shown to cause the over-expression of the *bla*$_{\text{OXA-58}}$ gene in *A. baumannii* (Ravasi *et al*., 2011).

1.5 Conclusion.

In the *Acinetobacter calcoaceticus- baumannii* complex, *Acinetobacter baumannii* is an emerging multi-drug resistant nosocomial pathogen, with other members of the complex *A. pittii* and *A. nosocomialis* also emerging along side.

A. baumannii has caused serious problems like ventilator associated pneumonia and septicaemia associated with a steady rise in infections seen in immuno-compromised patients over the past few decades.

A. baumannii is a major problem due to its evolving multi-drug resistant profile and is developing increasing antibiotic resistance to almost all classes of antimicrobials, parallel to the development of different antibacterial drugs.

Carbapenems are regarded as the last sustainable resort for treatment of *A. baumannii* infections but resistance to the carbapenems is an established problem in *A. baumannii* with different mechanisms of innate and acquired resistance phenotype in this species.

Carbapenem resistance due to presence of insertion sequences like IS*Aba1* present upstream of the intrinsic $bla_{\text{OXA-51-like}}$ gene has been observed in *A. baumannii*

A. baumannii has developed numerous mechanisms that make it a very strong nosocomial pathogen. These include IS elements governing the expression of carbapenemase genes, insertional inactivation or modifications of the outer membrane proteins, acquisition of metallo-carbapenemases leading to carbapenem resistance,

over-expression of acinetobacter derived cephalosporinases, over-expression of the efflux pumps and more recently it marks the beginning of emergence of resistance to colistin.

1.6 Aims and Objectives of this project

- To prove the importance of transposition of IS*Aba1* and its role in control of antibiotic resistance genes.
- To identify any novel insertion elements governing the bla_{ADC} gene expression
- To analyze multi-drug resistant profile of the isolates isolated from Cochabamba, Bolivia.
- To monitor and assess the trends in Aberdeen Royal Infirmary and to identify any novel $bla_{OXA\text{-}51\text{-}like}$ variants.
- To identify any novel insertion elements governing the $bla_{OXA\text{-}51\text{-}like}$ gene in isolates from the US
- To study the insertional inactivation of the $bla_{OXA\text{-}51\text{-}like}$ gene and to understand the mechanism of carbapenem resistance in *Acinetobacter baumannii* strain isolated in Carnaxide, Portugal
- To study the mechanism of ciprofloxacin resistance and the role of insertion sequences involved in the disruption or promotion of various genes involved in efflux pump regulation.
- To study the multi-drug resistant profile of isolates from Europe and from the US.

2.0 MATERIALS AND METHODS

2.1 Strain collection and identification

A total of 96 *Acinetobacter baumannii* isolates were used in this study. Thirty-six isolates each were from the collections of Benjamin Evans (Europe) and Susan Brown (United States), fifteen isolates from Bolivia (Lucía Gallego, University of the Basque Country, Bizkaia, Spain) and nine isolates from Aberdeen Royal Infirmary (Ian Gould).

The isolates had been previously identified by 16S-23S rRNA restriction analysis (Dolzani *et al*., 1995) or by the intrinsic $bla_{\text{OXA-51-like}}$ gene PCR (Héritier *et al*., 2005b) or by the *rpoB* gene sequencing (La Scola *et al*., 2006). They were reconfirmed by me by at least two of the above method.

The isolates were sub-cultured on MacConkey agar (Oxoid, Basingstoke) and incubated at 37^{o}C for 24hrs. A single colony was inoculated in IST broth and grown overnight at 37^{o}C in an orbital shaker. 900µL of the overnight culture was added to 100µL sterile glycerol in a cryovial and were stored at -85^{o}C for long-term storage.

2.2 Chemicals and Media

All the chemicals and antibiotics were purchased from Sigma-Aldrich Company (Poole, UK) Ltd unless otherwise stated. All media were purchased in powdered form from Oxoid (Basingstoke, UK). The broths and agar plates were prepared according to the manufacturer's instructions. They were sterilised at 121^{o}C/15psi for 15 minutes by autoclaving. Normal saline was prepared by dissolving 0.85 g of NaCl in 100mL of D/W and sterilized by autoclaving.

2.3 10X TAE buffer

48.44g of Trizma base (Sigma) and 3.72g of EDTA (Ethylene diamine tetra acetic acid) disodium salt dehydrate 99% were dissolved in D/W with the addition of 11.4 ml glacial acetic acid to give a final volume of one litre. The pH was adjusted to 8.0 and the solution was sterilized. For running the gels, 1:10 dilution was made to a final concentration of 1X and used for gel electrophoresis.

2.4 Gel electrophoresis and staining

1.5% gels (Gensieve LE agarose, Flowgen, UK) were prepared in 1X TAE buffer and used for running at 100V. 0.7% gels were prepared for the running of plasmid DNA and they were run at 60V for 19hrs for better resolution. All the gels were stained in a 100 mL of gel red (Biotium, US) staining solution (50 µL gel red dissolved in 100ml of 0.1M NaCl) and visualized by the Gel Doc system.

2.5 10X TBE buffer

Tris-base (108g) and 55g of boric acid were dissolved with 40mL of 20mM EDTA in one litre of D/W. The pH was adjusted to 8.0 with and the solution was sterilized. 0.5X TBE buffer was used for running of the PFGE gels.

2.6 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was performed on *Acinetobacter baumannii* strains isolated from the Aberdeen Royal Infirmary according to the procedure described by Seifert *et al*. (2005). The isolates were inoculated onto Iso-Sensitest agar (Oxoid, Basingstoke) and incubated overnight at 37°C in ambient air. A loopful of bacteria was removed from the agar surface with a sterile plastic loop and suspended in a glass or polystyrene round-

bottomed tube containing 2.5 ml of cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.0). Each cell suspension was adjusted to give a cell density of approximately 109 cells/ml. This was performed by using the spectrophotometer. The pellet was resuspended by vortexing, and the cell suspension was incubated at 55°C for 10 min a water bath. An aliquot of 25 µL proteinase K (20-mg/ml stock solution in ultrapure water) was added, and the suspension was mixed gently by inverting the tube two to four times. An equal volume of melted 1% CHEF genomic agarose (Bio-Rad, UK)-1% sodium dodecyl sulphate(Fisher Scientific, UK) in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was added to the cell suspension, and the suspension was mixed gently by inverting the tube 10 to 12 times. The agarose-cell suspension mixture was immediately dispensed into the wells of reusable plug moulds. The agarose plugs were allowed to solidify at room temperature for 5 min and at 4°C for another 5 min. A single plug was then transferred to disposable screw-cap 50-ml polypropylene tubes containing 5 ml of cell lysis buffer (50 mM Tris, 50 mM EDTA [pH 8.0], 1% sarcosine) and 25 µL of proteinase K (20 mg/ml stock solution). Lysis was performed at 55°C in a water bath for two hours with. After lysis, the buffer was carefully removed and the plugs were washed five times (15 min/wash) at 55°C (two times with sterile ultrapure water and three times with TE buffer; 10 ml for each washing step) in a water bath. The water and TE buffer were preheated at 50 to 55°C before each washing step. After the last wash, the TE buffer was poured off and 10 ml of fresh TE buffer was added to each tube. If the plugs were not used on the same day then they were refrigerated. For the restriction of the genomic DNA a single slice of the plug (4.0 by 5.5 mm) was cut with a scalpel or razor blade and transferred to a microcentrifuge tube containing 200 µL of the restriction buffer with 30 U *Apa*I (Promega, Southampton, UK). The plug slices were incubated at 37°C for 24 hours in

a water bath. 1.0% gels were prepared in 0.5X TBE buffer for PFGE and the gels were run with the plugs in CHEF apparatus with initial pulse 5 and final pulse 25 for 20 hours. The gel was stained with gel red on the completion of run. Cluster analysis was performed by the unweighted pair group method with mathematical averaging (UPGMA), and DNA relatedness was calculated by using the band-based Dice coefficient with a tolerance setting of 1.5% band tolerance and 1.5% optimization setting for the whole profile. Gel analysis was performed using the BioNumerics v4.0 software (Applied Maths, Sint-Martins-Latem, Belgium). The banding patterns were interpreted according to the criteria suggested by Tenover *et al*., 1995. A value of 80-85% was chosen as the threshold for the establishment of clonal relatedness of unknown isolates.

2.7 Determination of the minimum inhibitory concentration

The isolates were tested for their susceptibility to either some or all the antibiotics. The antibiotics used in this study were imipenem (IPM) (Merck, Sharp & Dohme Ltd, Hertfordshire, UK), meropenem (MEM) (AstraZeneca, Cheshire, UK), ceftazidime (CAZ) (Sigma), cefepime (FEP) (Bristol Myers Squibb, US), ciprofloxacin (CIP) (Bayer AG, Germany), gatifloxacin (GAT) (Sigma), gentamicin (CN) (Sigma), piperacillin/tazobactam (PIP/TZ) (Cyanamid, Gosport, UK).

The efflux pump inhibitor 1-(1-napthylmethyl) piperazine (NMP) was used at a concentration of 100 mg/L to check for the action of the efflux-mediated ciprofloxacin resistance in some strains.

Following overnight culture on MacConkey agar plates, a single colony was suspended in 5 mL of Iso-sensitest broth (Oxoid, Basingstoke) and grown overnight at 37°C/180 rpm in an orbital shaker. Minimum inhibitory concentrations (MICs) were determined by the agar double dilution technique in Iso-sensitest agar according to the British Society for Antimicrobial Chemotherapy (BSAC) methodology (British Society for Antimicrobial Chemotherapy, Version 6.1). The results were interpreted according to the BSAC guidelines. *Pseudomonas aeruginosa* NCTC 10662, *Escherichia coli* NCTC 10418 and *Staphylococcus aureus* NCTC 6571 were used as control strains.

2.8 Extraction of DNA

Following overnight sub-culture on MacConkey agar at 37^{o}C, DNA extraction was performed by boiling 5 identical colonies in 50 µL of sterile distilled water for about 15-20 minutes. The eppendorf tubes were centrifuged and the supernatant was used as the DNA template.

2.9 Amplification of genes by Polymerase chain reaction (PCR)

All the PCR's were performed in a total volume of 50 µL, with reagents supplied by Promega (Promega, Southampton, UK) according to the manufacturer's guidelines.

The primers used in this study were supplied by Eurofins MWG operon, UK. They were diluted to a concentration of 100 pmol/µL as stock standards. 12.5 pmol of primer was used for each PCR reaction.

All the primers used in this study were either from previously published papers or were designed using the biotools software

(http://biotools.umassmed.edu/bioapps/primer3_www.cgi, Last accessed, 9/8/2011)

2.10 The aims of the PCR reaction and purpose of the primers (5'-3') used in this study.

- A PCR described by Dolzani *et al*. (1995) using the primers 16S-23S primer 1 and 16S-23S primer 2, was used to re-identify and confirm the identity of some *A. baumannii* strains. The 975bp product amplified for *Acinetobacter* genospecies 2 was restricted with the enzymes *Alu*I and *Nde*II. Restriction of the product by the enzyme *Alu*I produced fragments of 50, 125, 135, 165, 175 and 330 bp specific to *A. baumannii*. Restriction of the same product produced fragment sizes 50, 110,145, 330 and 360 bp by the enzyme *Nde*II, specific to *A. baumannii*. The primers and expected fragment size were

16S-23S primer 1	TTG TAC ACA CCG CCC GTC A	975bp
16S-23S primer 2	GGT ACT TAG ATG TTT CAG TTC	

- A PCR described by La Scola *et al*. (2006) was used for the *rpoB* gene detection and sequencing for the molecular identification of *Acinetobacter* species. The primers and expected fragment size were

Ac696F	TAY CGY AAA GAY TTG AAA GAA G	350bp
Ac1093R	CMA CAC CYT TGT TMC CRT GA	

Y= C, T; M= A, C; R= A, G

- Multiplex PCR for detection of oxacillinases was performed according to the method of Woodford *et al*. (2006). The primers listed below were used for the PCR and the predicted fragment sizes are shown.

OXA-51-like F	TAA TGC TTT GAT CGG CCT TG	353bp
OXA-51-like R	TGG ATT GCA CTT CAT CTT GG	
OXA-23-like F	GAT CGG ATT GGA GAA CCA GA	501bp
OXA-23-like R	ATT TCT GAC CGC ATT TCC AT	
OXA-40-like F	GGT TAG TTG GCC CCC TTA AA	246bp
OXA-40-like R	AGT TGA GCG AAA AGG GGA TT	
OXA-58-like F	AAG TAT TGG GGC TTG TGC TG	599bp
OXA-58-like R	CCC CTC TGC GCT CTA CAT AC	

- PCR for the detection of *bla*$_{\text{OXA-143}}$ was performed using the primers OXA-143F and OXA-143R. The primers and expected fragment size were

OXA-143F	TTC TGT CAG TGC ATG CTC ATC	728bp
OXA-143R	CAG GCA TTC CTT GCT TCA TT	

PCR conditions were

Function	Temperature	Time	Cycle/s
Pre-denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	35
Annealing	57°C	40 sec	
Extension	72°C	50 sec	
Final extension	72°C	6 min	1
Cooling	4°C		

- PCR for the detection of the *bla*$_{\text{OXA-51-like}}$ gene and the upstream insertion, if any, was performed according to Héritier *et al*, (2005b). The primers used for this PCR anneal at the intergenic regions upstream and downstream of the *bla*$_{\text{OXA-51-like}}$ gene and hence are able to map the *bla*$_{\text{OXA-51-like}}$ gene and any insertion if present upstream of the *bla*$_{\text{OXA-51-like}}$ gene. The primers Oxa69-A and Oxa69-B were used for the PCR. The primers and expected fragment size were

Oxa69-A	CTA ATA ATT GAT CTA CTC AAG	975bp
Oxa69-B	CCA GTG GAT GGA TGG ATA GAT TAT C	

- PCR for the detection of the intergenic region downstream of the *bla*$_{\text{OXA-51-like}}$ gene were performed using the IntoxaF and IntaceR primers. The PCR was performed only for strains which were found to be positive for an insert upstream of the *bla*$_{\text{OXA-51-like}}$ gene (This study). The primers and expected fragment size were

IntoxaF	GGA ATA CCT AGC TCT GTT CG	250bp
IntaceR	GTT TTA CCC ACG CTG GTA CT	

PCR conditions were

Function	Temperature	Time	Cycle/s
Pre-denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	35
Annealing	57°C	40 sec	
Extension	72°C	50 sec	
Final extension	72°C	6 min	1

Cooling	4°C

- PCR for the analysis of the region upstream *bla*$_{\text{OXA-51-like}}$ gene was also performed by using the primers FxOxaF and FxOxaR (This study). The primers and expected fragment size were

FxOxaF	GAT ACC AGA CCT GGC AAC AT	889bp
FxOxaR	GCA CGA GCA AGA TCA TTA CC	

PCR conditions were

Function	Temperature	Time	Cycle/s
Pre-denaturation	94°C	7 min	1
Denaturation	94°C	1 min	35
Annealing	57°C	1 min	
Extension	72°C	2 min	
Final extension	72°C	7 min	1
Cooling	4°C		

- The primers for the sequencing of IS*Aba16* are as given below

ISAba16_F1	ATC CGG CTA ACA CAG CCT TA
ISAba16_R1	GAC CTG GCC TGA TTT AAA GC
ISAba16_F2	AGG CAC TGA TCC AAG GTT TG
ISAba16_R2	TAG TGC GAG TTC ATG CTC GT

- PCR for the detection of the entire *bla*$_{\text{OXA-58-like}}$ gene was performed using the primers preOxa-58prom+ and preOXA-58B designed by Héritier *et al*. (2005a). The primers and expected fragment size were

preOXA-58prom+	TTA TCA AAA TCC AAT CGG C	934bp
preOXA-58B	TAA CCT CAA ACT TCT AAT TC	

- PCR for the detection of upstream sequences of the *bla*$_{\text{OXA-58-like}}$ gene in certain *A. baumannii* strains was performed using a combination of primers, SM2 and OXA-58-like R. The primers are listed individually by Poirel and Nordmann (2006) and Woodford *et al*. (2006) respectively. The walk-58R primer was used for amplification and sequencing (This study). The primers were

SM-2	AAG TGT CTA TAT TCT CAC C	Variable
walk-58-R	CAG CAC AAG CCC CAA TAC TT	

PCR conditions were

Function	Temperature	Time	Cycle/s
Pre-denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	35
Annealing	55°C	40 sec	
Extension	72°C	50 sec	
Final extension	72°C	6 min	1
Cooling	4°C		

- PCR were performed for the detection of the whole *bla*$_{\text{OXA-40-like}}$ gene according to the primers described Afzal-Shah *et al*., (2001) using the Oxa-24A and Oxa-24B primers. The primers and expected fragment size were

Oxa-24A	GTA CTA ATC AAA GTT GTG AA	1023bp
Oxa-24B	TTC CCC TAA CAT GAA TTT GT	

- The *bla*$_{\text{OXA-40-like}}$ gene PCR was repeated for samples negative by Oxa-24A and Oxa-24B but positive by multiplex PCR, using the primers Oxa-24F and Oxa-24R by Jeon *et al*. (2005). The primers and expected fragment size were

Oxa-24F	ATG AAAAAATTTATACTTCCTATATTCAGC	825bp
Oxa-24R	TTA AAT GAT TCC AAG ATT TTC TAG C	

- The *bla*$_{\text{OXA-23-like}}$ gene PCR was performed using the primers 23A and 23B decribed by Afzal-Shah *et al*. (2001). The primers and expected fragment size were

Oxa-23F	GAT GTG TCA TAG TAT TCG TCG	1062bp
Oxa-23R	TCA CAA CAA CTA AAA GCA CTG	

- PCR for the detection of IS*Aba1* upstream of *bla*$_{\text{OXA-23-like}}$ gene was performed using a combination of primers, ISADC1 and Oxa-23R described individually by Ruiz *et al*. (2007) and Woodford *et al*. (2006) (This study). The primers and expected fragment size were

ISADC1	GTT GCA CTT GGT CGA ATG AAA A	1097
Oxa-23R	ATT TCT GAC CGC ATT TCC AT	

PCR conditions were

Function	Temperature	Time	Cycle/s
Pre-denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	35
Annealing	55°C	40 sec	
Extension	72°C	50 sec	
Final extension	72°C	6 min	1
Cooling	4°C		

- PCR for the detection of IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene was performed using ISAba1F and OxaR primers (This study). The primers and expected fragment size were

ISAba1F	TCA GGG TTC TGC CTT CTT AA	1365bp
OxaR	GCA CGA GCA AGA TCA TTA CC	

PCR conditions were

Function	Temperature	Time	Cycle/s
Pre-denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	35
Annealing	57°C	40 sec	
Extension	72°C	50 sec	
Final extension	72°C	6 min	1
Cooling	4°C		

❖ PCR for the detection of any insertion sequences upstream and downstream of *bla*$_{ADC}$ was performed using the primers FU, RU (annealing upstream of *bla*$_{ADC}$) and FD, RD (annealing downstream of *bla*$_{ADC}$) respectively (This study). The primers and expected fragment sizes were

FU	GCGCCGTGAATTCTTAAGTG	360bp
RU	AGCCATACCTGGCACATCAT	
FD	CAGCTT ATGCTGTGCTGGAT	267bp
RD	GAGCTGCCATATTGGGAAGA	

PCR conditions were

Function	Temperature	Time	Cycle/s
Pre-denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	35
Annealing	57°C	40 sec	
Extension	72°C	50 sec	
Final extension	72°C	6 min	1
Cooling	4°C		

❖ PCR for the detection of IS*Aba1* upstream of the *bla*$_{ADC}$ gene was performed according to Ruiz *et al*. (2007) using the ISADC1 and ISADC2 primers. The primers and expected fragment size were

ISADC1	GTT GCA CTT GGT CGA ATG AAA A	751bp
ISADC2	ACG TCG CGA GTT GAA GTA AGT T	

- PCR for the detection of ADC (AmpC) was performed according to Ruiz *et al*. (2007) using the ADC1 and ADC2 primers. The primers and expected fragment size were

ADC1	CCG CGA CAG CAG GTG GAT A	420bp
ADC2	TCG GCT GAT TTT CTT GGT T	

- A multiplex PCR for the detection of integrons was performed using the primers previously described by Dillon *et al*. (2005) using the primers int1F, int1R, int2F, int2R, and int3F and int3R. The primers and expected fragment sizes were

Int1F	CAG TGG ACA TAA GCC TGT TC	160bp
Int1R	CCC GAG GCA TAG ACT GTA	
Int2F	GTA GCA AAC GAG TGA CGA AAT G	788bp
Int2R	CAC GGA TAT GCG ACA AAA AGG T	
Int3F	GCC TCC GGC AGC GAC TTT CAG	979bp
Int3R	ACG GAT CTG CCA AAC CTG ACT	

- The primers CSa and CSb describer earlier by Lévesque *et al*. (1995) were used for the detection of conserved regions in class I integrons.

CSa	GGC ATC CAA GCA GCA AG	Variable
CSb	AAG CAG ACT TGA CCT GA	

- A multiplex PCR for the detection of metallo-β-lactamases was performed using the primers previously described by Ellington *et al*., (2007) using the

primers listed in the table below. The primers and expected fragment sizes were

Imp-F	GGA ATA GAG TGG CTT AAY TCT C	188bp
Imp-R	CCA AAC YAC TAS GTT ATC T	
Vim-F	GAT GGT GTT TGG TCG CAT A	390bp
Vim-R	CGA ATG CGC AGC ACC AG	
Gim-F	TCG ACA CAC CTT GGT CTG AA	477bp
Gim-R	AAC TTC CAA CTT TGC CAT GC	
Sim-F	TAC AAG GGA TTC GGC ATC G	570bp
Sim-R	TAA TGG CCT GTT CCC ATG TG	
Spm-F	AAA ATC TGG GTA CGC AAA CG	271bp
Spm-R	ACA TTA TCC GCT GGA ACA GG	

- Insertion causing disruption of the *carO* gene (29kDa OMP) was checked, using the established primers carOF and carOR described earlier by Mussi *et al*. (2005). The primers and expected fragment size were

carOF	CCA TGG CTG ACG AWG CAG TCG TAC ATG A	700bp
carOR	CCA TGG CAA AAG TAT TAA AAG TTT TAG CAG T	

- The efflux genes were amplified with the primers listed in the table below (This study). They were also used to record the levels of gene expression in strains having high antibiotic efflux, mainly to fluoroquinolones. The primers and expected fragment sizes were

adeS-F	TGC CGC CAA ATT CTT TAT TC	660bp
adeS-R	TTA GTC ACG GCG ACC TCT CT	

adeR-F	CGC TCT AGT GCA TCG CTA TC	313bp
adeR-R	GCA TTA CGC ATA GGT GCA GA	
adeA-F	CGC AAG TCG GAG GTA TCA TT	760bp
adeA-R	TAT ACC TGA GGC TCG CCA CT	
adeB-F	CCC TAA TCA AGG ACG TAT GC	381bp
adeB-R	TAG AGT GCA GCC AAG ACA AG	
adeC-F	AGC CTG CCA TTA CAT CTC AT	560bp
adeC-R	TGG CAC TTC ACT ATC AAT AC	
adeIF	ATC GCG CTT GTT GGT TGT AG	541bp
adeIR	AAG CAC CAG CCG TTA CTG AA	
adeJF	CTG GTG CTA TGG GCG TTA GT	500bp
adeJR	CGG AAT TGA CCA GCT TTC AT	
adeKF	CAC AGA ACA ACC AGC TTC CA	609bp
adeKR	CCG CAG CGC TTA AGT TGT AT	

PCR conditions were

Function	Temperature	Time	Cycle/s
Pre-denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	35
Annealing	57°C	40 sec	
Extension	72°C	50 sec	
Final extension	72°C	6 min	1
Cooling	4°C		

❖ The intergenic regions of *adeR* and *adeS*, *adeR* and *adeA* and the *adeA* and *adeB* were amplified using the primers listed in the table below (This study). The primers and expected fragment sizes were

adeRF	GCA TTA CGC ATA GGT GCA GA	790bp
adeSR	GAG GTC GCC GTG ACT AAT TT	
adeRA	TCA CGG GAG TCT GAG CTT TT	929bp
adeAB	AAT AGG CGC TCG AAC TGT TG	
adeAF	CAA CAG TTC GAG CGC CT ATT	999bp
adeBR	ACC TGA GCC ATT TCC ACA TC	

PCR conditions were

Function	Temperature	Time	Cycle/s
Pre-denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	35
Annealing	57°C	40 sec	
Extension	72°C	50 sec	
Final extension	72°C	6 min	1
Cooling	4°C		

❖ The primers described previously by Noppe-Leclercq *et al*. (1999) were used for the amplification of aminoglycoside resistance genes. The primers and expected fragment sizes were

AB1F	TGCCGATATCTGAATC	*aac(6')-Ih*	407bp
AB1R	ACACCACACGTTCAG		
AB2F	TATGAGTGGCTAAATCGAT	*aaa(6')-Ib*	395bp

AB2R	CCCGCTTTCTCGTAGCA		
AB3F	CGGAAACAGCGTTTTAGA	*aph(3')-VI*	716bp
AB3R	TTCCTTTTGTCAGGTC		
AB4F	CGAGCATCAAATGAAACTGC	*aph(3')-Ia*	623bp
AB4R	GCGTTGCCAATGATGTTACAG		
AB5F	TCT GCC GCT CTG GAT	*ant(2'')-Ia*	404bp
AB5R	CGAGCCTGTAGGACT		
AB6F	GACATAAGCCTGTTCGGTT	*aac(3)-Ia*	372bp
AB6R	CTCCGAACTCACGACCGA		
AB7F	ATGCATACGCGGAAGGC	*aac(3)-IIa*	822bp
Ab7R	TGCTGGCACGATCGGAG		

- The primers described previously by Valentine *et al*. (2008) were used for the amplification of QRDRs. The primers used were

gyrA-F	AAATCTGCTCGTGTCGTTGG	345bp
gyrA-R	GCCATACCTACAGCAATACC	
parC-F	AAGCCCGTACAGCGCCGTATT	327bp
parC-R	AAAGTTATCTTGCCATTCGCT	

- Primers were used for the detection of bla_{TEM} genes (Weill *et al*., 2004), $bla_{\text{CTX-M}}$ families (Woodford *et al*., 2005), bla_{SHV} (Findlay *et al*., 2012). The primers for bla_{PER}, bla_{VEB} and bla_{GES} were also used (This study). The primers and expected fragment sizes were

TEM-F	ATA AAA TTC TTG AAG ACG AAA	1080bp
TEM-R	GAC AGT TAC CAA TGC TTA ATC	
CTXMG1F	AAA AAT CAC TGC GCC AGT TC	415bp
CTXMG1R	AGC TTA TTC ATC GCC ACG TT	
CTXMG2F	CGA CGC TAC CCC TGC TAT T	522bp
CTXMG2R	CCA GCG TCA GAT TTT TCA GG	
CTXMG9F	CAA AGA GAG TGC AAC GGA TG	205bp
CTXMG9R	ATT GGA AAG CGT TCA TCA CC	
CTXMG8F	TCG CGT TAA GCG GAT GAT GC	666bp
CTXMG25F	GCA CGA TGA CAT TCG GG	327bp
CTXM8/25R	AAC CCA CGA TGT GGG TAG C	
SHV-F	CGC CGG GTT ATT CTT ATT TG	1069bp
SHV-R	CCA CGT TTA TGG CGT TAC CT	
PER-F	CCT GAC GAT CTG GAA CCT TT	715bp
PER-R	GCA ACC TGC GCA ATG ATA GC	
VEB-F	ATT TCC CGA TGC AAA GCG T	360bp
VEB-R	CCA ACA GCG ATG AAC AAA CT	
GES-F	ATG CGC TTC ATT CAC GCA C	840bp
GES-R	AAC TCA TCC TGA GCA CGG AC	

PCR conditions for the amplification of *bla*$_{PER}$, *bla*$_{VEB}$ and *bla*$_{GES}$ were

Function	Temperature	Time	Cycle/s
Pre-denaturation	94°C	5 min	1
Denaturation	94°C	30 sec	35
Annealing	57°C	40 sec	
Extension	72°C	50 sec	
Final extension	72°C	6 min	1
Cooling	4°C		

2.11 Primers used for analysis of gene expression

OXA-58-like F and OXA-58-like R primers described earlier by Woodford *et al*. (2006) were used for the expression analysis of the *bla*$_{OXA-58-like}$ gene.

Primers for the gene expression of the *bla*$_{ADC}$ gene (AmpC) were ADC1 and ADC2 primers, as mentioned earlier by Ruiz *et al*. (2007).

Primers 51-F and 51-R described earlier by Lopes *et al*. (2010). were used for the gene expression. The primers and expected fragment sizes were

51-F	TTT CAG CCT GCT CAC CTT	679bp
51-R	TTC CCT TGA GGC TGA ACA AC	
65A	CTC GTG CTT CGA CCG AGT AT	513bp
65B	GCT GAA CAA CCC ATC CAG TT	

Primers 16S-F and 16S-R, described earlier by Lin *et al*., (2009) were used as internal control primers for the constitutively expressed 16S-23S gene in *A. baumannii*. The primers and expected fragment size were

16S-F	GAC GTA CTC GCA GAA TAA GC	426bp
16S-R	TTA GTC TTG CGA CCG TAC TC	

2.12 Sequencing of the genes

The PCR products were purified by Qiagen PCR purification kit, UK and 5 µL of DNA was used with 1 µL of 3.2 pmol of primer for forward or reverse sequencing of the genes.

The PCR products were sequenced to check for any novel genes or mutations by the Sanger method using an ABI 373A DNA sequencer (PE Applied Biosystems, Warrington, UK). The BLAST and FASTA programs of the National Centre for Biotechnology Information (http://www.ncbi.nlm.nhi.gov) were used to search databases for similar nucleotide and amino acid similarities. The amino acid sequences were translated using the EXPASY translate tool (http://www.expasy.ch/tools/dna.html). Alignment of the DNA or amino acid sequences was performed with the Multalin tool to check for any changes in the sequences (http://www.toulouse.inra.fr/multalin.html).

2.13 Gradient plate technique

Some of the strains used in this study were treated with the frame-shift mutagen acriflavine after overnight growth in nutrient broth. The mutants were isolated using

the gradient plate technique as described previously by Hunt and Sandham (1969) using a concentration range between 0.03- 0.3 % .

A single colony at the highest concentration was selected and tested for further analysis.

2.14 Strain serial subculture

Strain subculture was performed daily on MacConkey agar for over 150 days in order to check for changes in the outer membrane proteins and the stability of IS*Aba1*.

2.15 RNA extraction

Bacterial cultures were grown to an optical density of 0.5-0.8 at OD_{600} and the total RNA was extracted with RiboPureTM-Bacteria Kit according to the manufacturer's instructions (Ambion, UK). Sterilization of the benches, gloves and the pipettes was done using RNAZAP (Ambion, UK). The RNA was measured using nanodrop spectrophotometer, ND-1000. 1500ng of RNA was taken forward and treated with DNase I at 37°C for 30 minutes to remove any traces of genomic DNA.

2.16 Reverse transcription

The treated RNA was re-estimated and 100ng of it was taken forward in order to perform reverse transcription. RNA was reverse transcribed to cDNA by incubation at 45 ^{o}C/1 hr, followed by specific PCR gene amplification according to the manufacturer's guidelines (Access RT-PCR System Kit, Promega). The reverse transcription was done according to the manufacturer's instructions.

2.17 Determination of outer membrane proteins (OMPs) by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The outer membrane proteins were extracted according to the method described by Bossi and Bossi. (2007). Briefly, bacterial cells were grown in 5 ml of LB to OD_{600} of 0.4 and collected by centrifugation at 3000 g (Sorvall RT 6000D) for 15 minutes at 4°C. The pellet was re-suspended in 1 ml of 10 mM Tris pH 8.0 and sonicated with cooling, using three pulses of 20 s with 30 s resting intervals (MSE Soniprep 150, MSE instruments, Crawley). The lysate was centrifuged at 7000 g for 5 minutes. The supernatant was recovered and centrifuged at 13 000 rpm for 45 minutes at room temperature. The pellet was re-suspended in 0.5 ml of freshly made 10 mM Tris-HCl pH 8.0, 10 mM MgCl2, 2% (v/v) Triton X-100. Furthermore, the suspension was incubated at 37°C for 30 minutes, and then centrifuged at 16000 g for 45 minutes at 20°C. Finally, the pellet was re-suspended in 50 µl of 100 mM Tris-HCl pH 8.0, 2% SDS and stored at -20°C if not used immediately.

2.18 Polyacrylamide gel electrophoresis

The percentages of separating and stacking gels were calculated as shown below. (http://www.changbioscience.com/calculator/sdspc.htm, Last accessed, 6/6/2010)

Stacking gel (4%)	Total Volume in µL	Separating gel (15%)	Total Volume in µL
Polyacrylamide (40%)	625	Polyacrylamide (40%)	3750
1M Tris (pH-6.8)	625	1M Tris (pH-6.8)	2500
10% Ammonium persulfate	12	10% Ammonium persulfate	22
TEMED	12	TEMED	22
D/W	3650	D/W	3550
10% SDS	50	10% SDS	100

Ammonium persulfate and TEMED were the last components to be added just before pouring the gel. The separating solution was poured into the pre-assembled Bio-Rad gel apparatus. Addition of ethanol ensured a fine uniform surface, which was allowed to solidify by cross-linking after which the stacking gel was added and a comb was placed in order to make wells to load the samples. The gel was allowed to polymerise for 30-45 mins in the presence of light.

The protein concentration was estimated using NanoDrop spectrophotometer (ND-1000) for the samples to be loaded. Approximately 50 to 100 µg of protein was mixed

in equal volume with the loading dye made up of 950 µl of Bio-Rad buffer and 50 µl β-mercaptoethanol. 5 µl of the protein sample was mixed with 5 µl dye and was heated at 100 ºC for 10 mins, cooled on ice in order to denature proteins.

Molecular weight markers (Bio Rad) were used for estimation of band size. The gel was inserted into a gel electrophoresis apparatus (Protean II, Bio Rad) and was electrophoresed at 150 V for approximately 90 minutes in 1X Tris-Glycine buffer.

The stacking gel was cut off and the separating gel was stained in 0.1% w/v Coomassie blue R-250, in 50% v/v ethanol, 1% v/v acetic acid in distilled water, for 50 minutes. Destaining of the membrane was carried out with the destaining solution (50% v/v ethanol, 1% v/v acetic acid in distilled water) for 50 minutes. The gel was washed in distilled water for 10 mins or left over-night followed by viewing under illuminated light surface.

2.19 Efflux assay

The efflux assays were either performed as described previously by Pannek *et al*. (2006). Bacterial cells were grown overnight on LB or IST agar plates and diluted in 1 mL of PBS + 0.4% glucose (pH 7.4) until an OD at 600 nm of 1 was reached. The cells were then transferred to a 96-well plate, and NMP (1mg/L) was added. Thereafter, EtBr was added to a final concentration of 1 mg/L, and the relative fluorescence intensity was measured over time at 37° C with a fluorescence spectrophotometer (Polarstar optima, BMG, labtech). Excitation and emission wavelengths of 544 and 612nm were used.

2.20 Detection of extended spectrum β-lactamases (ESBLs)

Some of the isolates were screened for ESBL production using the cephalosporin and co-amoxiclav synergy disc method as described previously by Livermore and Brown (2001). Discs containing co-amoxiclav (30µg) and ceftazidime (30µg) are applied 25-30 mm apart on IST agar swabbed with the test culture. A disc containing a different cephalosporin can be placed on the opposite side of the co-amoxiclav disc. The plate is incubated overnight at 37^{o}C and ESBL production was inferred when the cephalosporin zone was expanded by clavulanate. This method is cost-effective and the disadvantage is that the optimal separation of the discs may vary with the individual strain. The cephalosporins discs used for this assay were of ceftazidime, cefotaxime, cefpodoxime and cefepime.

2.21 Conjugation assay

Transconjugation assays were performed with *E.coli* J62.2 as the recipient as described earlier by Findlay *et al*. (2012). Overnight cultures of the recipient (*E. coli* J62.2) and donor strains were mixed in a 1:4 ratio, and cells were collected by centrifugation and resuspended in 30 µL of cold saline. Five microlitre aliquots of the resuspension were spotted onto a nutrient agar plate and incubated at 37°C for 6 h. Growth was collected and resuspended in cold saline and inoculated onto nutrient agar plates containing rifampicin at 16 mg/L and one of the selective antibiotics, ceftazidime or meropenem, at a range of concentrations.

2.22 S1-nuclease digestion

S1 nuclease is an endonuclease that is more active against DNA than RNA. Its reaction products are oligonucleotides or single nucleotides with 5' phosphoryl

groups. It is usually nicks the plasmid DNA and linearises it to form a distinct band. S1 nuclease digestion was performed for the PFGE plugs according to the manufacturer's instructions. A total of 10 units per plug were used for digestion with incubation at 37°C /45 mins. The plasmids were stained with gel red and visualized by the gel-doc system.

2.23 Plasmid curing

Plasmid curing is the selective removal of plasmids present in a specific species of bacteria. Plasmid curing can help in determining the presence of resistance genes, spreading in various bacterial species. Plasmid curing was performed using one or more of the curing agents like acriflavine (0.2-2%), SDS (4%) or ethidium bromide (1-2%) assisted with elevated temperature of incubation (reviewed by Trevors, 1986). The strains were serially subcultured for 14 days or more at 47°C.

2.24 Plasmid isolation (Joly, 1996)

A single well isolated colony was grown in 5mL IST broth at 37° C/24hrs. The culture was centrifuged and the supernatant was decanted. It was suspended in 100µL of bacterial resuspension solution [50 mM glucose, 50 mM Tris-HCI, pH 8.0, 10mMEDTA]. 200 µL of lysis solution (200 mM NaOH, 1% SDS) was added and the mixture was gently iverted and left at room temperature for 3 minutes. 150 µL of neutralizing solution (3M potassium/5M acetate solution) was then added and centrifuged at maximum speed for 5 minuted. The liquid phase was removed and transferred to another set of tubes containing chilled iso-propanol. The precipitate formed was recoved by centrifuging the tubes at maximum speed. Finally it was washed with 70% ethanol and resuspended in TE buffer (10 mM TrisHCI, pH 8.0,1

mMEDTA). Chromosomal DNA was removed with 4M ammonium acetate and 25:24:1 mix of TE-equilibrated phenol, chloroform, and isoamyl alcohol.

3.0 RESULTS AND DISCUSSION

3.1 SECTION 1:

IS*Aba1* transposition and control of resistance genes in *A. baumannii*.

3.1.1 Aims and objectives

To check if IS*Aba1* can transpose in the *Acinetobacter baumannii* genome using the frame-shift mutagen acriflavine.

To check if strain subculture leads to attenuation or weakening and whether this affects transposition of the IS*Aba1* element.

To observe any changes in the outer membrane protein profile which leads to resistance to carbapenems.

3.1.2 Identification and PCR amplification

The strains used in this study were previously identified as *A. baumannii* by Benjamin Evans in this laboratory and were designated as AB153, AB1225 (parent), AB153M, AB1225M (acriflavine derived mutant) and AB1225s (MacConkey agar serially sub-cultured strain for 150 days).

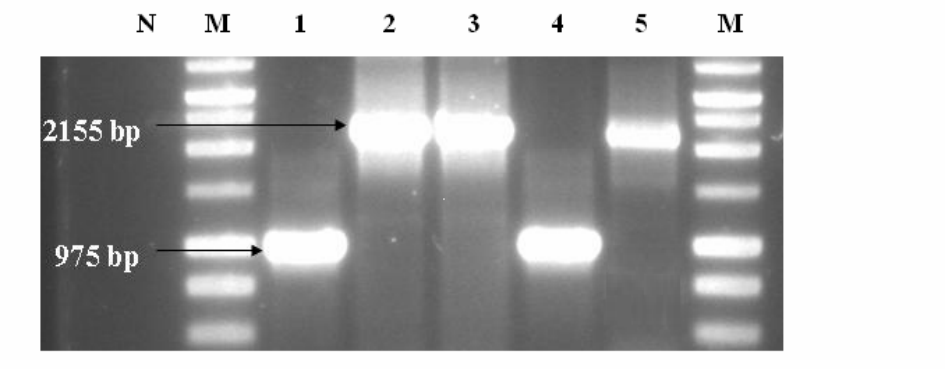
A PCR described by Dolzani *et al*. (1995) using the primers 16S-23S primer1 and 16S-23S primer 2, was used to re-identify and confirm the identity. The 975bp product amplified for *Acinetobacter* genospecies 2 was restricted with the enzymes *Alu*I and *Nde*II. Restriction of the product by the enzyme *Alu*I produced fragments of 50, 125, 135, 165, 175 and 330 bp specific to *A. baumannii*. Restriction of the same product produced fragment sizes 50, 110,145, 330 and 360 bp by the enzyme *Nde*II, specific to *A baumannii*.

They were reconfirmed by the *bla*$_{\text{OXA-51-like}}$ PCR using the 69A and 69B primers described earlier by Héritier *et al* (2005b). A product of 2155bp was AB1225 and AB1225s strains. Sequencing the fragment revealed the presence of IS*Aba1* upstream of the *bla*$_{\text{OXA-66}}$ gene. A fragment of 975bp was amplified for the strain AB153. Sequencing revealed the presence of the *bla*$_{\text{OXA-110}}$ gene in AB1225 and AB1225s strain

Primers ADC1 and ADC2 were used for the detection of *bla*$_{\text{ADC}}$ gene. AB153, AB1225 and AB1225s showed the presence of the *bla*$_{\text{ADC}}$ gene. Primers ISADC1 and ISADC2 detected the presence of insertion sequence IS*Aba1* upstream of the *bla*$_{\text{ADC}}$ gene in Ab1225 (Figure 20). They were described earlier by Ruiz *et al*. (2007).

After AB153 and AB1225 were treated with the frame-shift mutagen and isolated by the gradient plate technique, they were designated as AB153M and AB1225M. It was observed that isolate AB153M had gained IS*Aba1* upstream of the *bla*$_{\text{OXA-66}}$ gene whereas isolate AB1225M had lost the IS*Aba*1 sequence. The genetic rearrangement upstream of the *bla*$_{\text{OXA-110}}$ gene in strain AB1225s remained unchanged (Figure 19).

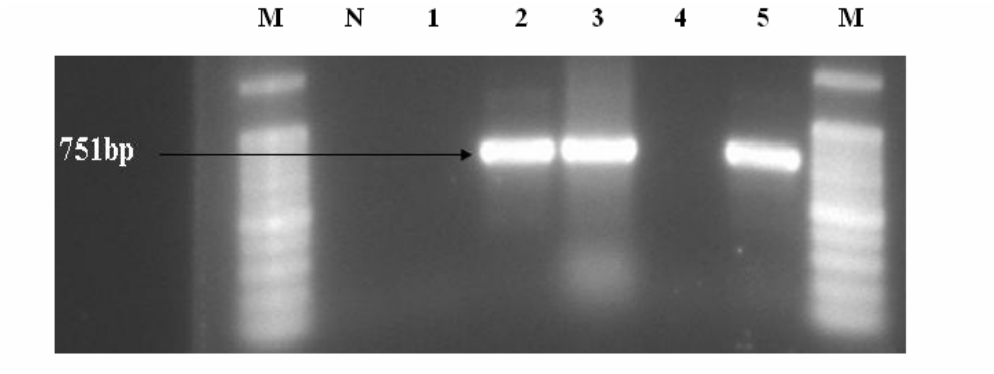
Figure 19: PCR for the detection of insertion element upstream of the *bla*$_{\text{OXA-51-like}}$ gene



N=negative control, M=1kb ladder, 1=AB153 parent, 2= AB153M (acriflavine mutant), 3= AB1225 parent 4=AB1225M (acriflavine mutant), 5=AB1225s, serial sub-cultured strain. The *bla*$_{\text{OXA-51-like}}$ gene in each mutant strain was sequenced in order to confirm its identity with the parent strain.

IS*Aba1* movement was seen upstream of *bla*$_{\text{ADC}}$ gene in the acriflavine treated AB153M but its loss was observed in the similarly treated strain AB1225M. IS*Aba1* movement was not seen in AB1225s (sub-cultured) and its features remained identical with respect to its parent strain AB1225 (Figure 20). The IS*Aba1* sequence of strain AB153M, AB1225 and AB1225s did not show any mutations or nucleotide substitutions.

Figure 20: PCR for the detection of insertion element upstream of the *bla*$_{ADC}$ gene



N=negative control, M=100bp ladder, 1=AB153 parent, 2= AB153M (acriflavine mutant), 3= AB1225 parent 4=AB1225M (acriflavine mutant), 5=AB1225s, serial sub-cultured strain.

3.1.3 Antimicrobial susceptibility testing

Table 5 shows changes in MICs of IPM, MEM, PIP/TZ and CAZ in *A. baumannii* parent, mutants and the serially sub-cultured strain. An 8-fold rise in MICs to imipenem was seen in AB153M and a 4-fold decrease was seen in AB1225M strains with respect to the parent strains, AB153 and AB1225. A 4-fold and 2–fold decrease in MIC of imipenem and meropenem was seen in AB1225s with respect to AB1225. There was negligible difference seen in MIC values of CAZ. No major difference was seen in the MIC values of PIP/TZ except that a 4-fold decrease was seen in isolate AB1225s compared to AB1225 parent strain. Furthermore as no IS*Aba1* change was observed in AB1225s strain, it was important to see if there were any significant changes related to the outer membrane proteins of this strain as it had a 4-fold and 2-fold decrease in MIC of imipenem and meropenem.

Table 5: MIC's of various antibiotics and the levels of gene expression in clinical isolates of *A. baumannii.*

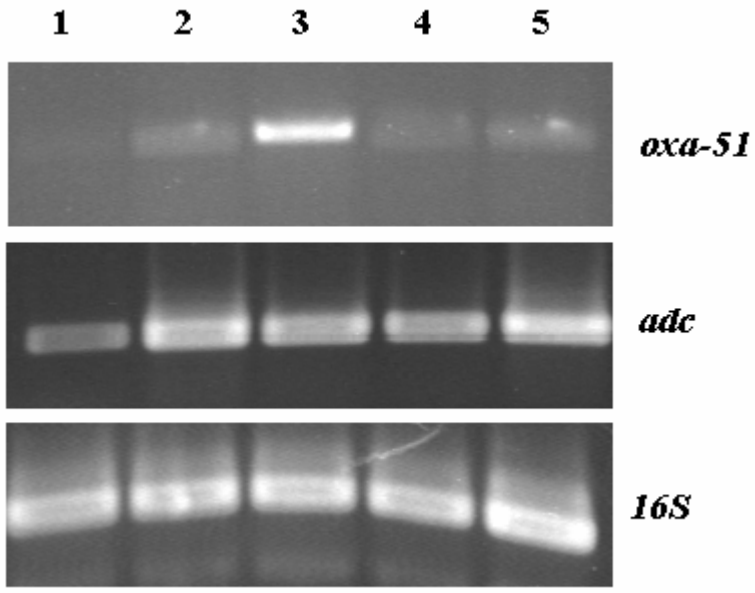
Strain	MIC (mg/L)					
	IPM	MEM	PIP/TZ	CAZ	Expression of $bla_{\text{OXA-51 like}}$ gene●	Expression of bla_{ADC} gene●
AB153	0.25	1	32	32	0	0
AB153M	2	4	64	128	+2.5 ± 0.11	+ 2.7 ± 0.105
AB1225	2	4	64	128	0 ± 0.057	0 ± 0.057
AB1225M	0.25	1	64	64	5.0 ± 0.106	-1.2 ± 0.57
AB1225s	0.5	2	16	128	-4.0 ± 0.15	0

● Fold increase/decrease (±Standard Deviation)

3.1.4 Gene expression analysis

The expression of the $bla_{\text{OXA-51-like}}$ and bla_{ADC} genes were analysed by RT-PCR (Promega) (Figure 21). The products obtained were quantified using Bio-Rad quantity one software 4.6.1. A 2.7-fold increase in expression of the bla_{ADC} gene was recorded in AB153M in comparison with AB153. There was a 1.2-fold decrease seen in the expression of the bla_{ADC} gene between AB1225M compared to AB1225. A negligible difference was seen in expression of the bla_{ADC} gene between AB1225 and AB1225s. It was observed that there was a 2.5-fold increased expression of $bla_{\text{OXA-51-like}}$ gene in AB153M in comparison to AB153 and a 5-fold decreased expression of $bla_{\text{OXA-51-like}}$ gene seen in AB1225M in comparison to AB1225. There was 4-fold decrease in expression of $bla_{\text{OXA-51-like}}$ gene seen in AB1225s as compared with AB1225. The expression was confirmed thrice using separate RNA preps and the results recorded are based on the average mean increase or decrease of individual strains. Structural organization of $bla_{\text{OXA-51-like}}$ and bla_{ADC} genes in *A. baumannii* genome is shown in Figure 22a and 22b.

Figure 21: Analysis of gene expression



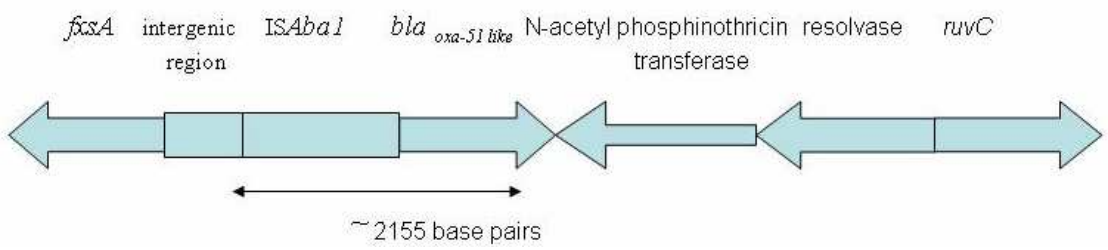
***oxa-51*:** Expression analysis of the $bla_{\text{OXA-51 like}}$ gene in *A. baumannii* using 51F and 51R internal primers, ***adc*:** Expression analysis of the bla_{ADC} gene in *A. baumannii* using the ADC1 and ADC2 primers, ***16S*:** PCR for 16S-23S rRNA gene in *A. baumannii* using 16S-F and 16S-R primers.

1= Ab153 (Parent strain), 2= Ab153M (acriflavine mutant strain), 3= Ab1225 (Parent strain) 4= Ab1225M (acriflavine mutant), 5= Ab1225s (serial sub-cultured strain).

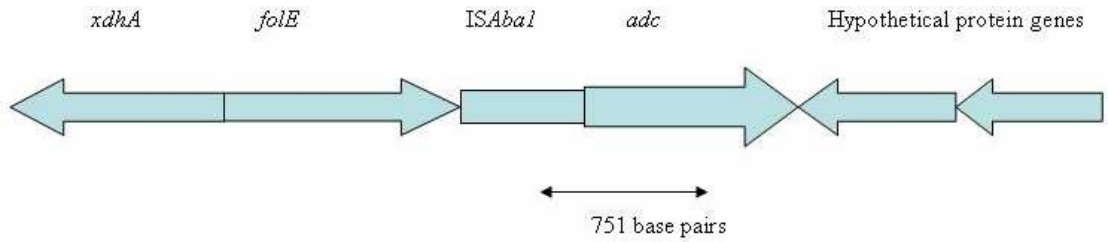
mcrA, *gyrB* or *rpoB* genes can also be used as 16S is highly abundant and widely distributed in the genome.

Figure 22: Structural organization of *bla*$_{\text{OXA-51-like}}$ (a) and *bla*$_{\text{ADC}}$ (b) genes in *A. baumannii* (Not to scale). IS*Aba1* (insertion element), *fxsA* (phage exclusion factor), *bla*$_{\text{OXA-51-like}}$ (oxacillinase gene), *adc* (acinetobacter derived cephalosporinase)

a)



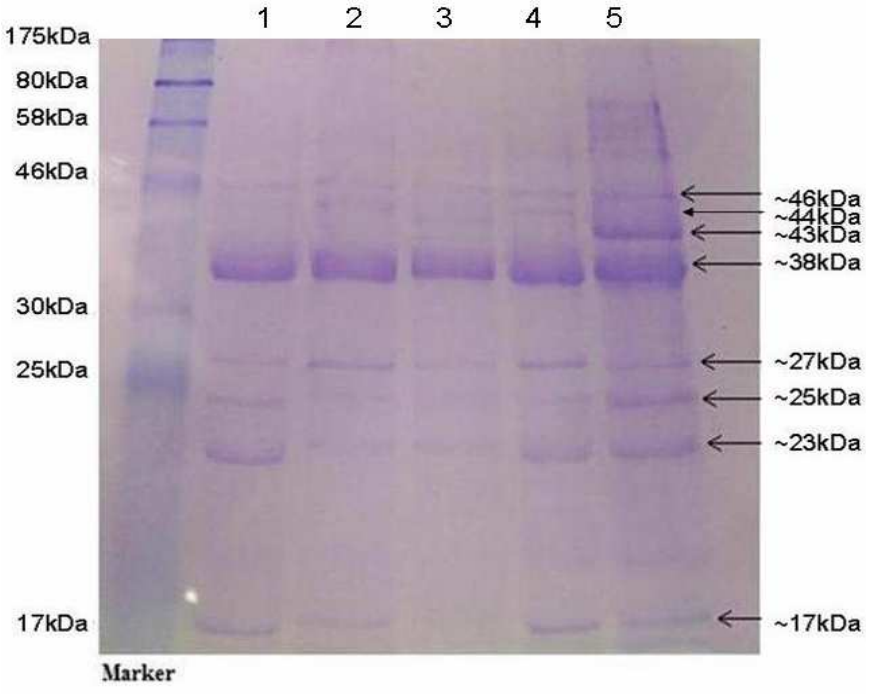
b)



3.1.5 Analysis of OMP profiles

Figure 23 shows the OMP profiles obtained for the *A. baumannii* used in this study. The analyses reveal significant differences between the AB153 parent and AB153M. Reduced expression of 17kDa, 23kDa and 25kDa OMPs was seen in the AB153M strain, which also had increased MIC values. AB1225M and AB1225s exhibited an increase in the expression of 17kDa, 23kDa, 25kDa and 27kDa OMPs and had a decrease in MICs values, rendering them more susceptible to some antibiotics. The outer membrane pattern produced by AB1225s showed significant over expression of a 43kDa OMP.

Figure 23: Outer Membrane profiles of *Acinetobacter baumannii* isolates.



1=AB153 parent, 2=AB1225 parent, 3=AB153M (acriflavine mutant), 4=AB1225M (acriflavine mutant), 5=AB1225s (serial sub-cultured strain).

3.1.6 DISCUSSION:

The different resistance profiles of *A. baumannii* correlate with the movement of IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ gene or the bla_{ADC} gene. The MIC values of AB153 and AB1225 parent and mutant strains suggest that IS*Aba1* may act as a promoter for $bla_{\text{OXA-51-like}}$ gene or the bla_{ADC} gene. It is noteworthy to say that the functionality of IS*Aba1* depends on it acting as a promoter rather than just being present upstream of an antibiotic resistance gene. This addresses the fact that IS*Aba1* may have a preferential role with respect to specific antibiotic resistance genes like bla_{OXA} or *ampC*. Resistance mechanisms to various antibiotics also depend on the usage of antibiotics in specific hospitals and hence the bacterium may recruit insertion

sequences like IS*Aba1*, which leads to over-expression of antibiotic resistance genes. This is also an important defence mechanism employed by the bacterium for its survival in the host and ability to resist antibiotic pressure. The selection of the gene for over-expression is pre-determined by the ability to hydrolyse antibiotics at a basal level first and second by strengthening its control by insertion elements, which are, at least, partly responsible for the multiresistant profile of *A. baumannii*.

Strain AB1225s had a negligible difference on expression of the bla_{ADC} gene with respect to AB1225, both of which retained IS*Aba1* upstream of the gene. This is supported by the fact that resistance to oxyiminocephalosporins is mainly due to hyperproduction of Amp-C-type-β-lactamases (Rodríguez-Martínez *et al*, 2010).

AB1225s had a 4-fold decrease in $bla_{\text{OXA-51-like}}$ gene expression concurring with lower MICs of carbapenems. The increased expression of OMPs in AB1225s can also be a factor that led to 4-fold decrease in the MIC of imipenem, a 2-fold decrease in the MIC of meropenem and 4-fold decrease in the MIC of piperacillin/tazobactam causing strain attenuation. It can be said that OMPs do play a role in antibiotic resistance depending on the stress load of the compound and they might be important for transport of the compound across the cell wall in either direction.

All the *A. baumannii* strains were resistant to ceftazidime and it can be said that IS*Aba1* may play a preferential role in increased expression of the bla_{ADC} gene making carbapenems the last resort for treatment of patients. This concurs with the fact that genetic organization of genes and their control is likely to play a crucial role in antibiotic resistance.

It was also observed that there was over-expression of OMPs in AB1225M, which correlated with a drop in MIC values of different antibiotics. Strains AB153 and AB153M also had changes with respect to their outer membrane protein profiles. Since an 8-fold increase in resistance to imipenem and 4-fold increase to meropenem was seen in the AB153M, it can be deduced that the resistance developed in AB153M may not only be due to the movement of IS*Aba1* upstream of $bla_{\text{OXA-51-like}}$ gene but also partly due to the reduced expression of three OMPs corresponding to 17kDa, 23kDa and 25kDa, all of which are poorly expressed in the mutant strain AB153M.

Over expression of OMP's was seen in AB1225M with drop in MIC values suggesting that they might have some implications with respect to the increased susceptibility in this strain, which lost the IS*Aba1* element upstream of $bla_{\text{OXA-51-like}}$ gene and bla_{ADC} genes. The MICs for ceftazidime and piperacillin/tazobactam in AB153 and AB1225 and the mutant strains may provide an insight into the development of resistance over a given period of time as no significant changes in MICs were seen in the parent and mutant strains.

This study presents an open view that with the movement of IS*Aba1* is a strong factor for conferring resistance provided it is crucial for the gene action; if there is over expression of primary surface structures of the bacterium, it can render the organism sensitive to a particular drug or poison and to switch the gene on may be advantageous to this bacterium when it is required since OMP changes do act synergistically as seen in AB1225s and other strains, and this would ideally depend on the action of promoters present for specific genes and their tight regulation by

repressor proteins. This undoubtedly contributes to the organism's plasticity making it resistant to different classes of antibiotics.

3.2 SECTION 2:

IS*Aba1* and IS*Aba125* govern the *bla*$_{ADC}$ gene expression in clinical *A. baumannii* isolates.

3.2.1 Aims and objectives

To examine the intergenic regions present upstream and downstream of the *bla*$_{ADC}$ gene of *A. baumannii* and to identify any novel insertion sequences, besides IS*Aba1* that could be responsible for the over-expression of *ampC* gene in *A. baumannii*.

The *A. baumannii* bacterial strains used this study were A2, A8, A16, and A21 which were of clinical origin and were chosen as representatives from a collection of 36 strains.

The isolates were from the collection of Benjamin Evans (Europe) and had been previously identified by the amplified fragment length polymorphism of the conserved 16S-23S rRNA intergenic spacer sequences described by Dolzani *et al*. (1995).

3.2.2 Identification and PCR amplification

All the strains had the intrinsic *bla*$_{OXA-51-like}$ gene. In addition to the *bla*$_{OXA-51-like}$ gene, strain 2 possessed the *bla*$_{OXA-58-like}$ gene as identified by multiplex PCR (Woodford *et al*., 2006). The primers oxa-69A and oxa-69B amplified the *bla*$_{OXA-51-like}$ gene without any insertion upstream of the gene in all the strains. Sequencing of the gene revealed that strains 2, 16 and 21 had the *bla*$_{OXA-66}$ gene. Strain 8 had the *bla*$_{OXA-69}$ gene. All the strains were also positive for the *bla*$_{ADC}$ gene by the PCR described by Ruiz *et al*. (2007). Strains 16 and 21 showed a positive result for the presence of IS*Aba1*

upstream of *bla*$_{ADC}$. Primers FU and RU used for the detection of sequence upstream of *bla*$_{ADC}$ showed the presence of IS*Aba125* upstream in strain 2 and IS*Aba1* in strains 16 and 21. Strain 8 did not have any insert upstream of the *bla*$_{ADC}$ gene. Primers FD and RD amplified specific region downstream of *bla*$_{ADC}$ but no insertion was detected in any of the strains.

3.2.3 Antimicrobial susceptibility testing

Table 6.1 shows the MICs of imipenem, meropenem, gatifloxacin, cefepime and ceftazidime with and without the efflux pump inhibitor 1-(1-napthylmethyl)-piperazine (NMP) for the strains used in this study. The MICs of meropenem were decreased 4-fold after addition of the efflux pump inhibitor NMP in strains 2, 16 and 21; however, there was no change seen in MICs of imipenem and all the strains were susceptible to this antibiotic. All strains, except 8, were resistant to gatifloxacin (BSAC guidelines for Enterobacteriaceae). A two-fold decrease in the MIC of gatifloxacin was seen in strain 2 and 21 after addition of NMP. All the strains were resistant to ceftazidime and cefepime and there was no decrease in MIC seen after addition of NMP. The degree of resistance for ceftazidime and cefepime varied for all the strains. The MICs of ceftazidime and cefepime for strain 2 and 16, were greater than that of strain 21, greater than strain 8.

Table 6.1: Antibiotic profile and the fold gene expression of *A. baumannii* clinical isolates.

Isolate	*bla*$_{\text{OXA-51-like}}$ gene	MIC (mg/L)										IS upstream of the *bla*$_{\text{ADC}}$ gene		Expression of *bla*$_{\text{ADC}}$ gene●
		IPM	IPM +NMP	MEM	MEM +NMP	CAZ	CAZ +NMP	FEP	FEP +NMP	GAT	GAT + NMP	IS*Aba1*	IS*Aba125*	± SD
2	66	0.25	0.25	1.0	0.25	>256	>256	32	32	8	4	-	+	6.2 ± 0.05
8	69	0.25	0.25	0.5	0.25	8	8	8	8	0.12	0.12	-	-	1.0 ± 0.10
16	66	0.5	0.5	1.0	0.25	>256	>256	32	32	16	16	+	-	3.0 ± 0.15
21	66	0.5	0.5	1.0	0.25	32	32	16	16	0.5	0.25	+	-	2.4 ± 0.10

● Fold increase/decrease (Standard Deviation ± 1)

IPM= Imipenem, MEM= Meropenem, CAZ= ceftazidime, FEP= Cefepime, GAT= Gatifloxacin, NMP=1-(1-napthylmethyl)-piperazine, SD=

SD= Standard Deviation

Table 6.2: Ethidium bromide accumulation assay for *A. baumannii* isolates.

Isolate	Ethidium bromide accumulation (arbitrary units)	
	-NMP ± SD	+NMP ± SD
2	16791 ± 106.4	30476 ± 109.0
8	13113 ± 87.90	12265 ± 90.6
16	20897 ± 101.3	48769 ± 108.7
21	17998 ± 108.2	37686 ± 101.3

3.2.4 Detection of extended spectrum β-lactamases (ESBLs)

Extended spectrum β-lactamases could not be detected by the double disc synergy assay as described previously by Livermore and Brown (2001).

3.2.5 Analysis of OMPs (Outer Membrane Proteins)

There were no significant changes seen in any of the outer membrane proteins patterns produced by any of the strains used in this study by the SDS PAGE analysis as described earlier by Bossi and Bossi (2007).The changes in *carO* a 29kDa outer membrane protein (OMP), loss of which is responsible for carbapenem resistance were also checked by PCR as described earlier by Mussi *et al.* (2005). Insertional inactivation of the *carO* was not seen in any of the strains, which also concurs with the fact that all the strains have very low MIC values of IPM, as one of the factor, leading to carbapenem resistance is the loss or disruption of this 29kDa OMP and this was not observed in any of the strains.

3.2.6 Efflux assay

The efflux activity of ethidium bromide, a substrate for AdeABC multidrug efflux pumps was measured in the presence and absence of the efflux pump inhibitor NMP using the method described earlier by Pannek *et al*. (2006). It was observed that strains 2, 16 and 21 demonstrated active efflux to a certain extent (Table 6.2). The fluorescence in strain 2, 16 and 21 increased gradually with respect to time except of that in strain 8 (Table 6.2).

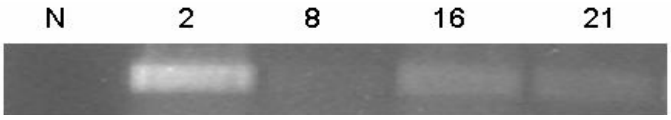
3.2.7 Analysis of gene expression

Figure 24 shows the level of expression of *bla*$_{ADC}$ after cDNA synthesis. Normalization of DNA was done with 16S-rRNA primers used as internal control. The expression studies on strain 2, 16, 21 and 8 indicate that strain 2, possessing IS*Aba125* upstream of *bla*$_{ADC}$, produced a significant over-expression of *bla*$_{ADC}$ which was 2.1-fold greater than strain 16 having IS*Aba1* upstream of *bla*$_{ADC}$ and 6.2-fold more than strain 8, which had no insertion element upstream of *bla*$_{ADC}$. Strain 16 had an MIC of CAZ > 256mg/L with IS*Aba1* upstream of *bla*$_{ADC}$ producing an expression 3-fold greater than strain 8, which had no insertion element upstream of *bla*$_{ADC}$. Strain 21 having MIC of CAZ =32mg/L with IS*Aba1* upstream of *bla*$_{ADC}$, had an expression 2.4-fold greater than strain 8, which had no insertion element upstream of *bla*$_{ADC}$. The results recorded are based on the average mean increase or decrease of individual strains (SD±1 unit). The gene expression was measured thrice under the same optimal conditions.

Figure 24:

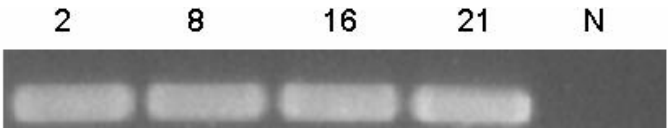
Expression of *bla*$_{ADC}$ gene for *Acinetobacter baumannii* strains 2, 8, 16 and 21.

N is the negative control.



Internal 16S-rRNA control for *Acinetobacter baumannii* strains 2, 8, 16 and 21.

N is the negative control.



3.2.8 DISCUSSION:

It has been previously reported by Corvec *et al*. (2003) and Héritier *et al*. (2006) that IS*Aba1* causes over-expression of *bla*$_{\text{ADC}}$ in *Acinetobacter baumannii*. All the strains reported here were resistant to ceftazidime (CAZ), cefepime (FEP) and gatifloxacin (GAT) but sensitive to imipenem (IPM) and meropenem (MEM). There were no changes in outer membrane proteins and they did not have high levels of resistance to carbapenems. Furthermore the strains did not have any insertion present upstream of *bla*$_{\text{OXA-51-like}}$ gene, which explains the low MIC values of IMP and MEM. Since active efflux was demonstrated by the efflux assay in strain 2, 16 and 21 (Table 6.2) although there was no significant decrease seen in MIC of GAT, they had a 4-fold decrease seen in MIC of MEM, which is also a known substrate for efflux pumps as described by Szabó *et al*. (2006). Efflux contributing to reduced ceftazidime and cefepime susceptibility was not observed in any of the strains. Furthermore expression of the *bla*$_{\text{ADC}}$ gene is the key factor in cephalosporin resistance in *A. baumannii* and it must be recognized that while NMP effects supports the existence of efflux pump, it does not necessarily indicate pump over-expression as efflux mediated resistance in absolute terms is determined in part by the permeability of the outer membrane.

All the strains had different resistance profiles to the antibiotic ceftazidime. It can be seen that strain 2 and 16 are very highly resistant to ceftazidime; on the other hand strains 21 and 8 were far less resistant.

Strain 2 had IS*Aba125* present upstream of the *ampC* (Figure)and the level of expression of the *bla*$_{\text{ADC}}$ gene was 6.2 times greater than strain 8 which has no insertion sequence present upstream of *bla*$_{\text{ADC}}$ gene. In contrast, strains 16 (MIC of

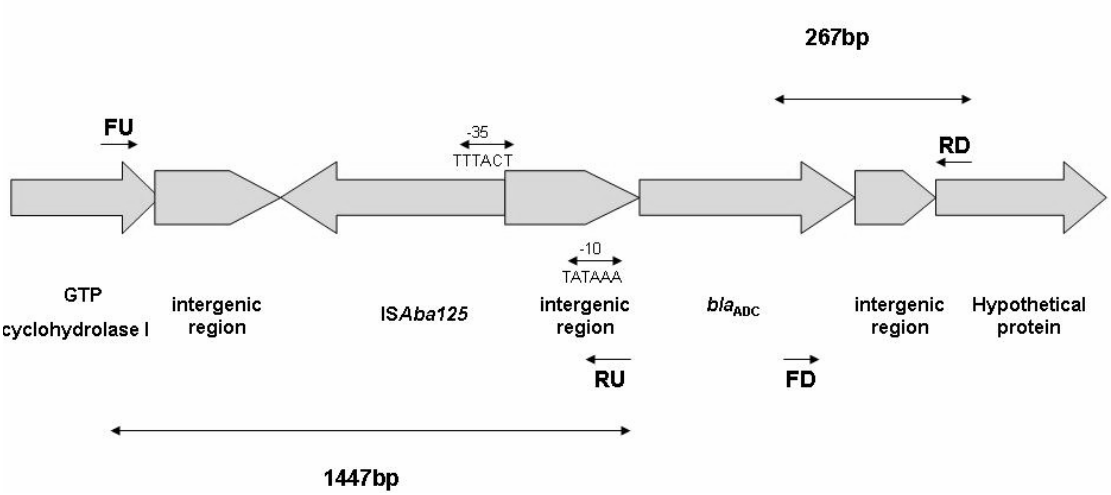
CAZ>256mg/L) and 21 (MIC of CAZ=32mg/L) had IS*Aba1* present upstream of the *bla*$_{ADC}$ but the level of expression in these two strain were 3- and 2.4-fold greater than strain 8 respectively, which did not harbour IS*Aba1* upstream of *bla*$_{ADC}$ gene. This indicates that IS*Aba125* may have a better promoter than IS*Aba1*.

It has been proven that -35 (TTAGAA) and -10 (TTATTT) sequences separated by 16bp constitute a promoter in IS*Aba1* and are located 41bp upstream of *bla*$_{ADC}$ (Héritier *et al*., 2006). The intergenic region also harbours -35 (TTGTTA) and -10 (TTTTTA) sequences separated by 18bp, which constitute a promoter for *bla*$_{ADC}$ gene without any insertion sequence (Héritier *et al*., 2006).

The IS*Aba125* was found 66bp upstream of the *bla*$_{ADC}$ gene (Figure 25) and was oriented in opposite direction against the *bla*$_{ADC}$ gene in strain 2. The right and the left inverted repeats showed 14/18 matches. A new -35 box having the sequence TTTACT was fused with the left inverted repeat and 17bp downstream the -10 box having the sequence TATAAA was seen, which was fused with the -35 promoter of *bla*$_{ADC}$. The *bla*$_{ADC}$ gene was present 39bp downstream of the new -10 box. The IS*Aba1* sequence has a 4/6 and 2/6 match respectively, with the *E. coli* consensus -35 (TTGACA) and -10 (TATAAT) whereas IS*Aba125* has a 4/6 and 5/6 match respectively, with the *E. coli* consensus. This may suggest that IS*Aba125* is able to provide a better promoter than IS*Aba1*. As the -10 box of IS*Aba125* is fused with the -35 box of *bla*$_{ADC}$ it can form a fusion promoter to drive a very strong expression of *bla*$_{ADC}$ and the expression results support this claim. IS*Aba1*, which drives the expression of *bla*$_{ADC}$, is also shown to be present in opposite orientation (3'-5') with the *bla*$_{ADC}$ as proven by Héritier *et al*. (2006). Therefore IS*Aba125*, present in opposite orientation (3'-5'),

may also act in a similar way to drive the expression of *bla*$_{ADC}$ gene. It has been proved that IS*Aba125* causes insertional inactivation of the CarO OMP in *A. baumannii* strains leading to a carbapenem resistant profile (Mussi *et al*., 2005).

Figure 25: Alignment of IS*Aba125* with various regions upstream and downstream of *Acinetobacter baumannii* chromosomal *bla*$_{ADC}$ gene. (Not to scale)



In this study, we demonstrate that IS*Aba125* can modulate the expression of the *bla*$_{ADC}$ gene as shown by the results obtained for strain 2. It is also shown by Evans *et al*. (2010) that IS*Aba125* is responsible for duplication of *bla*$_{OXA-58-like}$ gene and this provides a worrying scenario on the plasmid-borne dissemination of the *bla*$_{ADC}$ gene leading to high levels of cephalosporin resistance in other bacterial species. It is interesting to note that strain 2 not only has IS*Aba125* but also possesses the *bla*$_{OXA-58-like}$ gene as detected by the multiplex PCR (Woodford *et al*., 2006) and there may be a high possibility of the upstream integration and expression of the *bla*$_{OXA-58-like}$ gene mediated by IS*Aba125*, which, under carbapenem stress, would provide the ideal mechanism of carbapenem resistance as shown for *A. baumannii*

genospecies 3 by Evans *et al*. (2010). The results obtained in this study show that IS elements such as IS*Aba125* could modulate cephalosporin or carbapenem resistance depending on the antibiotic treatment of the patient as the isolates used in this study are of clinical origin. The movement of IS*Aba125* upstream of an antibiotic resistance gene can result in significant over-expression of the target resistance gene. If strong promoters are present in the intergenic region, as seen in this study, IS*Aba125* can also form a fusion promoter leading to significant over-expression of the resistance gene, as observed in strain 2, which has a very high expression of the bla_{ADC} gene. It is not surprising that IS elements can cause the spread of resistance genes between different species of bacteria, as it has been already shown by Mammeri *et al*. (2003) that IS*Our1* of *Oligella urethralis* is believed to have been co-transferred with the cephalosporinase gene $bla_{\text{ABA-1}}$ from *A. baumannii*. This study provides an insight into a novel insertion sequence IS*Aba125* belonging to the IS*30* family (Siguier *et al*., 2005), which is involved in significant over-expression of the bla_{ADC} gene, greater than that of the previously known sequence IS*Aba1* found upstream of the bla_{ADC} gene. These results also suggest that the repressor control in IS*Aba125* is different than that of IS*Aba1* and hence the contribution towards a greater the cephalosporin resistance with strong hydrolytic activity of ceftazidime.

3.3 SECTION 3:

Mechanisms of drug resistance from *A. baumannii* strains from Bolivia.

3.3.1 Aims and objectives

To study the drug resistance profile and the genetic features that influence the resilient adaptation in *Acinetobacter baumannii* isolates from Cochabamba, Bolivia

3.3.2 Identification and PCR amplification

Fifteen isolates thought to have a multiresistant profile were selected for this study. All the isolates were isolated from 3 hospitals in Cochabamba in the years 2008-2009 and were clonally related (Sevillano *et al*., 2012).

The strains were identified by the *bla*$_{\text{OXA-51-like}}$ PCR described by Héritier *et al*. (2005b) and by sequencing the *rpoB* PCR product described earlier by La Scola *et al*. (2006).

3.3.3 Antimicrobial susceptibility testing

Table 7 identifies the *bla*$_{\text{OXA-51-like}}$ enzyme and the MIC values of IPM, MEM, CAZ, CIP, CIP+NMP, CN and CN+NMP in each individual isolate. It shows that all the strains, except 2, are multi-resistant. The gentamicin MIC values did not change upon the addition of NMP whereas a 2 to 4-fold decrease was seen in all the isolates resistant to CIP suggesting the contribution of an efflux pump.

3.3.4 Screening for other antimicrobial resistance determinants

All the isolates were screened by PCR for the presence of genes of the *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-40-like}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58-like}}$ and *bla*$_{\text{OXA-143-like}}$ families by the multiplex

PCR described earlier by Higgins *et al*. (2010). All the strains showed the presence of *bla*$_{\text{OXA-51-like}}$ gene. All except, isolate 2, were also positive for the *bla*$_{\text{OXA-58-like}}$ gene.

The primers described by Héritier *et al*. (2005a) amplified the *bla*$_{\text{OXA-58-like}}$ gene, which was confirmed by sequencing. The primer SM2 described earlier by Poirel and Nordmann (2006b) was used in combination with walk-58-R showed the presence of IS*Aba3-like* element upstream of the *bla*$_{\text{OXA-58-like}}$ gene.

The IS*Aba-3*-like element had a codon change at position 25 (GAT to AAT) leading to an Aspartic acid to Asparagine amino acid change compared to the IS*Aba-3*-like structure deposited in the IS-finder (http://www-is.biotoul.fr/, last accessed 20/08/2011).

3.3.5 Screening for the disruption of the *carO* gene

The *carO* gene fragment was not disrupted by any insertion element as indicated earlier by Mussi *et al*. (2005), which would lead to reduced carbapenem susceptiblity.

3.3.6 Screening for the presence of aminoglycoside resistance genes

The mutiplex PCR described by Noppe-Leclercq *et al*. (1999), revealed that all the isolates, except isolate 2, were positive for the *aac(6')-Ib* and *aph(3')-VI* enzymes. Isolate 2 was positive for the *aph(3')-Ia*, *aac(3)-Ia*, and *ant(2'')-Ia* genes conferring resistance to aminoglycosides. They were negative for the 16s rRNA methylase genes such as *armA*, *rmtA*, *rmtB*, *rmtC* and *rmtD*.

3.3.7 Analysis of the genetic environment of the *bla*$_{ADC}$ gene

Primers FU and RU were used for amplifying the intergenic region upstream of *bla*$_{ADC}$ gene. They detected the presence of IS*Aba1* upstream of the *bla*$_{ADC}$ gene as confirmed by sequencing. Primers FD and RD were used for amplifying the intergenic region downstream of *bla*$_{ADC}$ gene which amplified a product of 267bp indicating that there was no insert present downstream of the *bla*$_{ADC}$.

3.3.8 Analysis of the quinolone resistance-determining region (QRDR) of the *gyrA* and *parC* genes

PCRs as described by Valentine *et al*. (2008), were used for the amplification of the QRDR's of the *gyrA* and the *parC* genes. The gene fragments were sequenced and checked for specific amino acid changes. The sequencing of the *gyrA* and *parC* gene revealed Serine-83 to Leucine and Serine-80 to Phenylalanine amino acid changes in all the isolates resistant to ciprofloxacin except in isolate 2, which was sensitive.

3.3.9 Analysis of the *adeABC* and *adeRS* genes involved in efflux

The intergenic regions of *adeRS* were amplified using the primers adeRF and adeSR which amplified a 790bp product indicating that there was no insertion. The primers adeRA and adeAB amplified a product of 929bp indicating there was no insert between these two genes. The primers adeAF, adeAR., adeBF, adeBR., and adeCF, adeCR detected the presence of the *adeA*, *adeB* and *adeC* genes respectively.

3.3.10 Analysis of gene expression

Expression of the *bla*$_{ADC}$, *bla*$_{OXA-58-like}$, *adeA*, *adeB* and *adeC* gene was studied by RT-PCR. The primers for the *bla*$_{ADC}$ gene were those defined previously by Ruiz *et al.* (2007) and those used for *bla*$_{OXA-58-like}$ gene expression were the same as the multiplex primers described by Woodford *et al.* (2006) (Table 7). The gene expression was measured thrice with different RNA preps under the same optimal conditions.

The RT-PCR for the *bla*$_{OXA-58-like}$ gene expression also recorded high levels of gene expression in isolates positive for the *bla*$_{OXA-58-like}$ gene (Table 7). RT-PCR for the analysis of the *adeA* (encoding for the membrane fusion protein) and the *adeB* (encoding the RND-transporter) genes revealed that both the genes were highly expressed as they are co-transcribed (Table 7). The *adeC* (encoding the outer membrane protein) was not as highly expressed as *adeA* and *adeB* and this may be because it has been shown previously that it is not necessary in conferring resistance (Marchand *et al.*, 2004) and when absent the bacteria can use alternative outer membrane proteins to export substrates for the functionality of the efflux pump.

Table 7: Activities of various antibiotics, the levels of gene expression and the amino acid changes in QRDR's in *A. baumannii* clinical isolates.

No	MIC (mg/L)							OXA-51	Gene expression* (Standard Deviation of ± 0.57 to 1)					*gyrA* ser83	*parC* ser80
	IPM	MEM	CN	CN+NMP	CIP	CIP+NMP	CAZ		bla_{OXA-58}	bla_{ADC}	*adeA*	*adeB*	*adeC*		
1	8	2	128	128	256	64	128	65	14	15	28	29	15	Leu	Phe
2	0.25	0.5	256	256	1	1	16	66	-	5	24	18	14	Ser	Ser
3	8	2	>256	256	256	64	128	65	14	14	31	31	14	Leu	Phe
4	8	2	256	256	256	64	128	65	14	15	25	29	14	Leu	Phe
5	8	2	64	64	128	32	128	65	15	15	27	29	13	Leu	Phe
6	16	4	64	64	128	32	128	65	14	16	26	28	13	Leu	Phe
7	8	2	128	128	256	64	128	65	15	15	27	30	11	Leu	Phe
8	8	2	64	64	256	64	128	65	15	15	26	29	14	Leu	Phe
9	8	2	>256	256	>256	64	128	65	14	14	31	36	15	Leu	Phe
10	8	2	64	64	128	32	128	65	14	15	26	28	15	Leu	Phe
11	8	2	128	128	256	64	128	65	15	14	27	29	14	Leu	Phe
12	16	2	128	128	256	64	128	65	14	15	27	27	13	Leu	Phe
13	8	2	256	256	128	64	128	65	15	14	28	27	15	Leu	Phe
14	8	2	256	256	128	64	128	65	15	14	27	28	17	Leu	Phe
15	8	2	256	256	128	64	128	65	14	15	28	28	15	Leu	Phe

* = Relative gene expression was determined by RT PCR and quantified by Bio-Rad quantity-I software
+ = present
- = absent
Ser = Serine
Leu = Leucine
Phe = Phenylalanine

3.3.11 DISCUSSION:

Carbapenem resistance is a growing phenomenon and it is a cause of much concern in the past few years. All strains, except isolate 2, examined in this study were not only carbapenem resistant but multi-drug resistant to the antibiotics that have established breakpoints according to the BSAC. They were previously confirmed to be clonally related according to their PFGE profiles (Sevillano *et al*., 2012).

The isolates did not have any insertion sequence present upstream of the $bla_{\text{OXA-51-like}}$ gene. All, except isolate 2, were also positive for the $bla_{\text{OXA-58-like}}$ gene by the multiplex PCR. The primers SM-2 and walk-58-R detected the insertion of IS*Aba3*-like structure, 17bp upstream of the $bla_{\text{OXA-58}}$ gene. Putative promoters with -35 (TTTATC) and -10 (TTTCTT) motifs was detected in the IS*Aba3*-like element. The promoters have been previously identified by Poirel and Nordman, (2006b). This explains the resistance to carbapenems.

The mutiplex PCR described by Noppe-Leclercq *et al*. (1999) revealed that all the isolates were positive for two or more aminoglycoside resistance enzymes. In a review by Shaw *et al*. (1993) the *aac(6')-Ib* class of aminoglycoside inactivating enzymes has been shown to confer resistance to tobramycin, dibekacin, amikacin, 5-episisomicin, netilmicin, 2'-N-ethylnetilmicin and sisomicin whereas the *aph(3')-VI* group of enzyme is characterized by resistance to kanamycin, neomycin, paromomycin, ribostamycin, butirosin, amikacin and isepamicin. The *aac(6')-Ib* and *aph(3')-VI* enzymes were found in all the strains except isolate 2.

Isolate 2 was positive for the enzymes *aph(3')-Ia*, which confers resistance to various aminoglycosides including gentamicin, *aac(3)-Ia* responsible for resistance to gentamicin and fortimicin, and *ant(2'')-Ia* which hydrolyzes tobramycin, dibekacin, sisomicin, kanamycin and gentamicin. The high MIC values of gentamicin in all the strains except 2 are due to other unknown mechanisms of resistance. Since the MIC values of gentamicin did not decrease after the addition of NMP it can be concluded that the multidrug resistance to gentamicin may not be efflux mediated, as aminoglycoside resistance is common in *Acinetobacter* and results primarily from inactivation of the antibiotic by specific modifying enzymes (Magnet *et al*., 2001). It may be beneficial to inactivate and hydrolyse the antibiotic rather than efflux it out of the cell as efflux mediated mechanisms are energy dependent and involve proton motive force (Magnet *et al*., 2001).

IS*Aba1* was found to govern the expression of the bla_{ADC} gene in all the strains except in isolate 2, which showed a low MIC of ceftazidime. There was no insertion detected downstream of the bla_{ADC} gene in any of the strains.

Even though IS*Aba1* was not found upstream of any efflux genes, the most likely cause of ciprofloxacin resistance is due to the mutations in the *gyrA* and *parC* genes, having Serine-83 to Leucine and Serine-80 to Phenylalanine amino acid changes in all the isolates resistant to ciprofloxacin with synergistic contribution of the increased expression of the efflux genes *adeA* and *adeB*.

This work analyzed the genetic environment and the multi-drug resistance profile of isolates isolated in Cochabamba, Bolivia. This report reflects on the emergence of

Acinetobacter baumannii as a multi-drug resistant pathogen in Latin American countries such as Bolivia. The role of efflux pumps and other antibiotic resistance mechanisms in mediating antibiotic resistance is evident in this bacterium and the continued study of these systems should contribute towards the better understanding of *A. baumannii*, which is evolving as a major threat. The high proportion of strains with resistance genes to the major groups of antibiotics used to control *A. baumannii* must be of great concern.

3.4 SECTION 4:

Study of *A. baumannii* isolates from Aberdeen Royal Infirmary.

3.4.1 Aims and Objectives

To study the resistance profile of *A. baumannii* strains isolated from Aberdeen Royal Infirmary and to classify novel $bla_{\text{OXA-51-like}}$ variants.

3.4.2 Identification and PCR amplification.

Nine distinct *A. baumannii* strains were isolated from diagnostic clinical specimens from patients in Aberdeen Royal Infirmary from the years 2006-2010. The strains were identified primarily by the $bla_{\text{OXA-51-like}}$ PCR described by Héritier *et al*. (2005b) and by sequencing the *rpoB* gene as described by La Scola *et al*. (2006). The source of the strains and the dates of the isolation are listed in Table 8.

3.4.3 Antimicrobial susceptibility testing

Table 8 outlines the MIC values of imipenem (IPM), meropenem (MEM), ceftazidime (CAZ), ciprofloxacin (CIP) and gentamicin (CN) in each individual isolate. Table 8 also identifies the $bla_{\text{OXA-51-like}}$ gene for each of the strains.

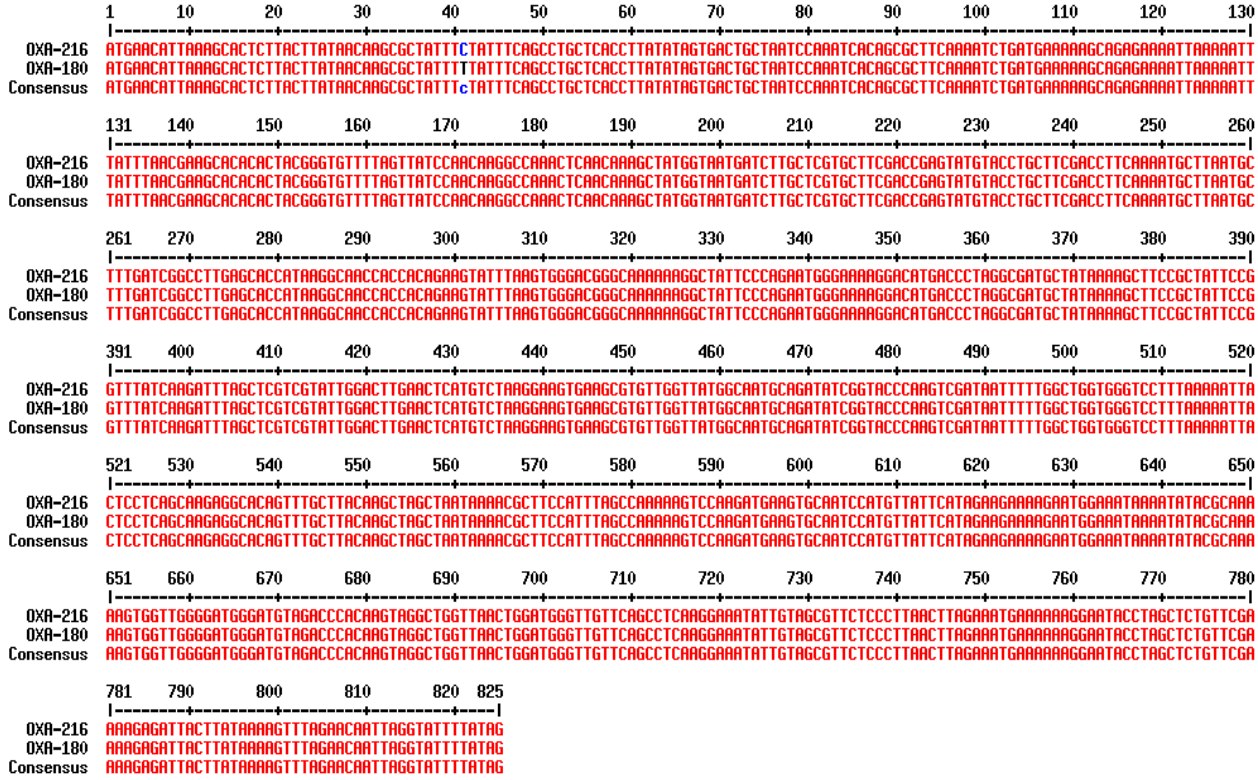
Table 8: Activities of various antibiotics in *A. baumannii* clinical strains.

Strain number	Source	Date of isolation	MIC (mg/L)					*int1*	bla_{ADC}	IS*Aba1*-bla_{ADC}	$bla_{OXA\text{-}51\text{-}like}$ gene
			MEM	IPM	CAZ	GEN	CIP				
3	blood	09.09.06	1	0.5	64	256	32	+	+	+	66
10	blood	08.06.08	0.06	0.06	1	0.12	0.12	-	-	-	65 (variant)
12	blood	25.08.08	16	16	64	8	32	+	+	+	66
14	blood	21.09.09	0.06	0.06	4	0.03	0.12	-	+	-	216
16	sputum	20.02.10	0.5	0.5	4	0.12	0.12	-	+	-	51
3n	blood	27.04.06	0.5	0.25	2	0.03	0.12	-	+	-	64
6n	blood	17.07.07	1	0.5	8	0.12	0.5	-	+	-	217
10n	blood	22.10.08	0.06	0.06	1	0.03	0.12	-	+	-	65 (variant)
14n	blood	14.09.09	0.5	0.25	8	0.12	0.5	-	+	-	89

3.4.4 PCR and sequencing of the $bla_{OXA\text{-}51\text{-}like}$ gene.

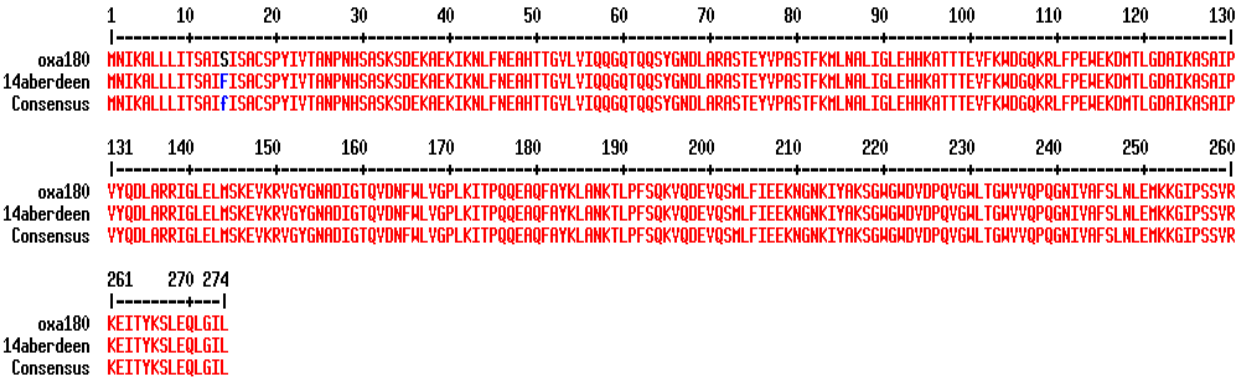
Two novel variants of the $bla_{OXA\text{-}51\text{-}like}$ gene were identified by sequencing. Strain 14 had Serine-14 (TCT) of the $bla_{OXA\text{-}180}$ gene replaced with Phenylalanine (TTT), and is now designated $bla_{OXA\text{-}216}$ (Figure 26a, 26b). Strain 6n had Threonine-255(ACA) of the $bla_{OXA\text{-}78}$ gene substituted to Isoleucine (ATA), which is now designated $bla_{OXA\text{-}217}$ (Figure 27a, 27b). Isolates 10 and 10n had variants of the $bla_{OXA\text{-}65}$ gene with silent mutations at positions 90 (C to T), 636 (C to T) and 663 (G to A). In addition, strain 12 also showed the presence of the $bla_{OXA\text{-}23\text{-}like}$ gene.

Figure 26a: OXA-216 nucleotide sequence (14 Aberdeen)



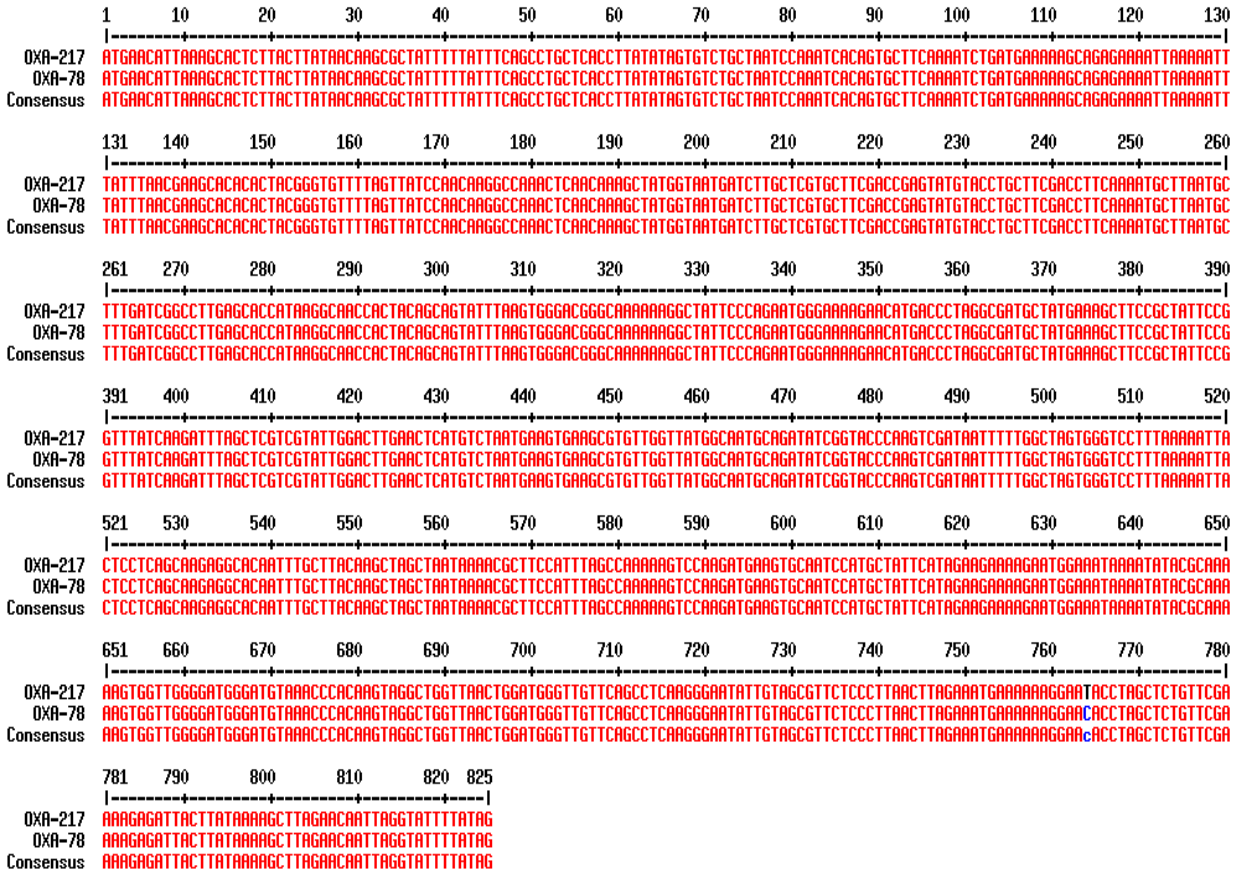
Cytosine=C and Thymine=T

Figure 26b: OXA-216 (14 Aberdeen) amino acid sequence



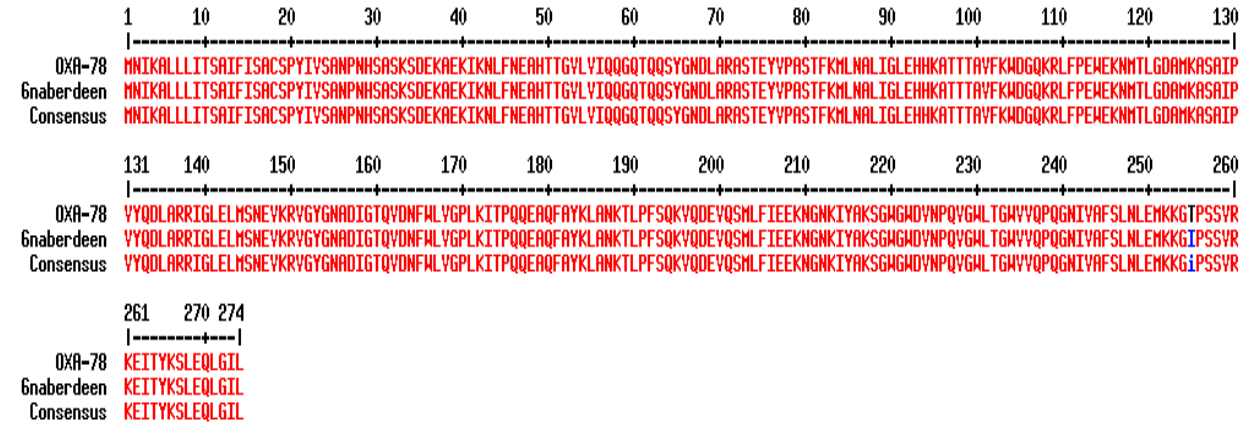
Serine (S) to Phenylalanine (F) change at position 14 in OXA-216 sequence.

Figure 27a: OXA-217 nucleotide sequence (6n Aberdeen)



Cytosine=C and Thymine=T

Figure 27b: OXA-217 amino acid sequence (6n Aberdeen)



Threonine (T) to Isoleucine (I) change at position 255 in OXA-217 sequence

3.4.5 Screening for other antimicrobial resistance determinants

All the isolates were screened by PCR for the presence of genes of the *bla*$_{\text{OXA-23-like}}$, *bla*$_{\text{OXA-40-like}}$, *bla*$_{\text{OXA-51-like}}$, *bla*$_{\text{OXA-58-like}}$ by multiplex primers described by Woodford *et al*. (2006). The *bla*$_{\text{OXA-143-like}}$ genes were screened for by using primers described in the materials and methods section of this thesis. All the strains showed the presence of a *bla*$_{\text{OXA-51-like}}$ gene. Isolate 12 was also positive for the *bla*$_{\text{OXA-23-like}}$ gene. ISADC1 primer used in conjunction with Oxa-23R detected the presence of IS*Aba1* upstream of the *bla*$_{\text{OXA-23-like}}$ gene.

3.4.6 Screening for the disruption of the *carO* gene

The PCR for the insertions causing disruption of c*arO* (29kDa OMP) did not detect the presence of any insertion element causing disruption of the *carO*, leading to carbapenem resistance. This was done to check for any changes in the OMPs of strains as one of them was carbapenem resistant and Mussi *et al*. (2005). have shown that the disruption of *carO* can lead to carbapenem resistance.

3.4.7 Screening for the presence of aminoglycoside resistance genes

The *aac(3')-Ia* gene was present in strains 3 and 12 and this was responsible for the resistance to gentamicin in these strains.

3.4.8 Analysis of the genetic environment of the *bla*$_{\text{ADC}}$ gene

Primers FU, RU and FD, RD were used for the amplification of the intergenic region upstream and downstream of the *bla*$_{\text{ADC}}$ gene. Strains 3 and 12 had high MICs of ceftazidime, which was due the presence of IS*Aba1* upstream of *bla*$_{\text{ADC}}$ gene. There was no insert observed downstream of the *bla*$_{\text{ADC}}$ gene making it a defunct

transposon. All strains were positive for the *bla*$_{ADC}$ gene, except isolate 10 but, as none of these had IS*Aba1* upstream, their MICs were 8mg/L or less.

3.4.9 Analysis of the quinolone resistance-determining region (QRDR**)** of the *gyrA* and *parC* genes.

PCRs as described by Valentine *et al*. (2008) were used for the amplification of the QRDRs of the *gyrA* and the *parC* genes. The gene fragments of the *gyrA* and *parC* were sequenced and checked for specific amino acid changes. Changes were seen in the *gyrA* (Serine83-Leucine) and the *parC* (Serine80-Leucine) genes in strains 3 and 12.

3.4.10 Detection of the integrase gene

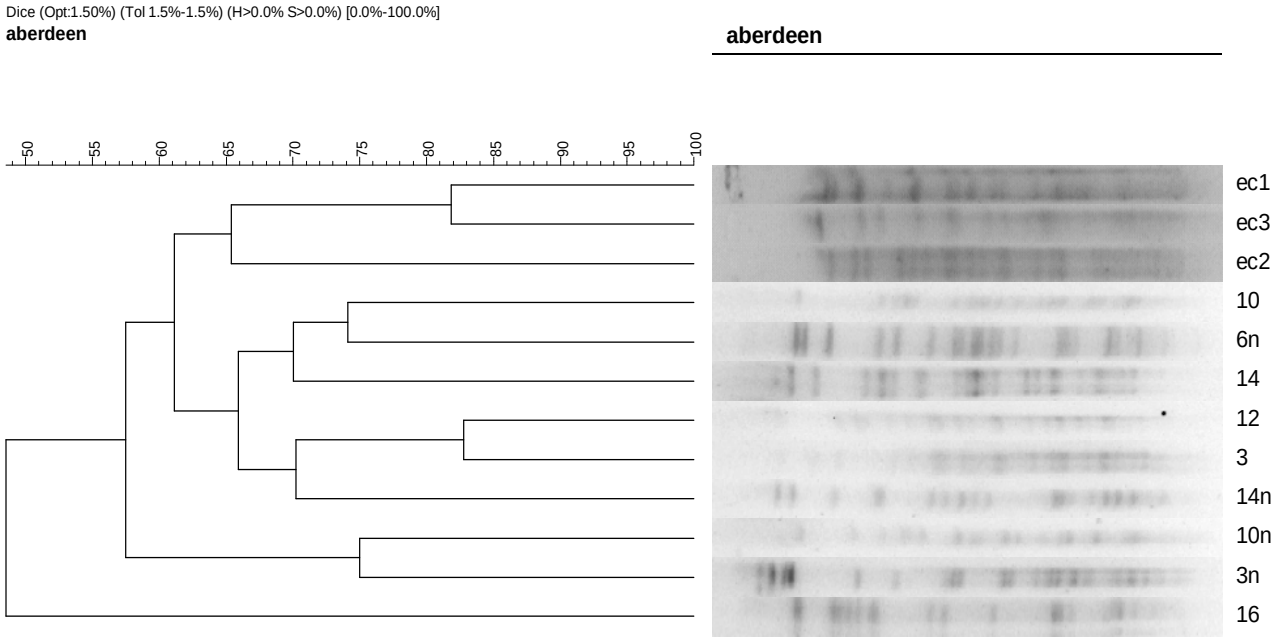
Strains 3 and 12 were positive for the integrase genes by PCR as described earlier by Lévesque *et al*. (1995). The sequencing of the gene revealed the presence of putative glucose dehydrogenase precursor that could be responsible for catabolism of glucose by oxidation as shown by Bouvet and Bouvet (1989).

3.4.11 Pulsed Field Gel Electrophoresis (PFGE)

The PFGE profiles of the strains showed that most were not clonally related as they had <80% similarity (Figure 28). Furthermore, they were not related to any of the European clones. PFGE analysis revealed that strains 3 and 12 had 83% similarity and showed some relationship to each other (Figure 28). Additionally it is important to note that strain 3, isolated in the year 2006, was negative for the *bla*$_{OXA-23}$ gene whereas strain 12 isolated in the year 2008 was positive for this gene and was also resistant to imipenem and meropenem. Otherwise, they had very similar drug

resistance profiles. This indicates the *A. baumannii* is actively acquiring resistance genes, probably through plasmid transfer.

Figure 28: PFGE profile for *A. baumannii* strains isolated from the Aberdeen Royal Infirmary.



EC1= European clone I, EC2= European clone II, EC3= European clone III

3.4.12 Conjugation and plasmid detection.

Transconjugation was performed as described in the materials and methods section using *E.coli* J62-2 (rif) as the recipient and it was unsuccessful.

S1 nuclease digestion described by Findlay *et al.*, (2012). and plasmid extraction procedure described by Joly (1996) failed to detect any plasmids. Furthermore, elimination studies did not remove the resistance determinants.

3.4.13 DISCUSSION:

The two *bla*$_{\text{OXA-51-like}}$ genes 216 (FR865168) and 217 (JN603240) are novel and they have arisen from point mutations in the existing *bla*$_{\text{OXA-51-like}}$ variants. They can provide a basal level of reduced susceptibility to carbapenems and, although this can be enhanced if an insertion is present upstream, this was not seen in our isolates. Clinical significance is related to the strain type and establishing clonal relatedness, which was seen in two strains, 3 and 12; strain 3 (isolated in 2006) did not have the *bla*$_{\text{OXA-23}}$ gene whereas strain 12 (isolated in 2008) harboured *bla*$_{\text{OXA-23}}$. There were no insertion sequences upstream to regulate the *bla*$_{\text{OXA-51-like}}$ genes and increase their resistance to carbapenems. The failure of transconjugation and plasmid extraction assays suggests that if *bla*$_{\text{OXA-23}}$ was plasmid borne, it was now integrated in the host chromosome of strain 12 endowing it with a stable mechanism of carbapenem resistance. Furthermore plasmid curing performed using acriflavine and with elevated temperature of incubation (47°C) described by Trevor (1986) failed to eliminate any plasmid borne *bla*$_{\text{OXA-23}}$ gene. Integration of the *bla*$_{\text{OXA-23}}$ in the host chromosome of *Acinetobacter baumannii* has been reported in strains from Latin America by Villegas *et al*. (2007). These results show that the clinical situation in the hospital in Aberdeen

is in a state of flux. New variant strains are emerging and, most importantly, a carbapenem-sensitive strain has become resistant through the acquisition of the *bla*$_{\text{OXA-23}}$ gene with an IS*Aba1* element upstream carrying a promoter allowing expression of the β-lactamase. The *bla*$_{\text{OXA-23}}$ was first found in Scotland more than 20 years ago (Donald *et al.*, 2000) and it has remained the sole mechanism of carbapenem resistance until this point (personal communication S. G. B. Amyes).

3.5 SECTION 5:

IS*Aba825* controls the *bla*$_{\text{OXA-65}}$ and *bla*$_{\text{OXA-58}}$ expression in four clinical isolates of *A. baumannii*.

3.5.1 Aims and Objectives

To identify novel insertion sequences that govern the expression of the *bla*$_{\text{OXA-51-like}}$ gene and to understand the carbapenem resistance in clinical strains isolated from the United States.

3.5.2 Identification and PCR amplification

Four consecutive distinct clinical strains, isolated in 2004 in the United States were confirmed as *A. baumannii* by PCR and sequencing of the *rpoB* gene using the primers Ac696F and Ac1093R described previously by La Scola *et al*. (2006).

3.5.3 Antimicrobial susceptibilty

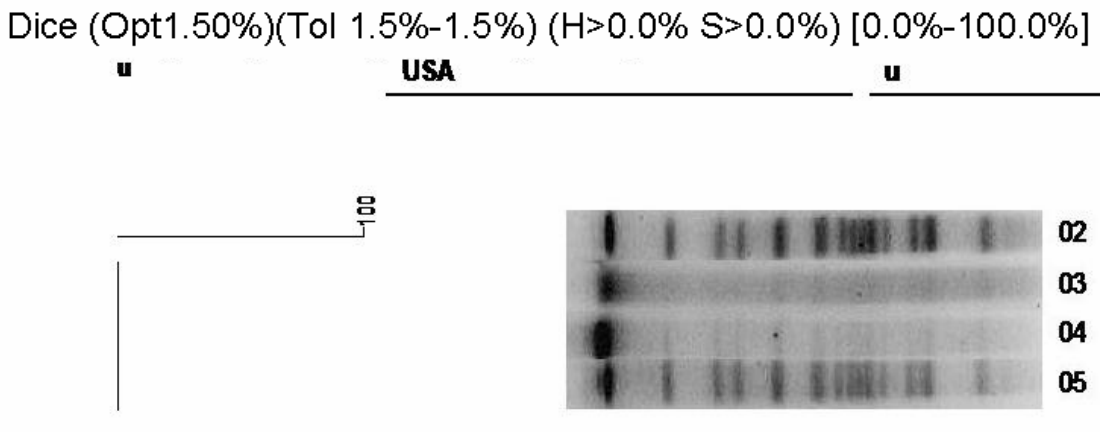
The MICs of imipenem and meropenem were determined by the agar double dilution method and the results were interpreted according to the BSAC guidelines.

All the strains were resistant to imipenem and meropenem (Table 9).

3.5.4 Pulsed-Field Gel Electrophoresis

ApaI macrorestriction anaylsis (Seifert *et al*., 2005) of the four isolates exhibited genetic similarities of 99.99% by the unweighted pair group method with arithmetic average method (Figure 29).

Figure 29: PFGE profile of *A. baumannii* clinical isolates from the United States.



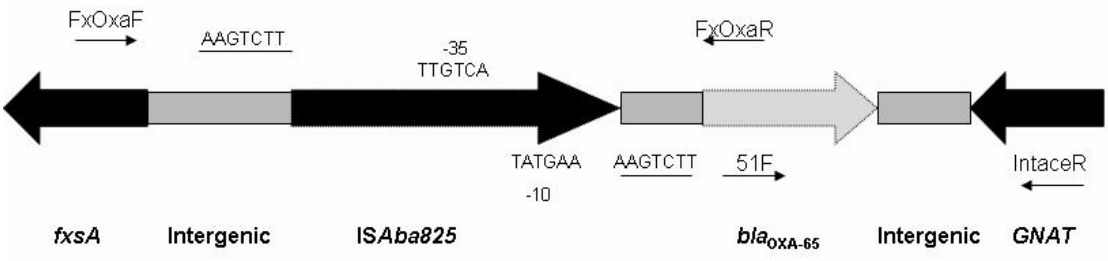
3.5.5 PCR for antimicrobial resistance determinants

Genes encoding known carbapenemases were investigated as described previously and the genes *bla*$_{\text{OXA-51-like}}$ and *bla*$_{\text{OXA-58-like}}$ were detected (Higgins *et al*., 2010). Genes encoding OXA-40-like and OXA-23-like carbapenemases were not detected. Neither were metallo-β-lactamases such as IMP, VIM, GIM, SIM, and SPM detected by multiplex PCR, as described previously by Ellington *et al*. (2007) in any of the isolates.

The primers FxOxaF and FxOxaR (Lopes *et al*., 2012) detected the presence of an insertion element upstream of the *bla*$_{\text{OXA-51-like}}$ gene in all the four isolates (Figure 30). The primers 51F and IntaceR (Lopes *et al*., 2012) were used for the amplification of the *bla*$_{\text{OXA-51-like}}$ gene. The sequencing results showed the presence of IS*Aba825*, present upstream of the *bla*$_{\text{OXA-65}}$ gene in all the four isolates. A putative promoter with -35 (TTGTCA) and -10 (TATGAA) were located 17bp apart from each other (BPROM, Softberry, Inc., Mount Kisco, NY) and were located 97bp and 74bp upstream of the *bla*$_{\text{OXA-65}}$ gene. A target site duplication of 7bp, AAGTCTT was seen

upstream and downstream of the IS*Aba825* sequence. No insertion was seen downstream making the IS*Aba825* a defunct transposon. The IS*Aba825* sequence was oriented in 5'-3' direction against the *bla*$_{\text{OXA-65}}$ gene in all the four isolates.

Figure 30: Genetic arrangement of the *bla*$_{\text{OXA-65}}$.controlled by upstream IS*Aba825* element. The arrows represent the primers used for detection and sequencing of genes (Not to scale). Arrows indicating FxOxaF, FxOxaR, 51F and IntaceR are the directon PCR primers.



The primers preOXA-58prom+ and preOXA-58B, described previously (Héritier *et al*., 2005a) were used to check for any variations within the *bla*$_{\text{OXA-58-like}}$ gene. The primers amplified a 934bp fragment which on sequencing confirmed that the *bla*$_{\text{OXA-58}}$ gene had no nucleotide substitutions.

The SM2 primer (Poirel and Nordmann, 2006a) and the walk-58-R (Lopes *et al*., 2012) primer amplified a fragment of IS*Aba825* oriented in 3'-5' direction. This has been previously reported by Ravasi *et al*. (2011). Putative promoter -35 (TTGAGA) present 148bp upstream of the *bla*$_{\text{OXA-58}}$ gene and -10 (TTTATA) present 127bp upstream of the *bla*$_{\text{OXA-58}}$ gene were identified 15 bp apart from each other. The *bla*$_{\text{OXA-58}}$ gene was highly over-expressed as determined by the RT-PCR assay.

Normalization of DNA was done with the 16S-rRNA primers described by Lin *et al*., (2009) with the appropriate amounts of serially diluted RNA used for c-DNA synthesis (Table 9).

3.5.6 Analysis of gene expression

The expression of the $bla_{\text{OXA-65}}$ gene for all the isolates was studied as described earlier (Lopes *et al*., 2010). The internal $bla_{\text{OXA-65}}$ gene primers, 65A and 65B showed a significant over-expression of the $bla_{\text{OXA-65}}$ gene expression (Table 9). OXA-58-like F and OXA58-like R primers described by Higgins *et al*. (2010) were used for the $bla_{\text{OXA-58}}$ gene expression.

PCR products were quantified using the Bio-Rad Quantity One Software 4.6.1 (Bio-Rad) (Table 9). Normalization of DNA was done with the 16S-rRNA primers described earlier by Lin *et al*. (2009) with the appropriate amounts of serially diluted RNA used for c-DNA synthesis.

3.5.7 Plasmid profiling

S1 nuclease digestion was performed for the PFGE plugs according to the manufacturer's instructions. A total of 10 units per plug were used for digestion with incubation at 37°C for 45 mins. The plasmid bands of approximately 40kb were excised from the gel and purified using the Qiagen gel extraction kit. Chromosomal DNA contamination if any was checked using the 16S-rRNA primers described earlier by Lin *et al*. (2009). The $bla_{\text{OXA-58}}$ was present to be on this 40kb plasmid.

3.5.8 Transconjugation assay

Transconjugation assays of *A. baumannii* strains having the *bla*$_{\text{OXA-58-like}}$ gene were performed with *E.coli* J62.2 as described earlier by Findlay *et al.* (2012). Transconjugation assays using *E.coli* J62.2 could not transfer the 40kb plasmid, which had the *bla*$_{\text{OXA-58}}$ gene.

3.5.9 Plasmid curing

Plasmid curing was performed using sodium dodecyl sulphate (SDS) with elevated temperature of incubation (Trevors, 1989). The strains were incubated in 4.8mL of nutrient broth with 200µL of 10% SDS at 46°C/48 hours. The loss of plasmid was observed in all the isogenic strains after the *bla*$_{\text{OXA-58}}$ gene was lost by the plasmid curing method as described in the Materials and Methods section. During this curing procedure the IS*Aba825*, which was present upstream of the *bla*$_{\text{OXA-65}}$ gene, was also lost, making the isolates carbapenem sensitive (Table 9). A 16-fold decrease in MIC of imipenem and an 8-fold decrease in MIC of meropenem were observed for all the sensitive isogenic strains. The isolates did retain the basal level of the *bla*$_{\text{OXA-65}}$ gene expression (Table 9).

Table 9: Activities of antibiotics and the levels of gene expression in clinical isolates of *A. baumannii*.

Strain	Minimum inhibitory concentration (mg/L)		Quantification of gene expression (arbitrary units)					
	Imipenem	Meropenem	Expression of the 16S rRNA gene		Expression of the bla_{OXA-65} gene		Expression of the bla_{OXA-58} gene	
Ab202	16	16	16S r-RNA	249	OXA-65	154	OXA-58	194
Ab203	16	16	16S r-RNA	250	OXA-65	167	OXA-58	189
Ab204	16	16	16S r-RNA	255	OXA-65	165	OXA-58	187
Ab205	16	16	16S r-RNA	251	OXA-65	165	OXA-58	189
Ab202s	1	2	16S r-RNA	240	OXA-65	74.0	OXA-58	-[a]
Ab203s	1	2	16S r-RNA	245	OXA-65	72.1	OXA-58	-[a]
Ab204s	1	2	16S r-RNA	255	OXA-65	67.5	OXA-58	-[a]
Ab205s	1	2	16S r-RNA	255	OXA-65	69.0	OXA-58	-[a]

-[a] denotes no transcript due to the absence of bla_{OXA-58} gene.

3.5j DISCUSSION:

The transposition of insertion sequence (IS) elements within a genome play important roles in the evolution of host cells (Kato *et al*., 2003, reviewed by Mahillon and Chandler, 1998). IS transposition usually completely inactivates a gene at a target site due to insertional inactivation (Lopes *et al*., 2012) but leads to the constitutive expression of an adjacently located cryptic or regulated gene by delivering IS-loaded promoter sequences (Poirel and Nordmann, 2006b). It has been observed that two or more copies of an identical IS element dispersed over a genome promotes various genetic rearrangements, including inversion, deletion, and duplication of the intervening region and fusion of two replicons residing in the same cell (reviewed by Mahillon and Chandler, 1998, Riehle *et al*., 2001). IS transposition is therefore one of the most important driving forces that enhance the variability and consequently the adaptive and evolutionary capacities of their hosts. This is demonstrated very well by

the acquisition of the plasmid borne *bla*$_{\text{OXA-58}}$ gene with IS*Aba825* and the loss of the same gene, leading to a sensitive phenotype.

The curing event promoted the loss of the *bla*$_{\text{OXA-58}}$ gene and its upstream IS*Aba825* promoter. However the same event also promoted the loss of the IS*Aba825* element upstream of the chromosomally encoded *bla*$_{\text{OXA-65}}$ gene thus making the strains carbapenem sensitive. These results suggest that control of the IS elements is coordinated by the activators and the repressors as the loss of the IS element occurs at the same step as the loss of the plasmid. Transposition activity is generally maintained at a low level and the reason for this is that high activities and the accompanying mutagenic effect of genome rearrangements would be detrimental to the host cell (reviewed by Mahillon and Chandler, 1998). Transposase (Tpase) promoters are generally weak, and many are partially located in the terminal IRs, enabling their autoregulation by Tpase binding. Many of the classical mechanisms of controlling gene expression, such as the production of transcriptional repressors (IS*1* and IS*2*) or translational inhibitors (antisense RNA in IS*10*), are known to operate in Tpase expression (reviewed by Mahillon and Chandler, 1998).

The temperature sensitivities of transposition in *E. coli* have been considered to be the intrinsic properties of the transposases (Haren *et al*., 1997). An increase in the transposition activities of the seven IS elements belonging to four different IS families of the *Burkholderia multivorans* ATCC 17616 cells suggested that some common host factors affect transposition at high temperatures (Ohtsubo *et al*., 2005). It is also suggested that some unknown factors that inhibit transposition might be depleted at higher temperatures (Ohtsubo *et al*., 2005). Alternatively, other unknown factors

might contribute to altering the donor and/or target DNA architecture into a form that enables the IS transposition machinery to work more effectively (Ohtsubo *et al*., 2005). The observed characteristics for the carbapenem resistant and sensitive strains indicate that the IS element examined here (IS*Aba825*) plays an important role in cells exposed to high temperatures thus generating genetic diversity in a population of *A. baumannii*. Expression studies also show the importance of this element in conferring carbapenem resistance by over-expression of the $bla_{\text{OXA-58-like}}$ and the $bla_{\text{OXA-65}}$ gene and it can be concluded that IS*Aba825* is also very crucial for the adaptation and evolution of carbapenem resistant *A. baumannii* besides the well-known IS*Aba1*. Our results concur with that of Ravasi *et al*. (2005) and additionally we prove that IS*Aba825* also governs the $bla_{\text{OXA-51-like}}$ gene expression and this is an essential mechanism of carbapenem resistance in *A. baumannii* resistant to imipenem and meropenem.

3.6 SECTION 6:

IS*Aba16* disrupts *bla*$_{\text{OXA-51-like}}$ gene in *A. baumannii* Ab244.

3.6.1 Aims and Objectives

This study was undertaken to understand alternative mechanisms of carbapenem resistance by the activation of the *bla*$_{\text{OXA-58}}$ gene in isolate Ab244 which had the *bla*$_{\text{OXA-51-like}}$ gene inactivated by a novel IS- element IS*Aba16*.

3.6.2 Identification and typing

The isolate Ab244 isolated from Carnaxide, Portugal (strain 12) was confirmed as *A. baumannii* by restriction of the conserved 16S-23S rRNA intergenic spacer sequences as described previously by Dolzani *et al*. (1995).

3.6.3 Antimicrobial susceptibility testing

The strain Ab244 had an MIC of imipenem of 4 mg/L and that of meropenem of 16 mg/L. The strain was classified as resistant to meropenem and intermediately resistant to imipenem (BSAC. http://www.bsac.org.uk/Resources/BSAC/version_6.1.pdf, 27 September 2011, date last accessed).

3.6.4 PCR analysis and sequencing

The multiplex PCR for the OXA-group of enzymes showed a positive result for *bla*$_{\text{OXA-51-like}}$ and *bla*$_{\text{OXA-58-like}}$ genes, but was negative for *bla*$_{\text{OXA-23-like}}$ and *bla*$_{\text{OXA-40-like}}$ genes. The expected product size was 889 bp without any insertion present upstream of the *bla*$_{\text{OXA-51-like}}$ gene, but the primers FxOxa_F (Figure 31: primer1) and FxOxa_R (Figure 31: primer 2) amplified a product of 3449bp.

Sequencing revealed that the chromosomal region had the conserved gene encoding a putative suppressor of F exclusion of phage T7 (*fxsA*) as observed by Chen *et al*. (2010) followed by the 399 bp intergenic fragment, followed by a new insertion sequence, named IS*Aba16* , located following the first 15 bp and disrupting the *bla*$_{\text{OXA-51-like}}$ gene. The primers ISAba16_F1 (Figure 31: primer 3), ISAba16_R1 (Figure 31: primer 4), ISAba16_F2 (Figure 31: primer 5) and ISAba16_R2 (Figure 31: primer 6) were used for sequencing of the insertion element. There was an 8 bp target site duplication of the *bla*$_{\text{OXA-51-like}}$ gene encoding the sequence CTCTTACT seen at the 5' and the 3' end of IS*Aba16* causing an insertional inactivation of the *bla*$_{\text{OXA-51-like}}$ gene (Figure 31). Analysis of the insertion sequence revealed that it had the left inverted repeat 5'-GTAAGCATCCGGCTAA-3' and the right inverted repeat 5'-TTCAGCGGACGCTTAC-3'. The IS*Aba16* sequence comprised 3 ORFs encoding transposase A, B and C respectively.

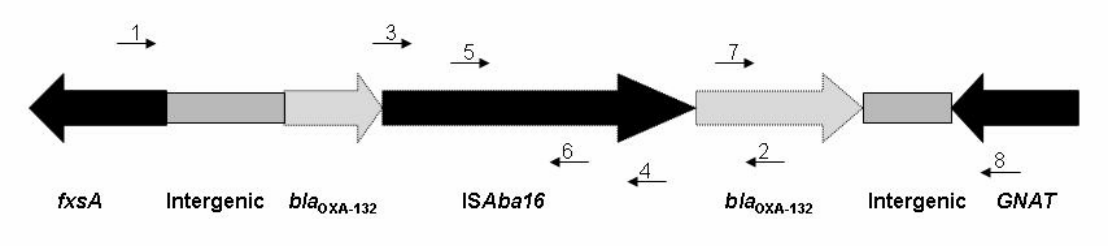


Figure 31: Genetic arrangement of the disrupted *bla*$_{\text{OXA-132}}$ gene due to the insertion of IS*Aba16*.

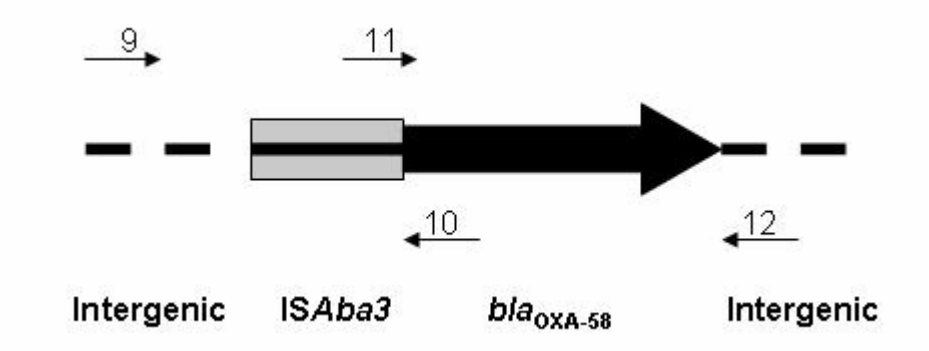
The primers 51F (Figure31: primer 7) and IntaceR (Figure 31: primer 8) amplified a product of 966 bp and the sequencing confirmed that the *bla*$_{\text{OXA-51-like}}$ gene was

identical to *bla*$_{OXA-132}$. There was no insertion present downstream of the *bla*$_{OXA-132}$ gene.

The primers preOXA-58prom+ (Figure 32: primer 11) and preOXA-58B (Figure 32: primer 12), previously described by Héritier *et al*. (2005a), (Figure 26) amplified a product of 934 bp of the *bla*$_{OXA-58-like}$ gene. The *bla*$_{OXA-58}$ gene had no nucleotide substitutions and this was confirmed by sequencing.

The primers SM2 (Figure 32: primer 9) and walk-58-R (Figure 32: primer 10) amplified a region of 575 bp, present upstream of the *bla*$_{OXA-58}$ gene, sequencing of which revealed an IS*Aba3*-like structure. A putative promoter with -35 (TTTATC) and -10 (TTTCTT) motifs was present 44 bp upstream of the *bla*$_{OXA-58-like}$ gene as described earlier by Poirel and Nordmann (2006b). The IS*Aba3*-like structure had a codon change at position 25 (GAT to TAT) causing the amino acid to change from Aspartic acid to Tyrosine and a second codon change at position 110 (AAT to AGT) leading to Asparagine to Serine change. The primers ISAba1A and ISAba1B detected the presence of IS*Aba1* in Ab244 (Poirel and Nordman, 2006b).

Figure 32: Genetic arrangement of the *bla*$_{OXA-58}$ gene in Ab244



3.6.5 Analysis of OMPs (Outer Membrane Proteins)

No change was seen in the outer membrane proteins after extraction and isolation on SDS-PAGE (Bossi and Bossi, 2007) in comparison with outer membrane proteins of *A. baumannii* (Ab2) (Data not shown). The primers described previously for the amplification of the *carO* gene did not identify any insert disrupting the *carO* gene (Mussi *et al*., 2005).

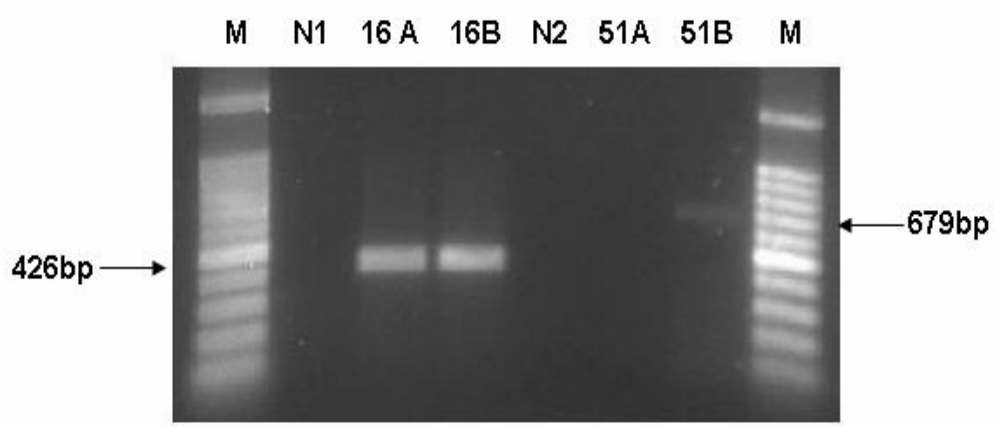
3.6.6 Analysis of gene expression

The expression studies on the $bla_{\text{OXA-51-like}}$ gene revealed that the gene in Ab244 was not expressed in comparison with strain Ab2 (strain 2), which had no insertion upstream of the $bla_{\text{OXA-132}}$ gene (Figure 33, Table 10). On the contrary, the $bla_{\text{OXA-58-like}}$ gene was highly expressed in Ab244 in comparison to the control strain Ab2 which had the $bla_{\text{OXA-58-like}}$ gene without any insertion upstream of the gene (Figure 34, Table 10).

Table 10: MICs and the level of expression for bla_{OXA} genes in *A. baumannii* Ab244

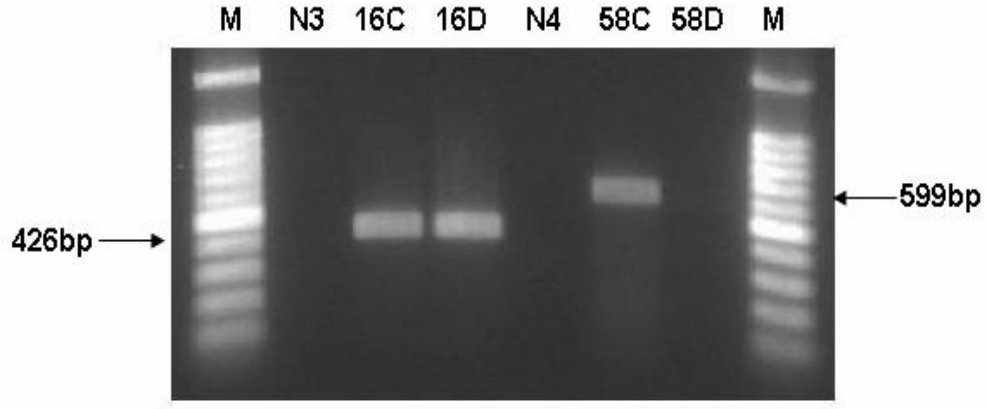
Strain	Minimum inhibitory concentration (mg/L)		Quantification of gene expression (arbitrary units) (Standard deviation ± 0.95)							
	Imipenem	Meropenem	$bla_{\text{OXA-51}}$ gene				$bla_{\text{OXA-58}}$ gene			
Ab244	4	16	16S r-RNA	209	OXA-51	0	16S r-RNA	155	OXA-58	154
Ab2	0.25	1	16S r-RNA	198	OXA-51	95.8	16S r-RNA	160	OXA-58	69.1

Figure 33: Expression of the *bla*$_{OXA-132}$ gene



N1, 16S-rRNA negative control., 16A, Expression of the 16S-rRNA gene in Ab244., 16B, Expression of the 16S-rRNA gene in the control strain Ab2., N2, *bla*$_{OXA-51-like}$ negative control., 51A, Expression of the *bla*$_{OXA-51-like}$ gene in Ab244., 51B, expression of the *bla*$_{OXA-51-like}$ gene in Ab2. The *bla*$_{OXA-51-like}$ gene is shown at 679bp and the 16S-rRNA gene is shown at 426bp

Figure 34: Expression of the *bla*$_{OXA-58}$ gene.



N3, 16S-rRNA negative control., 16C, Expression of the 16S-rRNA gene in Ab244., 16D, Expression of the 16S-rRNA gene in the control strain Ab2., N4, *bla*$_{OXA-58-like}$ negative control., 58C, Expression of the *bla*$_{OXA-58-like}$ gene in Ab244., 58D, expression of the *bla*$_{OXA-58-like}$ gene in Ab2. The *bla*$_{OXA-58}$ gene is shown at 599bp and the 16S-rRNA gene is shown at 426bp.

3.6.7 DISCUSSION:

The *A. baumannii* isolate Ab244 had the conserved F-like structure gene (*fxsA*) found to be present on the chromosome with the intergenic fragment followed downstream by IS*Aba16*, which was inserted within the *bla*$_{\text{OXA-51-like}}$ gene causing insertional inactivation of the gene. The sequencing of the region between *fxsA* and the phosphinothricin acetyltransferase (*GNAT*) gene revealed that there was no insert present in the intergenic region downstream of the *bla*$_{\text{OXA-51-like}}$ gene. The region mapped in this study was 4239 bp encompassing a part of the *fxsA*, intergenic fragment upstream of the *bla*$_{\text{OXA-51-like}}$ gene, the *bla*$_{\text{OXA-51-like}}$ gene itself, intergenic fragment downstream of the *GNAT* and a part of the phosphinothricin acetyltransferase. The inactivated *bla*$_{\text{OXA-51-like}}$ gene was identified as the *bla*$_{\text{OXA-132}}$ gene. Sequencing studies showed that the IS*Aba16* inserted after the first 15 nucleotides of the *bla*$_{\text{OXA-132}}$ gene, which encode the 'M N I K T' amino acid sequence. A target site duplication of 8bp having the sequence CTCTTACT was observed upstream and downstream of the IS*Aba16* element. The RT-PCR showed no expression of the *bla*$_{\text{OXA-132}}$ gene and this is because of the interruption caused by IS*Aba16* located following the first 15 bp and disrupting the *bla*$_{\text{OXA-132}}$ gene.

Analysis shows that the insertion element IS*Aba16* belongs to the IS*66* family. It has been reported that the IS*66* family elements may comprise of 3 or more ORFs (reviewed by Mahillon and Chandler, 1998). IS*Aba16* has the ability to transpose and duplicate an 8 bp target site sequence on its transposition, as observed by target site duplications present at the 5' and the 3' end of the insertion element. Transposases encoded by many IS elements belonging to the members of the IS families other than the IS*66* family have a DNA-binding domain with an α-helix–turn–α-helix DNA-

binding motif and a catalytic domain with a DDE motif (Han *et al*., 2001). The insertion element IS*Aba16* shows the presence of 3 ORFs where the TnpA protein has an α-helix–turn–α-helix DNA-binding motif, and the TnpC protein has a potential DDE motif located at the catalytic core domain. The transposases from several superfamilies possess a protein domain containing an acidic amino acid triad (DDE or DDD) that catalyzes the "cut and paste" transposition reaction (Yuan *et al*., 2011).

The TnpB proteins, however, seem to have no homology to any of the motifs identified in the transposases encoded by the IS elements of the different IS families (Han *et al*., 2001). Previous studies show that the TnpA, TnpB and TnpC proteins may be produced independently in appropriate amounts to form a complex, which acts as a transposase promoting the transposition of IS*66* family elements like IS*Aba16* in strain Ab244.

As the multiplex PCR was positive for the $bla_{\text{OXA-58-like}}$ gene alongside the $bla_{\text{OXA-132}}$ gene, it was essential to map the sequence of the $bla_{\text{OXA-58-like}}$ gene. Therefore a PCR was performed by using the primers described by Héritier *et al*. (2005a), sequencing of the amplicon revealed a similarity with the $bla_{\text{OXA-58}}$ gene without any nucleotide substitutions. The primers SM2 and walk-58-R amplified 575bp region, sequencing of which revealed an IS*Aba3*-like structure. A putative promoter with -35 (TTTATC) and -10 (TTTCTT) motifs was present 44 bp upstream of the $bla_{\text{OXA-58-like}}$ gene. The promoters had been previously identified by Poirel and Nordmann (2006b). The RT-PCR proved that the $bla_{\text{OXA-58-like}}$ gene was highly expressed. The frequent association between $bla_{\text{OXA-58}}$ genes and IS sequences could be associated with the low-levels of expression of the $bla_{\text{OXA-51-like}}$ genes in a wild-type strain having

insertion sequences dispersed in the genome. The mechanism of the insertion sequence activation may depend on activation complexes formed by repressor-inhibitory mechanisms under antibiotic stress conditions (Escoubas *et al*., 1991). Selection for insertion sequences which insert into sites that allow the bacterium to survive the antibiotic stress, such as upstream of β-lactamase genes where the IS elements provide strong promoters for β-lactamase gene expression, has provided the bacterium with a mechanism for transiently switching on resistance genes in response to the stress (Toleman and Walsh, 2011).

Ab244 was resistant to meropenem and intermediately resistant to imipenem although no change in the OMP pattern was seen. Furthermore no insertional activation of the *carO* gene was identified.

The results obtained in this study portray Ab244 as a carbapenem resistant pathogen, which can employ selective mechanisms of resistance through the expression of specific antibiotic resistance genes. The study shows a genetic structure IS*Aba16* inactivating the $bla_{\text{OXA-132}}$ gene and this could be due to the fact that the bacterium did not require a mechanism of resistance when it was not under clinical set conditions. The acquisition of the $bla_{\text{OXA-58}}$ gene under clinical settings facilitates the survival of the bacterium by helping it thrive more effectively under antibiotic stress. This clearly demonstrates the acclimatization of Ab244 to various drug environments by acquisition of acquired carbapenemases such as the $bla_{\text{OXA-58}}$ gene governed by IS*Aba*-3 like elements.

This is the first report describing the insertional inactivation of the *bla*$_{\text{OXA-132}}$ gene by IS*Aba16* (JN415682) and the employment of an alternate resistance mechanism by the over-expression of the *bla*$_{\text{OXA-58}}$ gene leading to carbapenem resistance in strain Ab244.

3.7 SECTION 7:

Role of IS elements involved in the disruption of the *adeR* gene in four isolates of *A. baumannii*.

3.7.1 Aims and Objectives

To understand the mechanism of resistance to ciprofloxacin mediated by the efflux pump genes and by the mutations in the *gyrA* and *parC* genes.

3.7.2 Identification and typing

A. baumannii isolates Ab12, Ab18 and Ab209 were identified by the $bla_{\text{OXA-51-like}}$ PCR (Héritier *et al*., 2005b) and *rpoB* sequencing (La Scola *et al*., 2006) and were used in this study. Ab12M was derived from Ab12 by treating it with sub-inhibitory concentrations of acriflavine (Hunt and Sandham, 1969).

3.7.3 Antimicrobial susceptibility testing

Table 11 outlines the MIC values of ciprofloxacin (CIP) and gentamicin (CN) in each individual isolate, with and without the efflux pump inhibitor (EPI) NMP. It was observed that strains 12 and 12M were not resistant to gentamicin; on the other hand Ab18 was less resistant to gentamicin (MIC=16mg/L) than Ab209, which had an MIC of gentamicin of >256mg/L. There was either two-fold decrease or no change seen in MIC of gentamicin for all strains in the presence of NMP (Table 11).

3.7.4 Aminoglycoside resistance determinants

All, but one, of the strains were negative for aminoglycoside resistance genes; the exception was Ab209, which was highly resistant to gentamicin and it harboured both the *ant (2'')-Ia* and *aph (3')-Ia* genes. It has been shown that *ant (2'')-Ia* is able to

inactivate tobramycin, dibekacin, sisomicin, kanamycin and gentamicin whereas *aph(3')-Ia* confers resistance to various aminoglycosides including gentamicin (Shaw *et al*., 1993). All the strains were highly resistant to ciprofloxacin and a ≥ 8-fold decrease in ciprofloxacin MIC was seen for all strains in the presence of NMP (Table 11).

3.7.5 Analysis of the quinolone resistance-determining region (QRDR) of the *gyrA* and *parC* genes

The amplification of the QRDR's of the *gyrA* and the *parC* genes was performed by PCR (Valentine *et al*., 2008) and amino acids sequence changes were found. All the strains had Serine83 to Leucine encoded by a change in the GyrA protein. Ab18 had Serine80 to Leucine change whereas Ab12, Ab12M and Ab209 had a novel mutation of Serine80 changing to Tryptophan in the ParC protein.

3.7.6 Screening of genes involved in efflux

The primers adeRF and adeSR were used to screen the intergenic regions of *adeR* and *adeS* genes. The intergenic primers adeRF and adeSR detected the presence of IS*Aba1* (5'-3') that disrupted the *adeR* gene after 530bp in Ab18 and after 588bp in Ab209. Ab12M showed the presence of IS*Aba16* (5'-3') disrupting the *adeR* gene after 522bp (Figure 35).

The primers adeRA and adeAB were used to screen for the intergenic regions of *adeR* and *adeA* genes. They detected the presence of another IS*Aba1* (5'-3') element 24bp upstream of the *adeA* gene in Ab209. It was located 709bp from the other IS*Aba1*

element disrupting the *adeR* gene. There was no insert seen for any of the other strains.

The primers adeA1F, adeA1R., adeBF, adeBR., adeCF, adeCR., adeIF, adeIR., adeJF, adeJR., adeKF and adeKR were used for the detection and expression of efflux genes. PCR was performed to check for the presence of *adeABC, adeRS* and *adeIJK* genes detected the presence of all the genes in all the strains except *adeC*, which was absent in Ab12 and Ab12M.

3.7.7 Fluorescent dye whole cell accumulation assays

The ethidium bromide (EtBr) dye accumulation assay of Pannek *et al*. (2006) was used. Table 11 outlines the mean quantification of relative expression of various efflux genes for all the isolates. It also outlines the mean fluorometric intracellular dye accumulation at 30 mins after the addition of EtBr (2mg/L) in the presence and absence of the efflux pump inhibitor, NMP. Dye accumulation experiments were consistent with EtBr intracellular concentrations.

3.7.8 Analysis of gene expression

The *adeSRAB* genes were expressed in Ab12 whereas only *adeSB* was expressed in Ab12M. This is due to the fact that *adeR* was disrupted by IS*Aba16* in Ab12. We have recently shown that IS*Aba16* is responsible for the disruption and inactivation of the $bla_{\text{OXA-51-like}}$ gene in isolate 12 (Ab244) (Lopes *et al*., 2012). It has been shown that insertional inactivation of *adeS,* the kinase component of the *adeRS* genes, results in susceptibility to aminoglycosides and other pump substrates (Marchand *et al*., 2004) and this would be important for the expression of the *adeAB* efflux pump. Our results,

however, suggest that the expression of *adeA* and *adeB* are independent, as *adeB* can be expressed at an effective level despite lack of expression of *adeA*. It was previously hypothesized that *adeAB* can recruit *adeK* in the absence of *adeC* (Marchand *et al*., 2004) and as *adeK* was very well expressed in Ab12 and Ab12M it could be a potential outer membrane protein for the efflux of antibiotics co-ordinated by *adeAB*.

In Ab18 *adeSC* and *adeIJK* were highly expressed, *adeR* was expressed at low levels and this is again due to the disruption of the *adeR* gene by the insertion element IS*Aba1*. Also the *adeAB* genes were not expressed and this is because in *A. baumannii* *adeS* may be involved in the transcription regulation of the *adeR* gene, which in turn would be responsible for regulation of the *adeA* and/or *adeB* as similarly shown by the *ompF* and *ompC* regulation by OmpR in *E. coli*. In *E. coli*, EnvZ (histidine kinase) responds to changes in the extracellular osmolarity of inner-membrane impermeable compounds and controls the phosphorylation of the response regulator *ompR*. Like EnvZ, AdeS functions as an autokinase, an *adeR* phosphotransferase and as a phospho-OmpR (OmpR-P) phosphatase (Batchelor *et al*., 2003). Hence the role of regulation by *adeS* is important. It has been suggested earlier that EvgA (regulator) is crucial for the modulation of multi-drug resistant phenotype in *E. coli* (Nishino and Yamaguchi, 2002). The efflux systems have not been extensively studied as it has been suggested that disruption of *adeS* can have serious consequences on the antibiotic resistance phenotype (Marchand *et al*., 2004). Our study shows that the disruption of *adeR* can be indirectly correlated with the decrease in expression of any of the *adeABC* genes as the primary signal passed on by AdeS is important in the auto-phosphorylation of the histidine residue, which can then transfer the phosphate group to an Aspartate residue of the response regulator AdeR. AdeR is responsible for

the modulation *adeABC* genes but, if *adeS* is disrupted, it could lead to the loss in the function of *adeR* and subsequent loss in the expression of one or more genes of the *adeABC* operon. The expression of *adeC* and *adeIJK* in Ab18 is suggestive that these highly expressed genes in part would contribute towards the ciprofloxacin resistance.

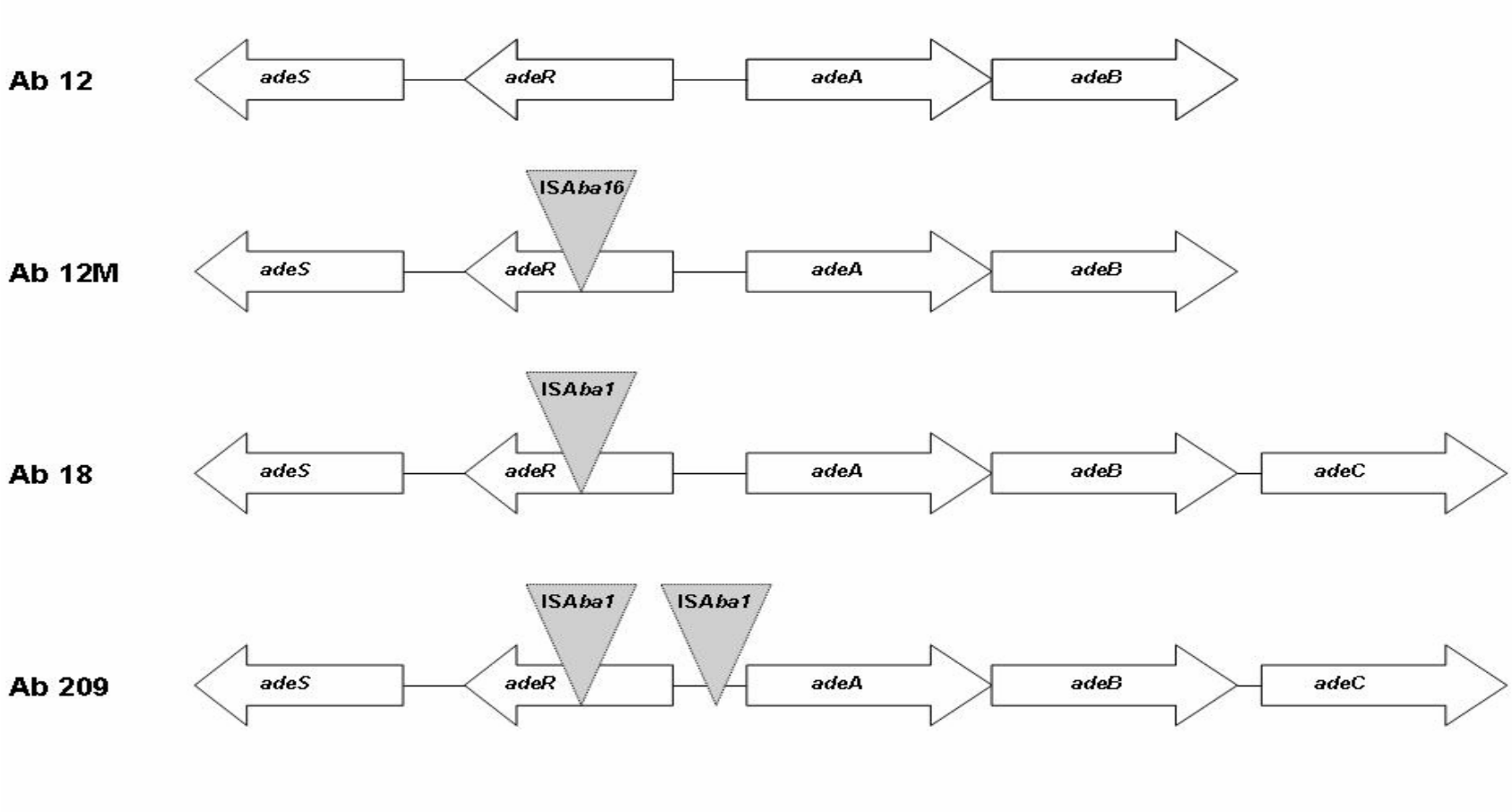
In Ab209, *adeR* gene was not expressed (Table 11) and this was because of IS*Aba1* causing gene disruption (Figure 35). The *adeSABC* and *adeIJK* genes were very well expressed leading to high levels of pump expression. The *adeA* gene was highly expressed because it had IS*Aba1* sequence (3'-5') present in the intergenic region of *adeR* (3'-5') and *adeA* (5'-3'), as opposed to Ab18 and Ab12M, which did not show any insert. IS*Aba1* is shown to govern the expression of bla_{ADC} and $bla_{\text{OXA-51-like}}$ genes when present upstream of the antibiotic resistance genes (Gordon and Wareham, 2010), hence it is not surprising that the *adeA* gene in strain 209 is so well expressed despite the inactivation of *adeR* gene by IS*Aba1*.

Table 11: Minimum inhibitory concentration (MIC) and the levels of gene expression for *A. baumannii* isolates.

Strain No	MIC (mg/L)				EtBr accumulation[a]		Gene expression (arbitrary units)[b]							
	CIP	CIP+NMP	CN	CN+NMP	-NMP	+NMP	*adeS*	*adeR*	*adeA*	*adeB*	*adeC*	*adeI*	*adeJ*	*adeK*
12	>256	32	1	1	16947	48192	2.42	2.56	2.43	2.29	-	1.35	1.53	1.42
12M	>256	32	1	0.5	15856	56052	2.40	1.23	0	2.31	-	1.27	1.62	1.31
18	256	32	16	8	20862	43783	2.52	1.44	0	0	2.26	1.55	1.51	1.24
209	>256	32	>256	>256	14847	49814	2.56	1.14	2.65	2.56	2.42	1.31	1.59	1.41
19606	0.5	0.12	8	8	16103	15235	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[a]Mean average of intracellular ethidium bromide (EtBr) dye accumulation measured by fluorometry at 30 mins (arbitrary units).
[b]Mean average of gene expression measured by RT-PCR and quantified by Bio-Rad Quantity-I software
CIP, Cirprofloxacin, CN, Gentamicin, +NMP (in the presence of 1-(1-napthylmethyl) piperazine), -NMP (in the absence of 1-(1-napthylmethyl) piperazine)
- No transcript detected due to the absence of the gene.
The values selected are based on the data obtained within permissible standard deviation range (±100 to 130 units for EtBr accumulation and ±0.55 to 1.0 units for gene expression)

Figure 35: Gnetic arrangement showing the efflux genes *adeABC* and its controllers *adeRS* with insertions at various positions.



3.7.9 DISCUSSION:

Over-expression of the *adeABC* efflux pump may be caused by the IS*Aba1* insertion to the upstream of *adeABC* operon (Ruzin *et al*., 2007). Although this point has been stated earlier, it has not been proved (Ruzin *et al*., 2007). In this study we show how insertion elements like IS*Aba1* and IS*Aba16* can cause disruption of the *adeR* gene and still lead to high expression of efflux pumps due to the various contributing factors highlighted in the Results section. Insertion elements will lead to the over-expression of a gene only if it can provide a strong promoter upstream of the gene. If insertion sequence forward (ISF) and Efflux gene reverse (EGR) primers were designed, they would have picked up an insert but the primers designed to span the intergenic sequences show how important it is to obtain a definitive result. It has been mentioned earlier (Ruzin *et al*., 2007) that an insert of IS*Aba1* had been found in the *adeS* gene but, in this study, its potential presence upstream of the gene was not studied. We have to consider the point that the *adeS* gene was disrupted by this element (Ruzin *et al*., 2007) and this should lead either to low levels of expression or no expression at all (Lopes *et al*., 2012) as it has been shown that the disruption of *adeS* can render the bacterium susceptible to a range of antibiotics (Marchand *et al*., 2004).

In this study, disruption of *adeR* gene leads to *adeR* gene inactivation but the expression of *adeS* shows that the primary signal passed on to *adeR* by phosphorylation is not only important for its transcriptional regulation but also for the regulation of *adeABC* genes.

In Ab209, even though the signal is not passed to *adeR*, because IS*Aba1* is present upstream of *adeA*, an expression driven by strong promoters of IS*Aba1* can be seen. This is in contrast to Ab12M and Ab18 where *adeA* gene is not expressed. The over-expression of the *adeB* gene in Ab12M may correlate with its high resistance to ciprofloxacin, compared with Ab18 where *adeB* was not expressed. We cannot necessarily infer that *adeA* and *adeB* are co-transcribed. This could be because of the extracytoplasmic function σ factors that are co-transcribed with anti-σ factors located in the cytoplasmic membrane and are released upon interaction with an extracytoplasmic signal (Marchand *et al*., 2004). The main contributing factor of ciprofloxacin resistance examined for the strains studied is largely due to the mutations in the *gyrA* and *parC*.

Also none of the point mutations in *adeR* (Proline116-Leucine) and *adeS* (Threonine153-Methionine or Glycine30-Aspartic acid) genes that have been reported previously (Marchand *et al*., 2004) were seen in any of the strains studied.

Inactivation of *adeR* does not exert a polar effect on the expression of *adeS* as shown in this study but previous studies have shown that inactivation of *adeS* may have a significant effect on the susceptibility to various antibiotics (Marchand *et al*., 2004). In two-component regulatory systems it has been established that the sensor kinase (*adeS*) autophosphorylates at an internal histidine (the H box) in response to a stimulus, the phosphate group of which is then transferred to an aspartate residue of the response regulator (*adeR*) (Marchand *et al*., 2004). The phosphorylated regulator may also be dephosphorylated by the phosphatase activity of the sensor (Marchand *et al*., 2004). The histidine kinases are bifunctional, in that they phosphorylate and

dephosphorylate their cognate response regulator, which leads to a switch between these two activities and directs the state of the regulators, thus governing expression of the genes on which they act (Marchand *et al*., 2004). It has been stated that, in *E.coli*, there is evidence that the operon consisting of *ompR* (regulator) and *envZ* (kinase) is subject to control by integration host factor, cAMP-catabolite activator protein, and the histone-like protein HNS (Batchelor *et al*., 2003). More work is needed in order to understand the role of efflux systems in control of resistance to various classes of antibiotics.

In this study, we demonstrated that over-expression of the *adeABC* efflux pump resulted in CIP non-susceptibility in the isolates studied by quantifying the transcripts of the efflux genes and their regulators. There was at least an 8-fold decrease in CIP susceptibility in the presence of the efflux pump inhibitor NMP. The fluorometric assays demonstrated active efflux in all the strains, which contributed, in part, to CIP resistance. The remaining contribution was largely due to commonly occurring mutations in the QRDR regions of the *gyrA* and the *parC* genes.

3.8 SECTION 8:

Multi-drug resistance of *A. baumannii* in the US and Europe.

3.8.1 Aims and objectives

To study the mechanisms of multi-drug resistance from clinical isolates of *Acinetobacter baumannii* isolated in the United States and Europe.

3.8.2 Identification and typing

The strains used in this study were previously identified either by 16S-23S rRNA restriction analysis or by the *rpoB* gene sequencing and by the intrinsic $bla_{\text{OXA-51-like}}$ gene PCR by Benjamin Evans (isolates from Europe) or by Susan Brown (isolates from the US). They were reconfirmed by me by the $bla_{\text{OXA-51-like}}$ gene PCR and by any of the above methods whenever necessary.

3.8.3 Carbapenem resistance and the detection of bla_{OXA} family genes

Table 12a and 12b outlines the MIC values of imipenem (IPM) and meropenem (MEM) for the 36 isolates from the Europe and 36 from the United States. The multiplex PCR was performed for each isolate (Woodford *et al*., 2006) (Figure 36a and 36b) and the upstream sequences of the $bla_{\text{OXA-51-like}}$ gene, $bla_{\text{OXA-58-like}}$ gene were determined using established primers (Lopes *et al*., 2012, Héritier *et al*., 2005b). The whole genes with their upstream sequences, if found, were sequenced. Isolates 2, 14, 206, 207 and 208 did not yield a positive band despite the OXA-58 multiplex PCR being positive. OXA 143-F and OXA 143-R primers failed to detect the $bla_{\text{OXA-143}}$ gene in any of the isolates. The primers OXA-24A and OXA-24B (Afzal-Shah *et al*., 2001), OXA- 24F and OXA-24R (Jeon *et al*., 2005), OXA-23F and OXA-23R (Afzal-Shah *et al*., 2001) were used for sequencing to detect any variation within the genes.

The primer ISADC1 (Ruiz *et al*., 2007) and OXA-23R were used for detection of insertion sequence IS*Aba1* upstream of the *bla*$_{\text{OXA-23-like}}$ gene, which was detected in all strains positive for the gene (Table 12). The primer IntoxaF and IntaceR did not detect the presence of insertion sequence downstream in any of the isolates which had an insert present upstream of the *bla*$_{\text{OXA-51-like}}$ gene. The primers ISAba1F and OxaR detected IS*Aba1* and *bla*$_{\text{OXA-51-like}}$ gene on a plasmid in some isolates. The gene was found to be present on a 110kb plasmid in strain 1, 90kb in strain 27 and 47kb in strain 29. The plasmid of strain 24 could not be isolated even on repeated trials. PCR performed with the multiplex primers (Ellington *et al*., 2007) for metallo-β-lactamases detected just two isolates positive for the *bla*$_{\text{IMP}}$ gene. They were Ab25 from the European collection and Ab97 from isolates from the US.

Fig 36a: Multiplex PCR (*bla*$_{OXA}$ families) for the European isolates

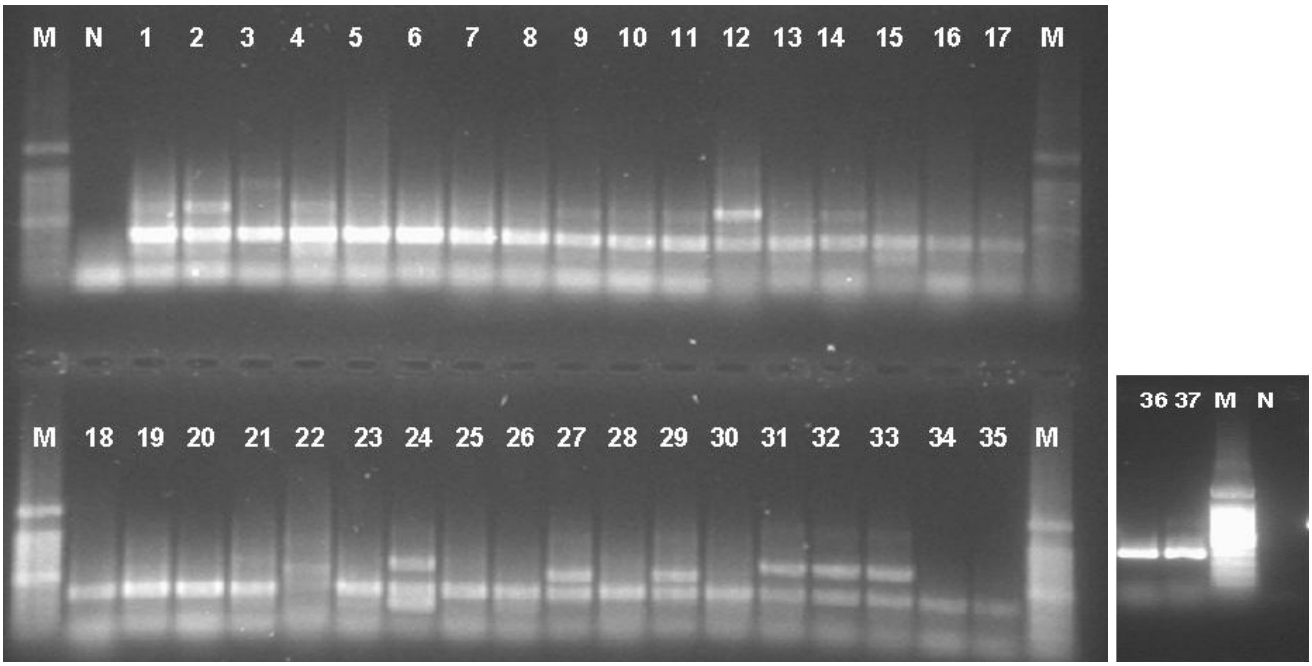
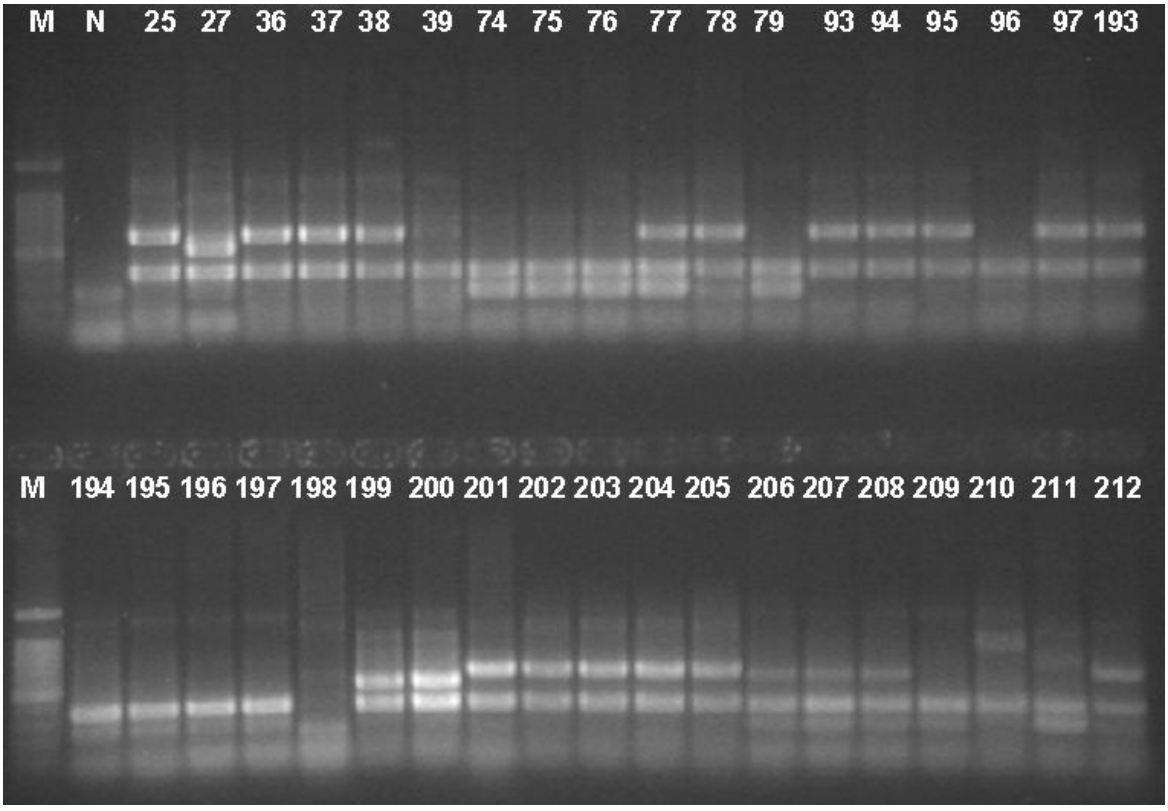


Fig 36b: Multiplex PCR (*bla*$_{OXA}$ families) for isolates from the US



***bla*$_{OXA-51-like}$ gene= 353bp, *bla*$_{OXA-40-like}$ gene= 246bp, *bla*$_{OXA-58-like}$ gene= 599bp**

***bla*$_{OXA-143-like}$ gene= 728bp, *bla*$_{OXA-23-like}$ gene= 501bp**

(Woodford *et al*., 2006).

Table 12a: MIC values, *bla*$_{OXA}$ genes and gene environment of *A. baumannii* isolates from Europe (EU).

EU	MIC (mg/L)								
Isolate/Strain	IMP	MER	*ISAba1*-OXA23	OXA-24like	OXA-58 like	IS upstream OXA-58	OXA-51like	IS upstream OXA-51-like	IS*Aba1*F-51R
1	32	64	N	N	N		66	N	Y
2	0.25	1	N	N	Y		66	N	N
3	0.25	0.25	N	N	N		64	N	N
4	8	4	N	N	N		108	IS*Aba1*	N
5	1	8	N	N	N		110	IS*Aba1*	N
6	1	2	N	N	N		69	N	N
7	0.25	1	N	N	N		65	N	N
8	0.25	0.5	N	N	N		69	N	N
9	16	16	N	N	N		82	IS*Aba1*	N
10	4	8	N	N	N		66	N	N
11	8	16	N	N	N		107	Y	N
12	4	16	N	N	Y	IS*Aba3*	Δ132	IS*Aba16*	N
13	0.25	1	N	N	N		71	N	N
14	0.25	2	N	N	Y		69	N	N
15	0.12	1	N	Y	N		68	N	N
16	0.5	1	N	N	N		66	N	N
17	0.25	2	N	N	N		71	N	N
18	0.12	1	N	N	N		112	N	N
19	0.5	1	N	N	N		106	N	N
20	0.25	0.5	N	N	N		69	N	N
21	0.5	1	N	N	N		66	N	N
A22									
23	4	8	N	N	N		69	N	N
24	32	64	N	Y	Y	IS*Aba3*	64	N	Y
25	2	4	N	N	N		208	N	N
26	0.12	0.25	N	N	N		98	N	N

EU Isolate/Strain	MIC (mg/L) IMP	 MER	*ISAba1*-OXA23	OXA-24like	OXA-58 like	IS upstream OXA-58	OXA -51like	IS upstream OXA-51-like	IS*Aba1*F-51R
27	16	32	Y	N	N		66	N	Y
28	0.25	0.12	N	N	N		68	N	N
29	16	8	Y	N	N		144	N	Y
30	2	2	N	N	N		65	N	N
31	4	4	N	N	Y	IS*Aba3*-IS*Aba2*-IS*Aba3*	66	N	N
32	1	2	N	N	Y	IS*Aba3*-IS*Aba2*-IS*Aba3*	92	N	N
33	4	4	N	N	Y	IS*Aba3*-IS*Aba2*-IS*Aba3*	66	N	N
34	8	16	N	N	N		107	**IS*Aba1***	Y
35	8	32	N	N	N		83	**IS*Aba1***	Y
36	0.5	0.5	N	N	N		66	N	N
37	8	64	N	N	N		83	**IS*Aba1***	Y

MIC (Minimum inhibitory concentration) values of imipenem (IPM) and meropenem (MEM): Highlighted in red= resistant, Highlighted in blue = intermediate, Highlighted in black= sensitive, Y=presence of a gene, N=absence of a gene, IS= Insertion Sequence

Table 12b: MIC values of imipenem, meropenem, *bla*$_{OXA}$ genes and gene environment of *A. baumannii* isolates from the US

US	MIC (mg/L)							IS upstream of	
Isolate/Strain	IMP	MER	IS*Aba1*-OXA-23	OXA-24-like	OXA-58-like	IS upstream of OXA-58	OXA-51-like	OXA-51-like	IS*Aba1*F-51R
Ab25	8	4	N	N	Y	IS*Aba825*	65	N	N
Ab27	16	16	Y	N	N	N	66	N	N
Ab36su	8	4	N	N	Y	IS*Aba825*	51	N	N
Ab37	8	4	N	N	Y	IS*Aba825*	51	N	N
Ab38	4	4	N	N	Y	IS*Aba825*	51	N	N
Ab39	0.25	0.5	N	N	N	N	67	N	N
Ab74	128	64	N	Y	N	N	66	N	N
Ab75	64	64	N	Y	N	N	71	N	N
Ab76	128	64	N	Y	N	N	71	N	N
Ab77	128	64	N	Y	Y	IS*Aba3*-IS*Aba2*-IS*Aba3*	68	N	N
Ab78	16	4	N	N	Y	IS*1008*	66	N	N
Ab79	4	8	N	Y	N	N	70	N	N
Ab93	8	8	N	N	Y	IS*1008*	71	N	N
Ab94	8	8	N	N	Y	IS*1008*	64	N	N
Ab95	8	4	N	N	Y	IS*1008*	71	N	N
Ab96	1	1	N	N	N	N	71	N	N
Ab97	4	8	N	N	Y	IS*Aba3*	78	N	N
Ab193	8	16	N	N	Y	IS*Aba3*	69	N	N
Ab194	0.25	0.25	N	N	N	N	66	N	N
Ab195	0.12	0.25	N	N	N	N	66	N	N
Ab196	0.25	0.25	N	N	N	N	66	N	N
Ab197	0.5	0.25	N	N	N	N	66	N	N
A198									
Ab199	16	16	Y	N	N	N	69	N	N
Ab200	16	16	Y	N	N	N	69	N	N

US Isolate/Strain	MIC (mg/L) IMP	 MER	IS*Aba1*-OXA-23	OXA-24-like	OXA-58-like	IS upstream of OXA-58	OXA-51-like	IS upstream of OXA-51-like	IS*Aba1*F-51R
Ab201	16	16	**N**	**N**	**Y**	IS*Aba825*	51	**N**	**N**
Ab202	16	4	**N**	**N**	**Y**	IS*Aba825*	65	IS*Aba825*	**N**
Ab203	16	4	**N**	**N**	**Y**	IS*Aba825*	65	IS*Aba825*	**N**
Ab204	16	4	**N**	**N**	**Y**	IS*Aba825*	65	IS*Aba825*	**N**
Ab205	16	4	**N**	**N**	**Y**	IS*Aba825*	65	IS*Aba825*	**N**
Ab206	4	4	**N**	**N**	**Y**	**N**	80	**IS*Aba1***	**Y**
Ab207	4	16	**N**	**N**	**Y**	**N**	79	**IS*Aba1***	**Y**
Ab208	8	8	**N**	**N**	**Y**	**N**	82	**IS*Aba1***	**Y**
Ab209	16	16	**N**	**N**	**N**	**N**	66	**N**	**N**
Ab210	16	16	**N**	**N**	**N**	**N**	69	**N**	**N**
Ab211	128	128	**N**	**Y**	**N**	**N**	66	**N**	**N**
Ab212	16	4	**N**	**N**	**Y**	IS*Aba825*	68	**N**	**N**

MIC (Minimum inhibitory concentration): Highlighted in red= resistant, Highlighted in blue = intermediate, Highlighted in black= sensitive, Y=presence of a gene, N=absence of a gene, IS= Insertion Sequence

3.8.4 Ceftazidime resistance

Table 13 outlines the MIC values of ceftazdime (CAZ) for the 36 strains isolated in Europe and 36 from the United States. The primers FU, RU detected insertion elements such as IS*Aba1* and IS*Aba125* upstream of the *bla*$_{ADC}$ gene. ISADC1 and ISADC2 primers detected IS*Aba1* sequence upstream of the *bla*$_{ADC}$ gene. ISADC1 and ISADC2 PCR was positive for isolate 24, and 29, which had low MIC values with no IS element detected by the FU, RU primers. Although the ISADC1 and 2 PCR for the detection of IS*Aba1*-*bla*$_{ADC}$ was positive for isolate 36su, it had IS*Aba125* detected upstream of the *bla*$_{ADC}$ with FU and RU primers. FD and RD primers failed to detect any insert downstream of the *bla*$_{ADC}$. Multiplex PCR for determination of integrase genes was performed (Dillon *et al*., 2005). Conjugation assays performed for isolate 24, 27 and 36su transferred plasmids with OXA-2 and OXA-10-like genes present on class one integrons as determined by the CSa and CSb primers. Hence this study was not pursued further. Isolates 194 (novel TEM-194), 195 (novel TEM-195) and 197 (novel TEM-193) did not have IS*Aba1* upstream of the *bla*$_{ADC}$ but tested positive for the *bla*$_{TEM}$ and *bla*$_{PER-1}$ genes. The primers TEMF, TEMR and PERF and PERR were used. The alignment of the nucleotides is seen in figure 37a and the amino acid translation in figure 37b. Isolate 196 was positive for *bla*$_{PER-1}$ gene. Isolates 193 and 195 had cysteine at position 165 that has been known to confer high resistance to ceftazidime (reviewed by Bradford, 2001). There were other amino acid changes at various different positions that could contribute towards resistance to ceftazidime. Isolate 25 having high MIC of ceftazidime had the OXA-2 gene within the integron structure.

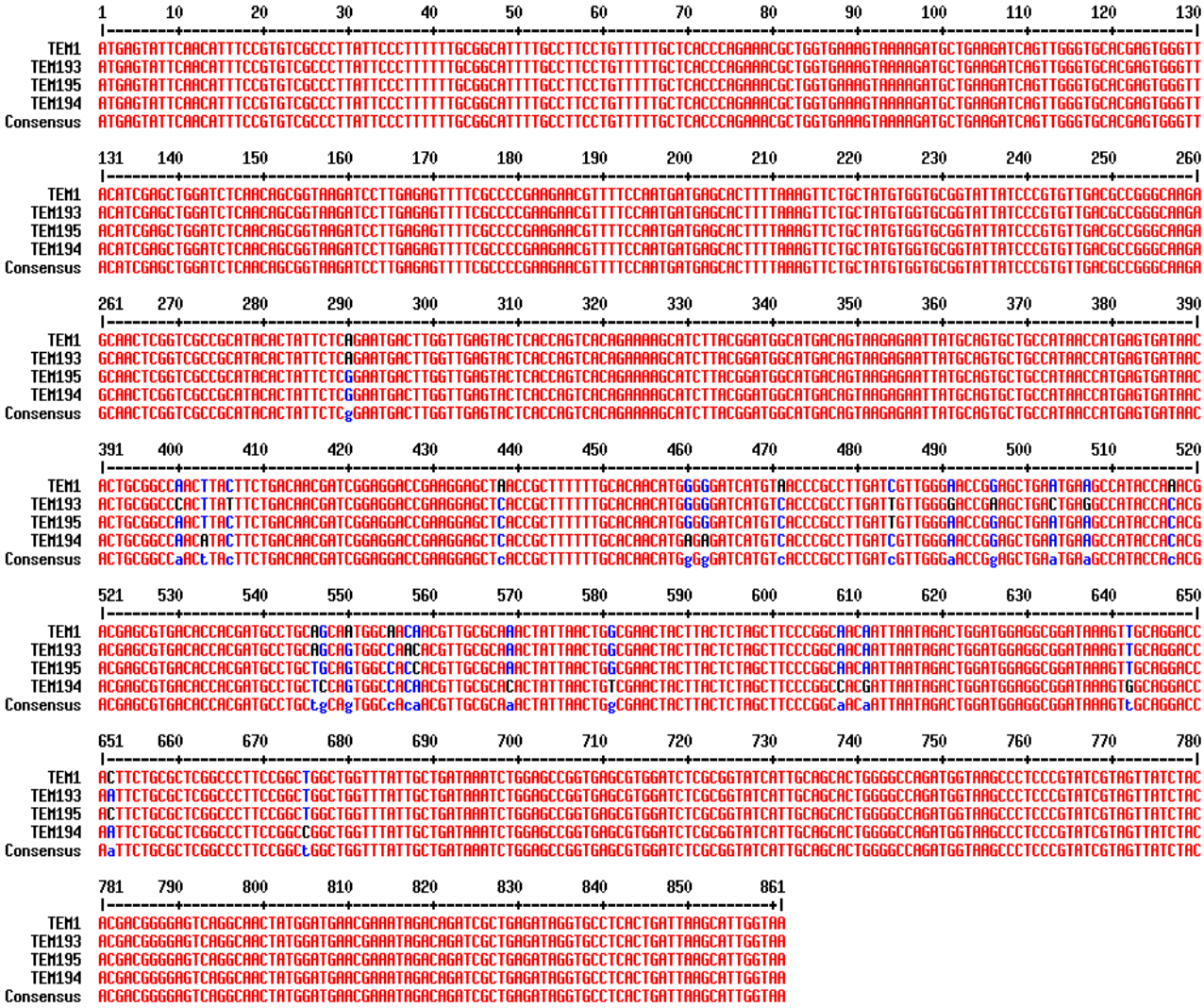
Table 13: MIC of Ceftazidime (CAZ), *bla*$_{ADC}$ gene environment

EU	MIC (mg/L)					US	MIC (mg/L)				
Isolate/Strain	CAZ	FD/RD	FU/RU	ISADC1 and 2	integrase	Isolate/Strain	CAZ	FU/RU	FD/RD	ISADC1 and 2	integrase
1	16	N	ISAba1	ISAba1	N						
2	>256	N	ISAba125	N	1	Ab25	8	N	N	N	2
3	128	N	ISAba1	ISAba1	2	Ab27	128	ISAba1	N	ISAba1	1
4	32	N	ISAba1	ISAba1	1	Ab36	128	ISAba125	N	ISAba1	1
5	64	N	ISAba1	ISAba1	1	Ab37	64	ISAba125	N	N	N
6	128	N	ISAba1	ISAba1	N	Ab38	64	ISAba125	N	N	N
7	32	N	ISAba1	ISAba1	2	Ab39	128	ISAba1	N	ISAba1	N
8	8	N	N	N	1	Ab74	>256	ISAba1	N	ISAba1	N
9	>256	N	ISAba1	ISAba1	1	Ab75	>256	ISAba1	N	ISAba1	1
10	32	N	ISAba1	ISAba1	1	Ab76	>256	ISAba1	N	ISAba1	1
11	128	N	ISAba1	ISAba1	N	Ab77	>256	ISAba1	N	ISAba1	N
12	8	N	N	N	2	Ab78	256	ISAba1	N	ISAba1	1
13	32	N	N	N	1	Ab79	8	N	N	N	N
14	16	N	N	N	1	Ab93	64	ISAba1	N	ISAba1	1
15	32	N	N	N	N	Ab94	>256	ISAba1	N	ISAba1	N
16	>256	N	ISAba1	ISAba1	N	Ab95	64	ISAba1	N	ISAba1	1
17	32	N	N	N	1	Ab96	64	ISAba1	N	ISAba1	1
18	128	N	ISAba1	ISAba1	N	Ab97	128	ISAba1	N	ISAba1	1 and 2
19	8	N	N	N	N	Ab193	64	N	N	N	N
20	>256	N	ISAba1	ISAba1	1	Ab194	>256	N	N	N	1
21	32	N	ISAba1	ISAba1	1	Ab195	>256	N	N	N	1
A22						Ab196	>256	N	N	N	1
23	8	N	N	N	1	Ab197	>256	N	N	N	1
24	8	N	N	ISAba1	1	A198					
25	>256	N	N	N	1	Ab199	128	ISAba1	N	ISAba1	2
26	4	N	N	N	1	Ab200	128	ISAba1	N	ISAba1	2

EU	MIC (mg/L)					US	MIC (mg/L)				
Isolate/Strain	CAZ	FD/RD	FU/RU	ISADC1 and 2	integrase	Isolate/Strain	CAZ	FU/RU	FD/RD	ISADC1 and 2	integrase
27	>256	N	**IS*Aba1***	**IS*Aba1***	1	Ab201	128	**IS*Aba1***	N	**IS*Aba1***	N
28	4	N	N	N	N	Ab202	128	**IS*Aba1***	N	**IS*Aba1***	2
29	16	N	N	**IS*Aba1***	N	Ab203	128	**IS*Aba1***	N	**IS*Aba1***	2
30	>256	N	N	N	N	Ab204	128	**IS*Aba1***	N	**IS*Aba1***	2
31	>256	N	IS*Aba125*	N	1	Ab205	128	**IS*Aba1***	N	**IS*Aba1***	2
32	>256	N	IS*Aba125*	N	1	Ab206	64	**IS*Aba1***	N	**IS*Aba1***	N
33	>256	N	IS*Aba125*	N	1	Ab207	128	**IS*Aba1***	N	**IS*Aba1***	1
34	>128	N	**IS*Aba1***	**IS*Aba1***	N	Ab208	128	**IS*Aba1***	N	**IS*Aba1***	1
35	>128	N	**IS*Aba1***	**IS*Aba1***	1	Ab209	128	**IS*Aba1***	N	**IS*Aba1***	2
36	128	N	**IS*Aba1***	**IS*Aba1***	1	Ab210	128	N	N	N	N
37	>128	N	**IS*Aba1***	**IS*Aba1***	1	Ab211	>256	**IS*Aba1***	N	**IS*Aba1***	N
						Ab212	16	N	N	N	N

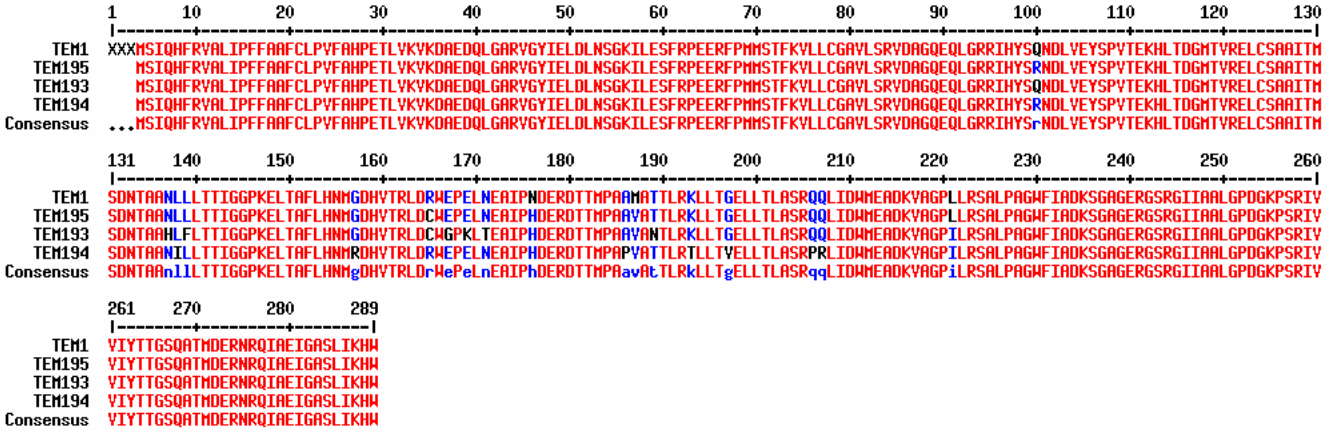
MIC (Minimum inhibitory concentration): Highlighted in red= resistant, Highlighted in blue = intermediate, Highlighted in black= sensitive, N=absence of a gene, 1= +ve for integrase 1, 2=+ve for integrase 2

Figure 37a: Nucleotide sequence of TEM-193, 194 and 195 aligned with TEM-1



The letters A denotes Adenine, T=Thymine, G=Guanine and C=Cytosine.

Figure 37b: Amino acid translated sequence of TEM-193, 194 and 195 aligned with TEM-1



Changes at position 165 Arginine (R) to Cysteine (C) seen in TEM-195 and TEM-193 enzymes explainin ceftazidime resistance (reviewed by Bradford, 2001). Gultamine (Q), Proline (P), Alanine (A), Threonine (T), Valine (V), Glycine (G), Histidine (H), Glutamic acid (E), lysine (K), Tryptophan (W), Isoleucine (I), Phenylalanine (F), Asparagine (N), Methionine (M), Leucine (L)

3.8.5 Ciprofloxacin resistance

PCR was performed using the primers described by Valentine *et al*. (2008) for the amplification of quinolone resistance determining regions (QRDRs). Table 14 shows the MIC values of ciprofloxacin and the mutations in the *gyrA* and *parC* genes.

Table 14: MIC values of ciprofloxacin and the mutations in *gyrA* and *parC*

EU Isolate	MIC(mg/L) CIP	*gyrA*	*parC*	US Isolate	MIC (mg/L) CIP	*gyrA*	*parC*
1	64	S83-L	S80-W				
2	16	S83-L	S80-S	Ab25	128	S83-L	S80- Y
3	64	S83-L	S80-L	Ab27	64	S83-L	S80-L
4	128	S83-L	Q84-K	Ab36	128	S83-L	S80-L
5	4	S83-L	S80-S	Ab37	128	S83-L	S80-L
6	128	S83-L	S80-L	Ab38	64	S83-L	S80-L
7	2	S83-L	S80-S	Ab39	64	S83-L	S80-F
8	1	N/D	N/D	Ab74	128	S83-L	S80-F
9	64	S83-L	S80-L	Ab75	128	S83-L	S80-F
10	32	S83-L	S80-W	Ab76	128	S83-L	S80-F
11	8	S83-L	S80-S	Ab77	128	S83-L	S80-F
12	>256	S83-L	S80-W	Ab78	32	S83-L	S80-S
13	128	S83-L	S80-L	Ab79	0.012	N/D	N/D
14	0.25	N/D	N/D	Ab93	128	S83-L	S80-L
15	0.5	N/D	N/D	Ab94	4	S83-S	S80-S
16	128	S83-L	S80-L	Ab95	128	S83-L	S80-L
17	128	S83-L	S80-L	Ab96	128	S83-L	S80-L
18	256	S83-L	S80-L	Ab97	8	S83-L	S80-S
19	0.12	N/D	N/D	Ab193	128	S83-L	D84-K
20	128	S83-L	S80-L	Ab194	128	S83-L	S80-F
21	4	S83-S	S80-S	Ab195	128	S83-L	S80-F
22	N/D	N/D	N/D	Ab196	0.5	N/D	N/D
23	64	S83-L	S80-L	Ab197	8	S83-L	S80-S
24	2	S83-L	S80-S	A198	N/D	N/D	N/D
25	0.12	N/D	N/D	Ab199	128	S83-L	S80-L
26	64	S83-L	S80-L	Ab200	128	S83-L	S80-L
27	256	S83-L	S80-L	Ab201	128	S83-L	S80-L
28	0.12	N/D	N/D	Ab202	128	S83-L	S80-F
29	64	S83-L	S80-L	Ab203	64	S83-L	S80-F
30	128	S83-L	S80-L	Ab204	64	S83-L	S80-F
31	16	S83-L	S80-L	Ab205	128	S83-L	S80-F
32	0.5	S83-L	S80-S	Ab206	32	S83-L	S80-S
33	16	S83-L	S80-S	Ab207	64	S83-L	S80-L
34	8	S83-L	S80-S	Ab208	16	S83-L	S80-S
35	16	S83-L	S80-S	Ab209	>256	S83-L	S80-W
36	32	S83-L	S80-L	Ab210	128	S83-L	S80-L
37	32	S83-L	S80-L	Ab211	256	S83-L	S80-L
				Ab212	128	S83-L	S80-L

MIC (Minimum inhibitory concentration) of Ciprofloxacin (CIP)

MIC values highlighted in red: Resistant

MIC values highlighted in black: Sensitive

N/D indicates not determined

***parC* changes highlighted in light green: novel changes**

gyrA, parC changes in blue: no change in the amino acid

gyrA, parC changes in black: amino acid changes

S= Serine, L=Leucine, W=Tryptophan, Q= Glutamine, K=Lysine, D= Aspartate, F=Phenylalanine

The numbers in *gyrA*, *parC* denote the position at which the amino acid change has occurred.

Highlighted in Blue: No change in the amino acid.

It was found that just 16% of the total European isolates and just 5% of the isolates from the US were sensitive to ciprofloxacin. Modifications of the DNA topoisomerase II and IV, due to in the mutations in the *gyrA* and *parC* have been well described in *A. baumannii* (Higgins *et al*., 2004). The AdeABC and AbeM efflux pumps have also been shown to play a role in ciprofloxacin resistance (Magnet *et al*., 2001; Su *et al*., 2005).

3.8.6 Gentamicin resistance

A multiplex PCR described by Noppe-Leclercq *et al.* (1999) was used for the identification of aminoglycoside resistance genes. Table 15 gives the gentamicin resistance profile with the detection of one or more aminoglycoside resistance genes.

Table 15: MIC of gentamicin (CN) and aminoglycoside resistance genes.

EU Isolate	MIC (mg/L) CN	aminoglycoside modifying enzymes	US Isolate	MIC (mg/L) CN	aminoglycoside modifying enzymes
1	0.5	*aph(3')-VI*			
2	32	*aph(3')-VI*	Ab25	>256	*aph(3')-VI*
3	64	*ant(2")-Ia*	Ab27	128	*aac(3)-Ia,ant(2")-Ia,aph(3')-Ia*
4	32	*ant(2")-Ia*	Ab36	4	*aac(3)-Ia,aac(6')-Ib*
5	128	*aac(3)-Ia, ant(3')-VI,aph(3')-Ia*	Ab37	128	*aac(3)-IIa,aph(3')-VI,ant(2")-Ia,*
6	0.25	*aph(3')-VI*	Ab38	>256	*aac(3)-IIa,aph(3')-VI*
7	>265	*ant(2")-Ia*	Ab39	128	*aac(3)-IIa*
8	>256	N	Ab74	32	*aac(3)-Ia*
9	>>256	*aac(3)-Ia*	Ab75	128	*aph(3')-VI,ant(2")-Ia*
10	32	*aac(3)-Ia*	Ab76	>256	*aph(3')-VI,ant(2")-Ia*
11	2	N	Ab77	128	*aph(3')-VI,ant(2")-Ia*
12	1	N	Ab78	2	*aph(3')-VI,ant(2")-Ia*
13	>256	*ant(2")-Ia*	Ab79	0.25	N
14	4	*ant(2")-Ia*	Ab93	256	*aac(3)-Ia,aph(3')-VI,ant(2")-Ia*
15	8	*ant(2")-Ia*	Ab94	128	*ant(2")-Ia*
16	64	*aac(3)-IIa*	Ab95	>256	*aac(3)-Ia*
17	256	*ant(2")-Ia*	Ab96	>256	*ant(2")-Ia*
18	16	N	Ab97	8	*aph(3')-VI,ant(2")-Ia*
19	0.06	N	Ab193	32	N
20	256	*aac(3)-Ia,ant(2")-Ia*	Ab194	16	*aac(3)-Ia,ant(2")-Ia,aph(3')-Ia*
21	>256	*aac(3)-Ia, aph(3')-Ia*	Ab195	32	*aac(3)-Ia,aph(3')-Ia*
22			Ab196	4	*aac(3)-Ia,ant(2")-Ia,aph(3')-Ia*
23	4	N	Ab197	4	*aac(3)-Ia,ant(2")-Ia,aph(3')-Ia*
24	1	*aac(3)-IIa,ant(2")-Ia,aph(3')-Ia*			
25	0.12	*ant(3')-VI,aph(3')-Ia*	Ab199	256	*aac(6')-Ib*
26	>256	*aac(3)-IIa,ant(2")-Ia*	Ab200	128	*ant(2")-Ia,aac(6')-Ib*
27	4	*aac(3)-Ia*	Ab201	128	*aac(3)-Ia,aph(3')-VI,ant(2")-Ia*
28	0.12	N	Ab202	0.25	*aph(3')-VI*
29	0.12	*aac(3)-IIa*	Ab203	0.12	*aph(3')-VI*
30	0.5	*aph(3')-VI*	Ab204	0.25	*aph(3')-VI*
31	8	*aph(3')-VI,aph(3')-Ia*	Ab205	0.12	*aph(3')-VI*
32	8	*aph(3')-VI,aph(3')-Ia*	Ab206	1	*aac(3)-Ia,ant(2")-Ia*
33	2	*aph(3')-VI,aph(3')-Ia*	Ab207	128	*aac(3)-Ia,ant(2")-Ia,aph(3')-VI*
34	2	*aph(3')-VI*	Ab208	>256	*aac(3)-Ia,ant(2")-Ia,aph(3')-Ia*
35	32	*aac(3)-Ia*	Ab209	>256	*ant(2")-Ia,aph(3')-Ia*
36	2	*aac(3)-Ia*	Ab210	>256	*ant(2")-Ia,aac(6')-Ib*
37	4	*aac(3)-Ia*	Ab211	128	*aac(3)-Ia,aph(3')-VI,ant(2")-Ia*
			Ab212	128	*aph(3')-VI,ant(2")-Ia*

Minimum inhibitory concentration (MIC) values of gentamicin (CN)

Highlighted in red= resistant, Highlighted in black= sensitive, N=absence of a resistance gene

The following description from Shaw *et al*., 1993

aac(3)-Ia is *N-acetyl transferase* and confers resistance to astromicin (fortimicin), gentamicin and sisomicin

aac(3)-IIa is *N-acetyl transferase* and confers resistance to gentamicn, tobramycin, dibekacin, 6'-*N*-ethylnetilmicin, 2'-*N*-ethylnetilmicin, netilmicin and sisomicin

aac(6')-Ib is *N-acetyl transferase* and confers resistance to tobramycin, dibekacin, 2'-*N*-ethylnetilmicin, netilmicin, sisomicin, amikacin, 5-episisomicin and isepamicin

ant(2'')-Ia is an aminoglycoside *adenylyl or nucleotidyltransferase* and confers resistance to gentamicn, tobramycin, dibekacin, sisomicin and kanamycin

aph(3')-Ia is a *phosphotransferase* and confers resistance to kanamycin, neomycin, paromomycin, ribostamycin, lividomycin, gentamicin B

aph(3')-VI is a *phosphotransferase* and confers resistance to kanamycin, neomycin, paromomycin, ribostamycin, butirosin, amikacin and isepamicin

The major classes of enzymes conferring resistance to amniglycoside in *A. baumannii* include acetyltransferases, nucleotidyltransferases and phosphotransferases (Noppe-Leclercq *et al*., 1999). Other mechanisms include the post-transcriptional rRNA methylation by 16S rRNA methylases such as *armA*, *rmtA*, *rmtB*, *rmtC* and *rmtD* (Doi *et al*., 2007; Yokoyama *et al*., 2003; Yamane *et al*., 2005). The PCR for 16S rRNA methylases was not sought due to time constraints, although the multiplex PCR by Noppe-Leclercq *et al*., (1999) explains the resistant profile of most of the isolates.

3.8.7 DISCUSSION:

Carbapenem resistance

Forty-seven percent of the strains from the US were carbapenem resistant in comparison with 14% resistant strains in Europe; 39% of the strains from the US had intermediate levels of carbapenem resistance in comparison to 28% strains isolated in Europe. These figures indicate that *A. baumannii* may be acquiring a resistant state slowly, but steadily, by looking at the trends studied for European isolates. The population structure of clinical isolates of *A. baumannii* is dominated by three international clonal lineages known as European clones I, II and III, corresponding to CC1 (comprising ST1, ST7, ST8, ST19 and ST20), CC2 (comprising ST2, ST45 and ST47) and CC3 (ST3 and ST14) in the MLST scheme of Diancourt *et al*. (2010), with most outbreak strains belonging to the first two of these lineages (Woodford *et al*., 2011). We observed that OXA-66 was the most predominant enzyme (28%), followed by OXA-69 (11%), OXA-71 (6%) and then other *bla*$_{\text{OXA}}$ enzyme variants in the European isolates.

A slightly different trend was observed for isolates from the US where OXA-66 was predominant (24%), followed by OXA-71 (14%), OXA-65 (13%), OXA-51 (11%), OXA-69 (11%) and other OXA enzyme variants. Seven percent of the total strains harboured the *bla*$_{\text{OXA-23-like}}$ with IS*Aba1*, immediately located upstream of the gene. Eleven percent of the strains harboured the *bla*$_{\text{OXA-40-like}}$ gene and 32% of the strains harboured the *bla*$_{\text{OXA-58-like}}$ gene linked to different insertion elements (Table12a and 12b) upstream of the gene. According to Woodford *et al*. (2011), even if the core genome of the clonal lineages is stable, the accessory genome is more fluid and carbapenem resistance among these isolates has become common, usually by

acquisition of OXA carbapenemase genes, although it may simply involve insertion sequence-mediated up-regulation of the intrinsic *bla*$_{\text{OXA-51-like}}$ gene, and this was seen for 23% of strains, which had their *bla*$_{\text{OXA-51-like}}$ gene upregulated by either IS*Aba825* or IS*Aba1*. The isolates with an insert upstream of the *bla*$_{\text{OXA-51-like}}$ gene did not have an insert downstream as tested by the intoxaF and intaceR primers. They formed defunct transposons. Carbapenem resistance due to metallo-β-lactamases was not detected at significant levels, with just one isolate (Ab97) being positive for the *bla*$_{\text{IMP-like}}$ gene.

Ceftazidime resistance

Resistance to ceftazidime was observed in 81% of European isolates and 94% of isolates from the US; 19% of the European isolates had intermediate levels of ceftazidime resistance and 6% from the US. There was no isolate sensitive to ceftazidime. Most of the isolates had either IS*Aba1* or IS*Aba125* governing the *bla*$_{\text{ADC}}$ gene (Table 13). These insertion elements have been shown to upregulate the expression of the *bla*$_{\text{ADC}}$ gene (Ruiz et al., 2007, Lopes *et al*., 2011. P601, ECCMID) The strains that did not have any insertion upstream but were still resistant to ceftazidime and were positive for at least one or more of either the *bla*$_{\text{TEM-like}}$, (TEM-F and TEM-R primers), *bla*$_{\text{PER-like}}$ (PER-F and PER-R primers) or *bla*$_{\text{OXA-2-like}}$ genes (CSa and CSb primers), certain variants of which provide high resistance to ceftazidime (reviewed by Bradford., 2001, Pasteran *et al*., 2006, Danel *et al*., 1997). The same isolates were negative for *bla*$_{\text{CTX-M}}$ families, *bla*$_{\text{VEB}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{GES}}$ genes as tested by the PCR with the primers described in the Material and Methods section.

Ciprofloxacin resistance

Resistance to ciprofloxacin was observed in 81% of European isolates and 94% of isolates from the US; 19% of the European isolates and 6% of isolates from the US were sensitive to ciprofloxacin. Commonly observed *gyrA* and *parC* mutations were seen in isolates having a ciprofloxacin resistance profile (Table 14). Fluoroquinolone resistance can be attributed to mutations in the *gyrA* and *parC* genes (Valentine *et al*., 2008) but also to up-regulation of efflux pumps, giving a lower level of resistance (Magnet *et al*., 2001)

Gentamicin resistance

Resistance to gentamicin was observed in 47% of European isolates and 28% of isolates from the US. A previous study by Henwood *et al* (2002) reports over 75% isolates as ceftazidime resistant followed by 30% isolates with gentamicin resistance profile and this concurs with the trends observed in this study. Aminoglycoside resistance due to acetyltransferases [*aac(6')-Ib*, *aac(6')- Ih*, *aac(3)-I*, *aac(3)-II*], nucleotidyltransferase [*ant(2''-I*)], and phosphotransferases [*aph(3')-I*, *aph(3')-VI*] has been predominantly identified within clinical *A. baumannii* isolates, with one or more gene(s) conferring aminoglycoside resistance (Noppe-Leclercq *et al*., 1999). In our study, we also observed some of the clinical isolates harbouring one or more aminoglycoside resistance but displaying a sensitive phenotype. This also hints towards the development of aminoglycoside resistance, which is evolving slowly but steadily with time.

4.0 DISCUSSION

The prevalence of insertion elements in *A. baumannii* is becoming a great concern in treating patients with multiresistant isolates. Insertion sequences are motors that regulate the expression of genes and drive their expression under conditions that are stressful for the survival of the bacterium. This study focused on exploring the role of insertion elements such as IS*Aba1*, IS*Aba2*, IS*Aba3*, IS*Aba16*, IS*Aba125* and many more which have contributed towards the multi-drug resistant nature of *A. baumannii*. These elements have assisted and enhanced profile of multi-drug resistance of *Acinetobacter baumannii* making it a resilient bacterium and an emerging pathogen. Insertion elements have added to the versatility, diversity, evolutionary capabilities, and virulence factors of *A. baumannii* (Bergone-Bérézin, 2008). The expression of various β-lactamse genes with the IS loaded promoters help in destroying most of the antibiotics. The insertion elements also act as vehicles of mobilization from one bacterial species to another. The entry of *Acinetobacter baumannii* in the military healthcare system and the civilian hospital environment, leading to numerous outbreaks is not surprising (Murray *et al*., 2008). The implementation of broad infection control measures including increased environmental cleaning, widespread availability and use of hand hygiene, active surveillance, and isolation of colonized and infected patients will help to reduce the spread of this bacterium among immuno-compromised patients (Murray *et al*., 2008). Novel strategies for control should be developed and applied to the combat the increasing rates of infections and the clonal spread of this pathogen. It is important to note that such advances in implementing infection control will have important implications for both military and civilian hospitals and will ultimately lead to better control of infections caused by various healthcare-associated pathogens (Murray *et al*., 2008). The work described in this

thesis adds to the better understanding of *A. baumannii* as the emerging pathogen of 21st century. It allows the reader to understand the mechanisms of resistance and gives an insight about the problems involved in treating multi-drug resistant *A. baumannii*. This study adds to the better understanding of antimicrobial resistance mechanisms in this emerging pathogen.

5.0 CONCLUSION

This study aimed to investigate in detail the mechanisms of resistance in *A. baumannii* strains isolated from various parts of the world:

Various hypotheses were tested and the following questions were successfully answered on completion of this study.

Section 1: Can IS*Aba1* modulate resistant and sensitive phenotypes? Yes. It can increase the beta-lactamase gene expression and contribute towards a phenotype that is favourable to the bacterium depending on the resistance load. Translational frameshifting of IS*Aba1* has already been shown (Mugnier et al., 2009) and the transposition of IS*Aba1* with frameshift mutants like acriflavine is likely to occur as it is a frameshift mutagen.

Section 2: Is there any other insertion element that can regulate bla_{ADC} gene expression? Yes. In the course of this study IS*Aba125* was identified. Though it is not as common as IS*Aba1*, which is found in most of the isolates, it is provides a very strong promoter for the bla_{ADC} gene up-regulation.

Section 3: Is *A. baumannii* emerging as a multiresistant pathogen in countries such as Bolivia? Yes. This study found that *A. baumannii* was resistant to almost all the antibiotics tested with the $bla_{\text{OXA-58-like}}$ gene, governed by IS*Aba3-like* element, conferring carbapenem resistance, and bla_{ADC} gene, governed by IS*Aba1*, conferring ceftazidime resistance. The mutations in the *gyrA, parC* were also high, thus conferring resistance to ciprofloxacin with synergistic contribution from the AdeABC

efflux pump. The mechanisms of gentamicin resistance remains unexplained in some of the isolates.

Section 4: What are the trends in Aberdeen Royal Infirmary? In this study we observed evolution of new variants of the *bla*$_{\text{OXA-51-like}}$ gene (OXA-216 and 217). We also established the clonal relatedness of 2 isolates isolated in different years and showed how the integration of *bla*$_{\text{OXA-23-like}}$ gene in the host chromosome can provide a stable mechanism of resistance.

Section 5: Are there any other insertion elements that govern the *bla*$_{\text{OXA-51-like}}$ gene expression? Yes. We found IS*Aba825*, which governed the expression of *bla*$_{\text{OXA-58-like}}$ and *bla*$_{\text{OXA-51-like}}$ genes in clinical isolates isolated in the US. We also demonstrated that the loss of this element is co-ordinated by a central mechanism that can render the bacterium sensitive to carbapenems.

Section 6: What was the mechanism of carbapenem resistance in strain Ab244 (isolates 12)? We observed the presence of *bla*$_{\text{OXA-58-like}}$ gene that was governed by IS*Aba-3-like* element and this provided intermediate levels of resistance in this isolate. We checked for the presence of insertion element upstream of the *bla*$_{\text{OXA-51-like}}$ gene to see if that was synergistically related to carbapenem resistance in this isolate but instead identified a novel insertion element IS*Aba16*, which disrupted the *bla*$_{\text{OXA-51-like}}$ gene in this isolate.

Section 7: Are efflux genes controlled by insertion elements? We observed a single isolate having IS*Aba1* sequence upstream of the *adeA* gene whereas other isolates had

disruptions of the *adeS* gene by insertion elements like IS*Aba1* and IS*Aba16*. Is fluoroquinolone resistance partially contributed by an efflux pump? We concluded that efflux does contribute towards multiresistant phenotype but *gyrA* and *parC* mutations are the primary targets that can make the organism easily resistant to fluroquinolones like ciprofloxacin.

Section 8: How do you compare *A. baumannii* resistance from Europe with that with the US? We saw that the emergence of *A. baumannii* as a multi-drug resistant pathogen was at a rapid rate in the US in comparison to Europe. During the course of the study we found that isolates belonging to sequence group 1 and sequence group 2 appear to be the most successful, certainly across Europe, and within these isolates it is the $bla_{\text{OXA-66}}$ and $bla_{\text{OXA-69}}$ genes that are most regularly identified. The trends in the US were a little different where OXA-66 was predominant, followed by OXA-71, OXA-65, OXA-51, and lastly OXA-69. The presence of IS*Aba1* within an isolate is a major contributing factor, as it has been shown in this study that isolates which encode an IS*Aba1* element upstream of an OXA-51-like gene have carbapenem MICs that are similar to those of isolates that encode an acquired OXA-type β-lactamase of the OXA-23, OXA-40 or OXA-58 families. The presence of IS*Aba1* or IS*Aba825* upstream of a $bla_{\text{OXA-51-like}}$ gene may thus greatly increase the ability of the isolate to survive carbapenem challenge. Our findings indicate that in certain isolates in this study the expression of an OXA-51-like enzyme is as important a threat to carbapenem use as the acquired OXA enzymes because due to the fluidity of the genome, the bacterium can recruit the help of insertion elements in order to enhance its gene expression, thus developing resistance to antibiotics. This is of great concern, as it presents the scenario that all *A. baumannii* have the potential to become resistant

to the carbapenems without needing to acquire additional β-lactamases. The insertion element IS*Aba1* was the most common element found in at least 60% of the isolates. Other elements such as IS*Aba2*, IS*Aba3*, IS*Aba825*, IS*1008*, IS*Aba125*, IS*Aba16* were found to govern the expression of β-lactamase genes or providing alternate mechanisms of resistance for the better fitness of the bacterium. It is noteworthy to point out that a variety of elements were associated with the expression the the $bla_{\text{OXA-58-like}}$ gene. The monitoring of the $bla_{\text{OXA-51-like}}$ to identify enzyme variants with increased substrate spectrums or higher levels of activity would be crucial if we want to preserve and use effectively the few remaining antibiotic treatment options that are available for this rapidly evolving multi-drug resistant pathogen.

6.0 REFERENCES

Actis, L. A., M. E. Tolmasky, L.M. Crosa, and J. H. Crosa. 1993. Effect of iron limiting conditions on growth of clinical isolates of *Acinetobacter baumannii*. *J Clin Microbiol*. **31:** 2812–2815.

Adams, M. D., G. C. Nickel, S. Bajaksouzian, H. Lavender, A. R. Murthy, M. R. Jacobs, and R. A. Bonomo. 2009. Resistance to colistin in *Acinetobacter baumannii* associated with mutations in the PmrAB two-component system. *Antimicrob Agents Chemother.* **53**: 3628-3634.

Afzal-Shah, M., N. Woodford, and D. Livermore. 2001. Characterization of OXA-25, OXA-26, and OXA-27, molecular class D β -lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **45**: 583–588.

Ambler, R. P. 1980. The structure of β -lactamases. *Philos Trans R Soc Lond B Biol Sci.* **289**: 321–331.

Amyes, S.G.B., and H.-K Young. 1996. Mechanisms of antibiotic resistance in *Acinetobacter* spp.-genetics of resistance. In Bergogne-Bérézin E., M. L. Joly-Guillou., and K. J. Towner (eds.) *Acinetobacter*: Microbiology, Epidemiology, Infection, Management. CRC Press, New-York, Chap 8, pp. 185-223.

Andersson, M. I., and A. P. MacGowan 2003. Development of the quinolones. *J Antimicrob Chemother.* **51**(Suppl 1): 1-11.

Anstey, N. M., B. J. Currie, M. Hassell, D. Palmer, B. Dwyer, and H. Seifert. 2002. Community-acquired bacteremic *Acinetobacter* pneumonia in tropical Australia is caused by diverse strains of *Acinetobacter baumannii*, with carriage in the throat in at-risk groups. *J Clin Microbiol*. **40**: 685–686.

Arakawa, Y., N. Shibata, K. Shibayama, H. Kurokawa, T. Yagi, H. Fujiwara, and M. Goto. 2000. Convenient test for screening metallo- β -lactamase- producing gram-negative bacteria by using thiol compounds. *J Clin Microbiol*. **38**: 40-43.

Audureau, A. 1940. Etude du genre *Moraxella*. *Ann Inst Pasteur*. **64**: 126-166.

Baraibar, J., H. Correa, D. Mariscal, M. Gallego, J. Valles, and J. Rello. 1997. Risk factors for infection by *Acinetobacter baumannii* in intubated patients with nosocomial pneumonia. *Chest*. **112**: 1050-1054.

Batchelor, E., and M. Goulian. 2003. Robustness and the cycle of phosphorylation and dephosphorylation in a two-component regulatory system. *Proc Natl Acad Sci, USA*. **100**: 691–696.

Baumann, P. 1968. Isolation of acinetobacter from soil and water. *J Bacteriol*. **96**: 39-42.

Baumann, P., M. Doudoroff, and R. Y. Stainer. 1968. A study of the *Moraxella* group. II. Oxidase negative species (genus *Acinetobacter*). *J Bacteriol*. **95**: 1520-1541.
Begg, E. J., and M. L. Barclay. 1995. Aminoglycosides--50 years on. *Br J Clin Pharmacol*. **39**: 597–603.

Beijerinck, M. W. 1911. Pigmenten als oxydatieproducten gevormd door bacterien. *Versl. Koninklijke Akad. Wetensch*. Amsterdam. **19**: 1092-1103.

Bellais, S., O. Mimoz, S. Leotard, A. Jacolot, O. Petitjean, and P. Nordmann. 2002. Efficacy of β-lactams for treating experimentally induced pneumonia due to a carbapenem-hydrolyzing metallo-β-lactamase-producing strain of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother*. **46**: 2032–2034.

Bergone-Bérézin, E. 2008. Importance of *Acinetobacter* spp. In Bergone-Bérézin, E., H. Friedman, and M. Bendinelli (eds). *Acinetobacter*: Biology and Pathogenesis. Springer science, New-York, Chap 1, pp. 1-18.

Berlau, J., H. Aucken, H. Malnick, and T. Pitt. 1999. Distribution of *Acinetobacter* species on skin of healthy humans. *Eur J Clin Microbiol Infect Dis*. **18**: 179–183.

Bertini, A., L. Poirel, S. Bernabeu, D. Fortini, L. Villa, P. Nordmann, and A. Carattoli. 2007. Multicopy $bla_{\text{OXA-58}}$ gene as a source of high-level resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **51**: 2324-2328.

Bogaerts, P., G. Cuzon, T. Naas, C. Bauraing, A. Deplano, B. Lissoir, P. Nordmann, and Y. Glupczynski. 2008. Carbapenem-resistant *Acinetobacter baumannii* isolates expressing the $bla_{\text{OXA-23}}$ gene associated with IS*Aba4* in Belgium. *Antimicrob Agents Chemother*. **52**: 4205-4206.

Bogaerts, P., T. Naas, I. Wybo, C. Bauraing, O. Soetens, D. Piérard, P. Nordmann, and Y. Glupczynski. 2006. Outbreak of infection by carbapenem-resistant *Acinetobacter baumannii* producing the carbapenemase OXA-58 in Belgium. *J Clin Microbiol*. **44**: 4189-4192.

Böhni, E. 1969. Chemotherapeutic activity of the combination of trimethoprim and sulfamethoxazole in infections of mice. *Postgrad Med J*. **45**: 18-21.

Bonnet, R., H. Marchandin, C. Chanal, D. Sirot, R. Labia, C. De Champs, E. Jumas-Bilak, J. Sirot. 2002. Chromosome-encoded class D beta-lactamase OXA-23 in *Proteus mirabilis*. *Antimicrob Agents Chemother*. **46**: 2004-2006.

Boo, T. W., F. Walsh, and B. Crowley. 2009. Molecular characterization of carbapenem-resistant *Acinetobacter* species in an Irish university hospital: predominance of *Acinetobacter* genomic species 3. *J Med Microbiol*. **58**: 209-216.

Bossi, L., and N. Figueroa-Bossi. 2007. A small RNA downregulates LamB maltoprotein in *Salmonella*. *Mol Microbiol*. **65**: 799-810.

Bou, G., A. Oliver, and J. Martínez-Beltrán. 2000a. OXA-24, a novel class D beta-lactamase with carbapenemase activity in an *Acinetobacter baumannii* clinical strain. *Antimicrob Agents Chemother*. **44**: 1556-1561.

Bou, G., G. Cervero, M. A. Dominguez, C. Quereda, and J. Martinez-Beltran. 2000b. Characterization of a nosocomial outbreak caused by a multiresistant *Acinetobacter baumannii* strain with a carbapenem-hydrolyzing enzyme: high-level carbapenem resistance in *A. baumannii* is not due solely to the presence of β-lactamases. *J Clin Microbiol*. **38**: 3299–3305.

Bou, G., and J. Martinez- Beltrán. 2000. Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC beta-lactamase in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **44**: 428–432.

Bouvet, P. J., and P. A. Grimont. 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 description of *Acinetobacter calcoaceticus* and *Acinetobacter lwoffii*. *Int J Syst Bacteriol*. **36**: 228-240.

Bouvet, P. J., and P. A. Grimont. 1987. Identification and biotyping of clinical isolates of *Acinetobacter*. *Ann Inst Pasteur Microbiol*. **138**: 569-578.

Bouvet, P. J. M., and O. M. M. Bouvet. 1989. Glucose dehydrogenase activity in *Acinetobacter* species. *Res Microbiol*. **140**: 531-540.

Bouvet, P. J., and S. Jeanjean. 1989. Delineation of new proteolytic genomic species in the genus *Acinetobacter*. *Res Microbiol*. **140**: 291-299.

Brade, H., and C. Galanos. 1983. Biological activities of the lipopolysaccharide and lipid A from *Acinetobacter calcoaceticus*. *J Med Microbiol*. **16**: 211–214.

Bradford., P. A. 2001. Extended-spectrum β-Lactamases in the 21st Century: Characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Infect*. **14**: 933-951

Braun, G. 2008. Virulence mechanisms in *Acinetobacter*. In Bergone-Bérézin, E., H. Friedman, and M. Bendinelli (eds). *Acinetobacter*: Biology and Pathogenesis. Springer science, New-York, Chap 8, pp 145-154.

Brisou, J. 1953. Essay on the system of the genus of the genus *Achromobacter*. *Ann Inst Pasteur* (Paris) **84**: 812-814.

Brisou, J., and A. R. Prévot. 1954. Studies on bacterial taxonomy. X. The revision of species under *Achromobacter* group. *Ann Inst Pasteur* (Paris) **86**: 722-728.

Brown, H. J., H. W. Stokes, and R. M. Hall. 1996. The integrons In0, In2, and In5 are defective transposon derivatives. *J Bacteriol*. **178**: 4429-4437.

Brown, S., and S. Amyes. 2006. OXA β-lactamases in *Acinetobacter*: the story so far. *J Antimicrob Chemother*. **57**: 1-3.

Brown, S., and S. G. B. Amyes. 2005. The sequences of seven class D β-lactamases isolated from carbapenem-resistant *Acinetobacter baumannii* from four continents. *Clin Microbiol Infect.* **11**: 326-329

Brown, S., H. K. Young, and S. G. B Amyes. 2005. Characterisation of OXA-51 a novel class D carbapenemase found in genetically unrelated clinical strains of *Acinetobacter baumannii* from Argentina. *Clin Microbiol Infect*. **11**: 15-23.

BSAC. Methods for Antimicrobial Susceptibility Testing—Version 6.1. http://www.bsac.org.uk/Resources/BSAC/version_6.1.pdf (27 September 2011, date last accessed).

Burchall, J. 1973 Mechanism of action of trimethoprim- sulfamethoxazole: II. *J Inf Dis*. **128**: S437-S441.

Bush, K. 2010. The coming of age of antibiotics: discovery and therapeutic value. *Ann N Y Acad Sci*. **1213**: 1-4.

Bush, K., G. A. Jacoby, and A. A. Medeiros. 1995. A functional classification scheme for β -lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother*. **39**: 1211-1233.

Bushby, S. R., and G.H. Hitchings. 1968. Trimethoprim, a sulphonamide potentiator. *Br J Pharmacol Chemother*. **33**: 72-90.

Castanheira, M., M. A. Toleman, R. N. Jones, F. J. Schmidt, and T. R. Walsh. 2004. Molecular characterization of a β-lactamase gene, bla_{GIM-1},encoding a new subclass of metallo-β-lactamase. *Antimicrob Agents Chemother*. **48**: 4654-4661.

Catalano, M., L. S. Quelle, P. E. Jeric, A. Di Martino, and S. M. Maimone. 1999. Survival of *Acinetobacter baumannii* on bed rails during an outbreak and during sporadic cases. *J Hosp Infect.* **42**: 27-35.

Campbell, N. A, and J. B. Reece. 2002. *Biology* (6th ed.) Benjamin Cummings, San Francisco, pp. 345-346.

Chau, S. L., Y. W. Chu, and E. T. Houang. 2004. Novel resistance-nodulation-cell division efflux system AdeDE in *Acinetobacter* genomic DNA group 3. *Antimicrob Agents Chemother.* **48**: 4054-4055.

Chen, H. Y., and D. M. Livermore. 1994. In-vitro activity of biapenem, compared with imipenem and meropenem, against *Pseudomonas aeruginosa* strains and mutants with known resistance mechanisms. *J Antimicrob Chemother*. **33**: 949-958.

Chen, S. F., W. W. Chang, Y. C. Chuang, H. H. Tsaï, N. W. Tsaï, H. W. Chang, P. Y. Lee, C. C. Chien, C. R. Huang, and T.G. Young. 2005. Adult *Acinetobacter* meningitis and its comparison with non *Acinetobacter* Gram negative bacterial meningitis. *ACTA neurol Taïwan*. **14**: 131–137.

Chen, T. L., Y. T. Lee, S. C. Kuo, P. R. Hsueh, F. Y. Chang, L. K. Siu, W. C. Ko, and C. P. Fung. 2010. Emergence and distribution of plasmids bearing the bla_{OXA-51}-like gene with an upstream IS*Aba1* in carbapenem-resistant *Acinetobacter baumannii* isolates in Taiwan. *Antimicrob Agents Chemother*. **54**: 4575-4581.

Choi, C. H., E. Y. Lee, Y. C. Lee, T. I. Park, H. J. Kim, S. H. Hyun, S. A. Kim, S. K. Lee, and J. C. Lee. 2005. Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the mitochondria and induces apoptosis of epithelial cells. *Cell Microbiol*. **7**: 1127–1138.

Choi, S. H., E. J. Choo, Y. G. Kwak, M. Y. Kim, J. B. Jun, M. N. Kim, N. J. Kim, J. Y. Jeong, Y. S. Kim, and J. H. Woo. 2006. Clinical characteristics and outcomes of bacteremia caused by *Acinetobacter* species other than *A. baumannii*: comparison with *A. baumannii* bacteremia. *J Infect Chemother*. **12**: 380-386.

Chu, Y. W., C. M. Leung, E. T. Houang, K. C. Ng, C. B. Leung, H. Y. Leung, and A. F. Cheng. 1999. Skin carriage of acinetobacters in Hong Kong. *J Clin Microbiol*. **37**: 2962–2967.

Chu, Y. W., M. Afzal-Shah, E. T. Houang, M. I. Palepou, D. J. Lyon, N. Woodford, and D. M. Livermore. 2001. IMP-4, a novel metallo-beta-lactamase from nosocomial *Acinetobacter* spp. collected in Hong Kong between 1994 and 1998. *Antimicrob. Agents Chemother*. **45**: 710–714.

Chu, Y. W., S. L. Chau, and E. T. Houang. 2006. Presence of active efflux systems AdeABC, AdeDE and AdeXYZ in different *Acinetobacter* genomic DNA groups. *J Med Microbiol*. **55**: 477-478.

Clark, R. B. 1996. Imipenem resistance among *Acinetobacter baumannii*: association with reduced expression of a 33-36 kDa outer membrane protein. *J Antimicrob Chemother*. **38**: 245-251.

Coates, A.,Y. Hu, R. Bax, and C. Page. 2002. The future challenges facing the development of new antimicrobial drugs. *Natu Rev Drug Disc*. **1**: 895-910.

Coelho, J. M., J. F. Turton, M. E. Kaufmann, J. Glover, N. Woodford, M. Warner, M. F. Palepou, R. Pike, T. L. Pitt, B. C. Patel, and D. M. Livermore. 2006. Occurrence of carbapenem-resistant *Acinetobacter baumannii* clones at multiple hospitals in London and Southeast England. *J Clin Microbiol*. **44**: 3623-3627.

Collis, C. M., M. J. Kim, S. R. Partridge, H. W. Stokes, and R. M. Hall. 2002. Characterization of the class 3 integron and the site-specific recombination system it determines. *J Bacteriol*. **184**: 3017-3026.

Conrad, R. S., and C. Galanos. 1989. Fatty acid alterations and polymyxin B binding by lipopolysaccharides from *Pseudomonas aeruginosa* adapted to polymyxin B resistance. *Antimicrob Agents Chemother*. **33**: 1724-1728.

Corrigan, K. M., N. Y. Harmis, and M. D. Willcox. 2001. Association of *Acinetobacter* species with contact lens-induced adverse responses. *Cornea*. **20**: 463-466.

Corvec, S., L. Poirel, T. Naas, H. Drugeon, and P. Nordmann. 2007. Genetics and Expression of the Carbapenem-Hydrolyzing Oxacillinase Gene bla_{OXA-23} in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **51**: 1530-1533.

Corvec, S., N. Caroff, E. Espaze, C. Giraudeau, H. Drugeon, and A. Reynaud. 2003. AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. *J Antimicrob Chemother*. **52**: 629-635.

Coudron, P. E. 2005. Inhibitor-based methods for detection of plasmid-mediated AmpC β -lactamases in *Klebsiella* spp., *E. coli*, and *P. mirabilis*. *J Clin Microbiol*. **43**: 4163-4167.

Craven, G. R., R. Gavin, and T. Fanning. 1969. The transfer RNA binding site of the 30S ribosome and the site of tetracycline inhibition. *Cold Spring Symp Quant Biol*. **34**: 129-137.

Dalla-Costa, L. M., J. M. Coelho, H. A. Souza, M. E. Castro, C. J. Stier, K. L. Bragagnolo, A. Rea-Neto, S. R. Penteado-Filho, D. M. Livermore, and N. Woodford. 2003. Outbreak of carbapenem-resistant *Acinetobacter baumannii* producing the OXA-23 enzyme in Curitiba, Brazil. *J Clin Microbiol*. **41**: 3403-3406.

Damier-Piolle, L., S. Magnet, S. Brémont, T. Lambert, and P. Courvalin. 2008. AdeIJK, a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **52**: 557-562.

Danel, F., L. M. Hall, D. Gur, and D. M. Livermore. 1997. OXA-15, an extended-spectrum variant of OXA-2 β-lactamase, isolated from a *Pseudomonas aeruginosa* strain. *Antimicrob Agents Chemother*. **41**: 785–790.

Daniel, C., S. Haentjens, M.C. Bissinger, and R. J. Courcol. 1999. Characterization of the *Acinetobacter baumannii* Fur regulator: cloning and sequencing of the fur homolog gene. *FEMS Microbiol Lett*. **170**: 199–209.

Da Silva, G. J., S. Quinteira, E. Bértolo, J. C. Sousa, L. Gallego, A. Duarte, L. Peixe, and Portugese Resistance Study Group. 2004. Long-term dissemination of an OXA-40 carbapenemase-producing *Acinetobacter baumannii* clone in the Iberian Peninsula. *J Antimicrob Chemother*. **54**: 255-258.

DeBord, G. 1939. Organisms invalidating the diagnosis of gonorrhoeae by the smear method. *J Bacteriol*. **38**: 119-120

DeBord, G. 1942. Descriptions of Mimeae trib now.with three genera and three species and two new species of *Neisseria* from conjunctivitis and vaginitis. *Iowa State Coll J Sci*. **16**: 471-480.

del Mar Tomas, M., A. Beceiro, A. Perez, D. Velasco, R. Moure, R. Villanueva, J. Martinez-Beltran, and G. Bou. 2005. Cloning and functional analysis of the gene encoding the 33-to 36-kilodalton outer membrane protein associated with carbapenem resistance in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **49**: 5172-5175.

Derrien, M., M. W. van Passel, J. H. van de Bovenkamp, R. G. Schipper, W.M. de Vos, and J. Dekker. 2010. Mucin-bacterial interactions in the human oral cavity and digestive tract. *Gut Microbes*. **1**: 254-268.

Dijkshoorn, L., E. van Aken, L. Shunburne, T. J. van der Reijden, A. T. Bernards, A. Nemec, and K. J. Towner. 2005. Prevalence of *Acinetobacter baumannii* and other *Acinetobacter* spp. in faecal samples from non-hospitalised individuals. *Clin Microbiol Infect*. **11**: 329–332.

Diancourt, L., V. Passet, A. Nemec, L. Dijkshoorn and S. Brisse S. 2010. The population structure of *Acinetobacter baumannii*: expanding multiresistant clones from an ancestral susceptible genetic pool. *PLoS One* **5**: e10034.

Dijkshoorn, L., A. Nemec, and H. Seifert. 2007. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. *Nat Rev Microbiol*. **5**: 939-951.

Dixon, R. A., and I. Chopra. 1986. Polymyxin B and polymyxin B nonapeptide alter cytoplasmic membrane permeability in *Escherichia coli*. *J Antimicrob Chemother*. **18**: 557-563.

Docobo-Pérez, F., P. Nordman, J. Domínguez-Herrera, R. López-Rojas, Y. Smani, L. Poirel, and J. Pachón. 2012. Efficacies of colistin and tigecycline in mice with experimental pneumonia due to NDM-1-producing strains of *Klebsiella pneumoniae* and *Escherichia coli*. *Int J Antimicrob Agents*. **39**: 251-254.

Doi, Y., J. M. Adams, K. Yamane, and D. L. Paterson. 2007. Identification of 16S rRNA methylase-producing *Acinetobacter baumannii* clinical strains in North America. *Antimicrob Agents Chemother*. **51**: 4209-4210.

Dolzani, L., E. Tonin, and C. Lagatolla. 1995. Identification of Acinetobacter isolates in the *A. calcoaceticus-A. baumannii* complex by restriction analysis of the 16S-23S rRNA intergenic spacer sequences. *J Clin Microbiol*. **33**: 1108–1113.

Donald, H. M., W. Scaife, S. G. Amyes and H. K. Young. 2000. Sequence analysis of ARI-1, a novel OXA β-lactamase, responsible for imipenem resistance in *Acinetobacter baumannii* 6B92. *Antimicrob Agents Chemother*. **44**: 196–199.

Dorsey, C. W., P. A. Tomaras, P. L. Connerly, M. E. Tolmasky, J. H. Crosa, and L. A. Actis. 2004. The siderophore-mediated iron acquisition systems of *Acinetobacter baumannii* ATCC 19606 and *Vibrio anguillarum* 775 are structurally and functionally related. *Microbiology.* **150**: 3657-3667.

Drlica, K., and X. Zhao. 1997. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol Mol Biol R.* **61**: 377-392.

Duggar, B. M. 1948. Aureomycin; a product of the continuing search for new antibiotics. *Ann N Y Acad Sci*. **51**: 177-181.

Dupont, M., Pages, J. M., Lafitte, D., Siroy, A. and Bollet, C. 2005. Identification of an OprD homologue in *Acinetobacter baumannii*. *J Proteome Res*. **4**: 2386-2390.

Durán, M., A. Faljoni-Alario, and N. Durán. 2010. *Chromobacterium violaceum* and Its Important Metabolites – review. *Folia Microbiol*. **55**: 535–547.

Echenique, J. R., H. Arienti, M. E. Tolmasky, R. R. Read, R. J. Staneloni, J. H. Crosa, and L.A. Actis. 1992. Characterization of a high-affinity iron transport system in *Acinetobacter baumannii*. *J Bacteriol*. **174**: 7670–7679.

Ellington, M. J., J. Kistler, D.M. Livermore, and N. Woodford. 2007. Multiplex PCR for rapid detection of genes encoding acquired metallo-β-lactamases. *J Antimicrob Chemother*. **59**: 321–322.

Ennis, D. M., and C. G. Cobbs. 1995. The newer cephalosporins: aztreonam and imipenem. *Infect Dis Clin North Am*. **95**: 687-713.

Erridge, C., O. L. Moncayo-Nieto, R. Morgan, M. Young, and I. R. Poxton. 2007. *Acinetobacter baumannii* lipopolysaccharides are potent stimulators of human monocyte activation via Toll-like receptor 4 signalling. *J Med Microbiol*. **56**: 165-171.

Escoubas, J. M., M. F Prère, O. Fayet, I. Salvignol, D. Galas, D. Zerbib, and M. Chandler. 1991. Translational control of transposition activity of the bacterial insertion sequence IS*1*. *EMBO J*. **10**: 705-712.

Evans, B. A., A. Hamouda, K. J. Towner and S. G. B. Amyes. 2008. OXA-51-like β-lactamases and their association with particular epidemic lineages of *Acinetobacter baumannii*. *Clin Microbiol Infect*. **14**: 268-275.

Evans, B. A., A. Hamouda, K. J. Towner, and S. G. Amyes. 2010. Novel genetic context of multiple $bla_{\text{OXA-58}}$ genes in *Acinetobacter* genospecies 3. *J Antimicrob Chemother*. **65**: 1586-1588.

Evans, B. A., S. Brown, A. Hamouda, J. Findlay, and S. G. B. Amyes. 2007. Eleven novel OXA-51-like enzymes from clinical isolates of *Acinetobacter baumannii*. *Clin Microbiol Infect.* **13**: 1137-1138.

Fagon, J. Y., J. Chastre, Y. Domart, J. L. Trouillet, J. Pierre, C. Darne, and C. Gibert. 1989. Nosocomial pneumonia in patients receiving continuous mechanical ventilation. Prospective analysis of 52 episodes with use of a protected specimen brush and quantitative culture techniques. *Am Rev Respir Dis*. **139**: 877-884.

Falagas, M. E., A. P. Grammatikos, and A. Michalopoulos. 2008. Potential of old-generation antibiotics to address current need for new antibiotics. *Expert Rev Anti Infect Ther*. **6**: 593–600.

Falagas, M. E., I. A. Bliziotis, and I. I. Siempos. 2006. Attributable mortality of *Acinetobacter baumannii* infections in critically ill patients: a systematic review of matched cohort and case-control studies. *Crit Care*. **10**: R48.

Fernandez-Cuenca, F., L. Martinez-Martinez, M. C. Conejo, J. A. Ayala, E. J. Perea, and A. Pascual. 2003. Relationship between beta-lactamase production, outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates of *Acinetobacter baumannii*. *J Antimicrob Chemother*. **51**: 565-574.

Figueiredo, S., L. Poirel, and H. Seifert. 2010. OXA-134, a naturally-occurring carbapenem-hydrolyzing class D β-lactamase from *Acinetobacter lwoffii*. *Antimicrob Agents Chemother* **54**: 5372-5375.

Figueiredo, S., L. Poirel, J. Croize, C. Recule, and P. Nordmann. 2009. In vivo selection of reduced susceptibility to carbapenems in *Acinetobacter baumannii* related to IS*Aba1*-mediated overexpression of the natural $bla_{\text{OXA-66}}$ oxacillinase gene. *Antimicrob. Agents Chemother*. **53**: 2657–2659.

Findlay, J., A. Hamouda, S. J. Dancer, and S. G. B. Amyes. 2012. Rapid acquisition of decreased carbapenem susceptibility in a strain of *Klebsiella pneumoniae* arising during meropenem therapy. *Clin Microbiol Infect*. **18**: 140-146.

Fontana, R., G. Cornaglia, M. Ligozzi, and A. Mazzariol. 2000. The final goal: penicillin-binding proteins and the target of cephalosporins. *Clin Microbiol Infect*. **6**: 34-40.

Fournier, P. E., D. Vallenet, V. Barbe, S. Audic, H. Ogata, L. Poirel, H. Richet, C. Robert, S. Mangenot, C. Abergel, P. Nordmann, J. Weissenbach, D. Raoult, and J. M. Claverie. 2006. Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. *PLoS Genet*. **2**: e7.

Francey, T., F. Gaschen, J. Nicolet, and A. P. Burnens. 2000. The role of *Acinetobacter baumannii* as a nosocomial pathogen for dogs and cats in an intensive care unit. *J Vet Intern Med*. **14**: 177-183.

Frere, J. M. 1995. β-lactamases and bacterial resistance to antibiotics. *Mol Microbiol*. **16**: 385-395.

Freter, C. E., and M. C. Perry. 2008. Principles of Chemotherapy. In Perry M. C (ed.) The chemotherapy source book. Lippincott, Williams and Wilkins press, USA, Chap 4, pp. 30-36

Fu, Y., J. Zhou, H. Zhou, Q. Yang, Z. Wei, Y. Yu, and L. Li. 2010. Wide dissemination of OXA-23-producing carbapenem-resistant *Acinetobacter baumannii* clonal complex 22 in multiple cities of China. *J Antimicrob Chemother*. **65**: 644-650.

Gaddy, J. A., A. P. Tomaras, and L. A. Actis. 2009. The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infect Immun*. **77**: 3150-3160.

Garcia-Garmendia, J. L., C. Ortiz-Leyba, J. Garnacho-Montero, F. J. Jimenez-Jimenez, C. Perez-Paredes, A. E. Barrero-Almodovar, and M. Gili-Miner. 2001. Risk factors for *Acinetobacter baumannii* nosocomial bacteremia in critically ill patients: a cohort study. *Clin Infect Dis*. **33**: 939-946.

Gaynes, R., and J. R. Edwards. 2005. Overview of nosocomial infections caused by gram-negative bacilli. *Clin Infect Dis*. **41**: 848-854.

Georgopapadakou, N. H., S. A. Smith, and R.B. Sykes. 1982. Mode of action of aztreonam. *Antimicrob Agents Chemother*. **21**: 950-956.

Gin, A., L. Dilay, J. A. Karlowsky, A. Walkty, E. Rubinstein, and G.G. Zhanel. 2007. Piperacillin-tazobactam: a beta-lactam/beta-lactamase inhibitor combination. *Expert Rev Anti Infect Ther*. **5**: 365-383.

Girlich, D., T. Naas, and P. Nordmann. 2004. Biochemical characterization of the naturally occurring oxacillinase OXA-50 of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. **48**: 2043-2048.

Glew, R. H., R. C. Moellering, Jr., and L. J. Kunz. 1977. Infections with *Acinetobacter calcoaceticus* (*Herellea vaginicola*): clinical and laboratory studies. *Medicine* (Baltimore) **56**: 79-97.

Goel, V. K., and A. Kapil. 2001. Monoclonal antibodies against the iron regulated outer membrane proteins of *Acinetobacter baumannii* are bactericidal. *BMC Microbiol*. **1**: 16.

Gonzalez, R. H., A. Nusblat, and B. C. Nudel. 2001. Detection and characterization of quorum sensing signal molecules in Acinetobacter strains. *Microbiol Res*. **155**: 271-277.

Gordon, N. C., and D. W. Wareham. 2010. Multidrug-resistant *Acinetobacter baumannii*: mechanisms of virulence and resistance. *Int J Antimicrob Agents*. **35**: 219-226

Gospodarek, E., A. Grzanka, Z. Dudziak, and J. Domaniewski. 1998. Electron-microscopic observation of adherence of *Acinetobacter baumannii* to red blood cells. *Acta Microbiol Pol*. **47**: 213-217.

Grener-Smidt, P. 1992. Ribotyping of the *Acinetobacter calcoaceticus- Acinetobacter baumannii* complex. *J Clin Microbiol*. **30**: 2680-2685.

Grener-Smidt, P., I. Tjernberg, and J. Ursing. 1991. Reliability of phenotypic tests for identification of *Acinetobacter* species. *J Clin Microbiol*. **29**: 227-282.

Gribun, A., Y. Nitzan, I. Pechatnikov, G. Hershkovits, and D. J. Katcoff. 2003. Molecular and structural characterization of the HMP-AB gene encoding a pore-forming protein from a clinical isolate of *Acinetobacter baumannii*. *Curr Microbiol*. **47**: 434-443.

Griffith, R. S., and H. R. Black.1964. Cephalothin- a new antibiotic. *JAMA*. **189**: 823-828.

Grotiuz, G., A. Sirok, P. Gadea, G. Varela, and F. Schelotto. 2006. Shiga toxin 2-producing *Acinetobacter haemolyticus* associated with a case of bloody diarrhea. *J Clin Microbiol*. **44**: 3838-3841.

Gu, B., M. Tong, W. Zhao, G. Liu, M. Ning, S. Pan, and W. Zhao. 2007. Prevalence and characterization of class I integrons among *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolates from patients in Nanjing, China. *J Clin Microbiol*. **45**: 241-243.

Guardabassi, L., L. Dijkshoorn, J. M. Collard, J. E. Olsen, and A. Dalsgaard. 2000. Distribution and in-vitro transfer of tetracycline resistance determinants in clinical and aquatic *Acinetobacter* strains. *J Med Microbiol*. **49**: 929-936.

Gur, D., V. Korten, S. Unal, L. M. Deshpande, and M. Castanheira. 2008. Increasing carbapenem resistance due to the clonal dissemination of oxacillinase (OXA-23 and OXA-58)-producing *Acinetobacter baumannii*: report from the Turkish SENTRY Program sites. *J Med Microbiol*. **57**: 1529-1532.

Hall, R. M., and C. M. Collis. 1998. Antibiotic resistance in gram-negative bacteria: the role of gene cassettes and integrons. *Drug Resist Updat*. **1**: 109-119.

Hamouda, A., and S. G. Amyes. 2004. Novel *gyrA* and *parC* point mutations in two strains of *Acinetobacter baumannii* resistant to ciprofloxacin. *J Antimicrob Chemother*. **54**: 695-696.

Han, C. G., Y. Shiga, T. Tobe, C. Sasakawa, and E. Ohtsubo. 2001. Structural and functional characterization of IS*679* and IS*66*-family elements. *Antimicrob Agents Chemother*. **183**: 4296-4304.

Haren, L., M. Betermier, P. Polard, and M. Chandler. 1997. IS*911*-mediated intramolecular transposition is naturally temperature sensitive. *Mol. Microbiol*. **25**: 531–540.

Harrison, C. J., and D.Bratcher. 2008. Cephalosporins: A Review. *Ped in Rev*. **29**: 264-273.

Haseley, S. R., and S.G.Wilkinson. 1998. Structure of the O-7 antigen from *Acinetobacter baumannii*. *Carbohydr Res*. **306**: 257-263.

Henwood, C. J., T. Gatward, M. Warner, D. James, M. W. Stockdale, R. P. Spence, K. J. Towner, D. M. Livermore, and N. Woodford. 2002. Antibiotic resistance among clinical isolates of Acinetobacter in the UK, and in vitro evaluation of tigecycline (GAR-936). *J Antimicrob Chemother*. **49**: 479-487.

Héritier, C., A. Dubouix, L. Poirel, N. Marty, and P. Nordmann. 2005a. A nosocomial outbreak of *Acinetobacter baumannii* isolates expressing the carbapenem-hydrolysing oxacillinase OXA-58. *J Antimicrob Chemother*. **55**: 115-118.

Héritier, C., L. Poirel, D. Aubert, and P. Nordmann. 2003. Genetic and functional analysis of the chromosome-encoded carbapenem-hydrolyzing oxacillinase OXA-40 of *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **47**: 268-273.

Héritier, C., L. Poirel, P. E. Fournier,J. M. Claverie, D. Raoult, and P. Nordmann. 2005b. Characterization of the naturally occurring oxacillinase of *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **49**: 4174-4179.

Héritier, C., L. Poirel, and P. Nordmann. 2006. Cephalosporinase overexpression resulting from insertion of IS*Aba1* in *Acinetobacter baumannii*. *Clin Microbiol Infect*. **12**: 123-130.

Héritier, C., L. Poirel, T. Lambert, and P. Nordmann. 2005c. Contribution of acquired carbapenem-hydrolyzing oxacillinases to carbapenem resistance in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **49**: 3198-3202.

Higgins, P. G., H. Wisplinghoff, D. Stefanik, and H. Seifert. 2004. Selection of topoisomerase mutations and overexpression of *adeB* mRNA transcripts during an outbreak of *Acinetobacter baumannii*. *J Antimicrob Chemother*. **54**: 821-823.

Higgins, P. G., L. Poirel, M. Lehmann, P. Nordmann, and H. Seifert. 2009. OXA-143, a novel carbapenem-hydrolyzing class D beta-lactamase in *Acinetobacter baumannii*. *Antimicrob Agents Chemother.* **53**: 5035-5038.

Higgins, P. G., M. Lehmann, and H. Seifert. 2010. Inclusion of OXA-143 primers in a multiplex polymerase chain reaction (PCR) for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. *Int J Antimicrob Agents*. **35**: 305

Hirakata, Y., R. Srikumar, K. Poole, N. Gotoh, T. Suematsu, S. Kohno, S. Kamihira, R. E. Hancock, and D. P. Speert. 2002. Multidrug efflux systems play an important role in the invasiveness of *Pseudomonas aeruginosa*. *J Exp Med*. **196**: 109-118.

Hooper, D. C. 2005. Efflux pumps and nosocomial antibiotic resistance: a primer for hospital epidemiologists. *Clin Infect Dis*. **40**: 1811–1817.

Houang, E. T., Y. W. Chu, C. M. Leung, K. Y. Chu, J. Berlau, K. C. Ng, and A. F. Cheng. 2001. Epidemiology and infection control implications of *Acinetobacter* spp. in Hong Kong. *J Clin Microbiol*. **39**: 228–234.

Houang, E. T. S., R. T. Sormunen, L. Lai, C. Y. Chan, and A. S. Y. Leong. 1998. Effect of desiccation on the ultrastructural appearances of *Acinetobacter baumannii* and *Acinetobacter lwoffii*. *J Clin Microbiol*. **51**: 786-788.

Hujer, K. M., A. M. Hujer, E.A. Hulten, S. Bajaksouzian, J. M. Adams, C. J. Donskey, D. J. Ecker, C. Massire, M. Eshoo, R. Sampath, J. M. Thomson, P. N. Rather, D. W. Craft, J. T. Fishbain, A. J. Ewell, M. R. Jacobs, D. L. Paterson, and R. A. Bonomo. 2006. Analysis of antibiotic resistance genes in multidrug resistant *Acinetobacter* sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. *Antimicrob Agents Chemother*. **50**: 4114–4123.

Hujer, K. M., N. S. Hamza, A. M. Hujer, F. Perez, M. S. Helfand, C. R. Bethel, J. M. Thomson, V. E. Anderson, M. Barlow, L. B. Rice , F. C. Tenover, and R. A. Bonomo. 2005. Identification of a new allelic variant of the *Acinetobacter baumannii* cephalosporinase, ADC-7 beta-lactamase: defining a unique family of class C enzymes. *Antimicrob Agents Chemother*. **49**: 2941-2948.

Hunt, D. E., and H. J, Sandham. 1969. Improved Agar Gradient-Plate Technique. *Applied Microbiology*, **17.2**: 329-330

Jacoby, G.A. 2009. AmpC- β- lactamases. *Clin Microbiolo Rev*. **22**: 161-182.

Jawad, A., A.M. Snelling, J. Heritage, and P. M. Hawkey. 1998. Exceptional desiccation tolerance of *Acinetobacter radioresistens*. *J Hosp Infect*. **39**: 235-240.

Jawad, A., J. Heritage, A. M. Snelling, D. M. Gascoyne-Binzi, and P. M. Hawkey. 1996. Influence of relative humidity and suspending menstrua on survival of *Acinetobacter* spp. on dry surfaces. *J Clin Microbiol*. **34**: 2881-2887.

Jeon, B. C., S. H. Jeong, I. K. Bae, S. B. Kwon, K. Lee, D. Young, J. H. Lee, J. S. Song, and S.H. Lee. 2005. Investigation of a nosocomial outbreak of imipenem-resistant *Acinetobacter baumannii* producing the OXA-23 beta-lactamase in Korea. *J Clin Microbiol*. **43**: 2241-2245.

Johnson, E. N., T. C. Burns, R. A. Hayda, D. R. Hospenthal, and C. K. Murray. 2007. Infectious complications of open type III tibial fractures among combat casualties. *Clin Infect Dis*. **45**: 409-415.

Joly, E. 1996. Preparation of plasmid DNA using alkaline lysis. In A Harwood (ed). Methods in Molecular Biology Vol 58, Basic DNA and RNA Protocols, Humana Press Inc, Totowa, NJ. Chap 31, pp. 257-263.

Joly-Guillou, M. L. 2005. Clinical impact and pathogenicity of *Acinetobacter*. *Clin Microbiol Infect*. **11**: 868-873.

Joly-Guillou, M. L. 2008. Nosocomial and Community-acquired *Acinetobacter* Infections. In Bergone-Bérézin, E., H. Friedman, and M. Bendinelli (eds). *Acinetobacter*: Biology and Pathogenesis. Springer science, New-York, Chap 9, pp 155-165

Joly-Guillou, M. L., and M. Wolff. 2008. Experimental models of *Acinetobacter* infection. In Bergone-Bérézin, E., H. Friedman, and M. Bendinelli (eds). *Acinetobacter*: Biology and Pathogenesis. Springer science, New-York, Chap 10, pp. 167-174.

Juni, E. 2005. Genus II *Acinetobacter* Brisou and Prévot 1954. In Brenner, D. J., N. R. Krieg, and J. T. Stanely (eds). Bergey's Manual of Systematic Bacteriology. East Lansing: Bergey's Manual Trust, pp 425-437.

Kahan, J. S., F.M. Kahan, R. Goegleman, S.A. Currie, M. Jackson, E.O. Stapley, T.W. Miller, A.K. Miller, D. Hendlin, S. Mochales, S. Hernandez, H. B. Woodruff, and J. Birnbaum. 1979. Thienamycin, a new β-lactam antibiotic. I: discovery, taxonomy, isolation and physical properties. *J Antibiot*. **32**: 1-12.

Kanatani, M. S., and B. J Guglielmo. 1994. The new macrolides. Azithromycin and clarithromycin. *West J Med*. **160**: 31-37.

Karthikeyan, K., M. A. Thirunarayan, and P. Krishnan. 2010. Coexistence of $bla_{\text{OXA-23}}$ with $bla_{\text{NDM-1}}$ and *armA* in clinical isolates of *Acinetobacter baumannii* from India. *J Antimicrob Chemother* **65**: 2253-2254

Kato, N., K. Yamazoe, C. G. Han, and E. Ohtsubo. 2003. New insertion sequence elements in the upstream region of *cfiA* in imipenem-resistant *Bacteroides fragilis* strains. *Antimicrob Agents Chemother*. **47**: 979–985

Kees, F., and H. Grobecker. 1995. Systematics of beta-lactams: chemical properties and structure activity relationship of oral cephalosporins. *Antibiot Chemother*. **47**: 1-7.

Kim, C. K., Y. Lee, H. Lee, G. J. Woo, W. Song, M. N. Kim, W. G. Lee, S.H. Jeong, K. Lee, and Y. Chong. 2010. Prevalence and diversity of carbapenemases among imipenem-nonsusceptible *Acinetobacter* isolates in Korea: emergence of a novel OXA-182. *Diagn Microbiol Infect Dis*. **68**: 432-438.

Kim, J. W., S. T. Heo, J. S. Jin, C.H. Choi, Y.C. Lee, Y.G. Jeong, S. J. Kim, and J. C. Lee. 2008. Characterization of *Acinetobacter baumannii* carrying $bla_{\text{OXA-23}}$, $bla_{\text{PER-1}}$ and *armA* in a Korean hospital. *Clin Microbiol Infect*. **14**: 716-718.

Knapp, S., C. W. Wieland, S. Florquin, R. Pantophlet, L. Dijkshoorn, N. Tshimbalanga, S. Akira, and T. van der Poll. 2006. Differential roles of CD14 and Toll-like receptors 4 and 2 in murine *Acinetobacter* pneumonia. *Am J Respir Crit Care Med*. **173**: 122–129.

Koch, A. L. 2000. Penicillin binding proteins, beta-lactams, and lactamases: offensives, attacks, and defensive countermeasures. *Crit Rev Microbiol*. **26**: 205-220.

Koeleman, J. G., J. Stoof, M. W. van der Bijl, C. M. Vandenbroucke-Grauls, and P. H. Savelkoul. 2001. Identification of epidemic strains of *Acinetobacter baumannii* by integrase gene PCR. *J Clin Microbiol*. **39**: 8-13.

Koh, T. H., L. H. Sng, G. C. Wang, L. Y. Hsu, and Y. Zhao. 2007. IMP-4 and OXA β-lactamases in *Acinetobacter baumannii* from Singapore. *J Antimicrob Chemother*. **59**: 627-632.

Kohonen, A., and J. Tarkanen. 1969. Cochlear damage from ototoxic antibiotics by intratympanic application. *Acta Otolaryngol*. **68**: 90-97.

Kollef , M. H. 2009. New antimicrobial agents for methicillin- resistant *Staphylococcus aureus*. *Crit Care Resusc*. **11**: 282–286.

Koomanachai, P., A. Kim, and D. P. Nicolau. 2009. Pharmacodynamic evaluation of tigecycline against Acinetobacter baumannii in a murine pneumonia model. *J Antimicrob Chemother*. **63**: 982-987.

Kumagai, T., S. Tamai, T. Abe, and M. Hikida. 2002. Current status of oral carbapenem development. *Current Medicinal Chemistry Anti Infective Agents*. **1**: 1-14.

Labeda, D. P. 1987. Transfer of the type strain of *Streptomyces erythraeus* (Waksman 1923) Waksman and Henrici 1948 to the Genus *Saccharopolyspora* Lacey and Goodfellow 1975 as *Saccharopolyspora erythraea* sp. nov., and Designation of a Neotype Strain for *Streptomyces erythraeus*. *Int J Sys Bacteriol*. **37**: 19-22.

La Scola, B., and D. Raoult. 2004. *Acinetobacter baumannii* in human body louse. *Emerg Infect Dis*. **10**: 1671–1673.

La Scola, B., V. A. K. B. Gundi, A. Khamis, and D. Raoult. 2006. Sequencing of the *rpoB* gene and flanking spacers for molecular identification of *Acinetobacter* species. *J Clin Microbiol*. **44**: 827-832.

Lee, H., D. Yong, J. H. Yum, K. H. Roh, K. Lee, K. Yamane, Y. Arakawa, and Y. Chong. 2006a. Dissemination of 16S rRNA methylase-mediated highly amikacin-resistant isolates of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in Korea. *Diagn Microbiol Infect Dis*. **56**: 305–312.

Lee, J. C., H. Koerten, P. van den Broek, H. Beekhuizen, R. Wolterbeek, M. van den Barselaar, T. van der Reijden, J. van der Meer, J. van de Gevel, and L. Dijkshoorn. 2006b. Adherence of *Acinetobacter baumannii* strains to human bronchial epithelial cells. *Res Microbiol*. **157**: 360–366.

Lee, J. S., C.H. Choi., J. W. Kim, and J. C. Lee. 2010. *Acinetobacter baumannii* outer membrane protein A induces dendritic cell death through mitochondrial targeting. *J Microbiol*. **48**: 387-392.

Lee, K., J. H. Yum, D. Yong, H. M. Lee, H. D. Kim, J. D. Docquier, G. M. Rossolini, and Y. Chong. 2005. Novel acquired metallo-beta-lactamase gene, $bla_{\text{SIM-1}}$, in a class 1 integron from *Acinetobacter baumannii* clinical isolates from Korea. *Antimicrob. Agents Chemother*. **49**: 4485–4491.

Lee, K., M. N. Kim, T. Y. Choi, S. E. Cho, S. Lee, D. H. Whang, D. Yong, Y. Chong, N. Woodford, D. M. Livermore, and the KONSAR Group. 2009a. Wide dissemination of OXA-type carbapenemases in clinical *Acinetobacter* spp. isolates from South Korea. *Int J Antimicrob Agents*. **33**: 520–524.

Lee, K., W. G. Lee, Y. Uh, G. Y. Ha, J. Cho, and Y. Chong. 2003. VIM- and IMP-type metallo-beta-lactamase-producing *Pseudomonas* spp. and *Acinetobacter* spp. in Korean hospitals. *Emerg Infect Dis*. **9**: 868–871.

Lee, N. Y., T. C. Chang, C. J. Wu, C. M. Chang, H. C. Lee, P. L. Chen, C. C. Lee, Nai.Ying Ko, and W. C, Ko. 2010. Clinical manifestations, antimicrobial therapy, and prognostic factors of monomicrobial *Acinetobacter baumannii* complex bacteremia. *J Inf*. **61**: 219-227.

Lee, Y. T., J. F. Turton, T. L. Chen, R. C. Wu, W. C. Chang, C. P. Fung, C. P. Chen, W. L. Cho, L. Y. Huang, and L. K. Siu. 2009b. First identification of $bla_{\text{OXA-51}}$-like in non-baumannii *Acinetobacter* spp. *J Chemother*. **21**: 514-520.

Lemoigne, M., H. Girad, and G. Jacobelli. 1952. Soil bacteria easily utilizing 2-3, butanediol. *Ann. Inst. Pasteur* (Paris) **82**:389-398

Leung, W. S., C. M. Chu, K. Y. Tsang, F. H. Lo, K. F. Lo, and P. L. Ho. 2006. Fulminant community-acquired *Acinetobacter baumannii* pneumonia as a distinct clinical syndrome. *Chest*. **129**: 102–109.

Lévesque, C., L. Piché, C. Larose, and P. H. Roy. 1995. PCR mapping of integrons reveals several novel combinations of resistance genes. *Antimicrob Agents Chemother*. **39**: 185-191

Li, J., C. R. Rayner, R. L. Nation, R. J. Owen, D. Spelman, K. E. Tan, and L. Liolios. 2006a. Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **50**: 2946-2950.

Li, J., R. L. Nation, J. D. Turnidge, R. W. Milne, K. Coulthard, C. R. Rayner,and D. L. Paterson. 2006b. Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet Infect Dis*. **6**: 589-601.

Lim, Y. M., K. S. Shin, and J. Kim. (2007). Distinct antimicrobial resistance patterns and antimicrobial resistance-harboring genes according to genomic species of *Acinetobacter* isolates. *J Clin Microbiol*. **45**: 902-905.

Limansky, A. S., M. A. Mussi, and A. M. Viale. 2002. Loss of a 29-kilodalton outer membrane protein in *Acinetobacter baumannii* is associated with imipenem resistance. *J Clin Microbiol*. **40**: 4776–4778.

Lin, L., B. D. Ling, and X. Z. Li. 2009. Distribution of the multidrug efflux pump genes, *adeABC*, *adeDE* and *adeIJK*, and class 1 integron genes in multiple-antimicrobial-resistant clinical isolates of *Acinetobacter baumannii–Acinetobacter calcoaceticus* complex. *Int J Antimicrob Agents*. **33**: 27-32.

Lin, R. Y. 1992. A perspective on penicillin allergy. *Arch Intern Med*. **152**: 930-937.

Livermore, D. M. 1995. β-Lactamases in Laboratory and Clinical Resistance. *Clin Microbiol Rev*. **8**: 557-584.

Livermore, D. M, and D. F. J. Brown. 2001 Detection of β-lactamase-mediated resistance. *J Antimicrob Chemother*. **48**: 59-64.

Livermore, D. M., and N. Woodford. 2006. The beta-lactamase threat in Enterobacteriaceae, *Pseudomonas* and *Acinetobacter*. *Trends Microbiol*. **14**: 413-420.

Livermore, D. M., S. Mushtaq, and M. Warner. 2009. Activity of the anti-MRSA carbapenem razupenem (PTZ601) against Enterobacteriaceae with defined resistance mechanisms. *J Antimicrob Chemother*. **64**: 330-335.

Lopes, B., A. Hamouda, S. Amyes. 2011. The role of IS*30* in the expression of the bla_{ADC} gene in *Acinetobacter baumannii*. 21st ECCMID / 27th ICC, 7th-10th May 2011. Milano: Italy.

Lopes, B. S., A. Hamouda, J. Findlay, and S. G. B. Amyes. 2010. Effect of frame-shift mutagen acriflavine on control of resistance genes in *Acinetobacter baumannii. J Med Microbiol*. **60**: 211-15.

Lopes, B. S., B. A. Evans, and S. G. B. Amyes. 2012. Disruption of the $bla_{OXA-51-like}$ gene by IS*Aba16* and activation of the bla_{OXA-58} gene leading to carbapenem resistance in *Acinetobacter baumannii* Ab244. *J Antimicrob Chemother*. **67**: 59-63.

Lopez-Otsoa, F., L. Gallego, K. J. Towner, L. Tysall, N. Woodford, and D. M. Livermore. 2002. Endemic carbapenem resistance associated with OXA-40 carbapenemase among *Acinetobacter baumannii* isolates from a hospital in northern Spain. *J Clin Microbiol*. **40**: 4741-4743.

Magnet, S., P. Courvalin, and T. Lambert. 2001. Resistance-nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *Acinetobacter baumannii* strain BM4454. *Antimicrob Agents Chemother*. **45**: 3375-3380.

Mahillon, J., and M. Chandler. 1998. Insertion Sequences. *Microbiol Mol Biol Rev*. **62**: 725-774.

Mammeri, H., L. Poirel, N. Mangeney, and P. Nordmann. 2003. Chromosomal integration of a cephalosporinase gene from *Acinetobacter baumannii* into *Oligella urethralis* as a Source of Acquired Resistance to β-Lactams. *Antimicrob Agents Chemother*. **47**: 1536-1542 .

Mannheim, W., and W. Stenzel. 1962. Zur Systematik der obligate aeroben gram-negativen Diplobakterien des Menschen. *Zentralbl Bakteriol*. **198**: 55-83.

Marchaim, D., S. Navon-Venezia, D. Schwartz , J. Tarabeia , I. Fefer, M. J. Schwaber, and Y. Carmeli. 2007. Surveillance cultures and duration of carriage of multi-drug-resistant *Acinetobacter baumannii*. *J Clin Microbiol*. **45**: 1551–1555.

Marchand, I., Damier-Piolle, L., Courvalin, P. and Lambert, T. 2004. Expression of the RND-type efflux pump AdeABC in *Acinetobacter baumannii* is regulated by the AdeRS two-component system. *Antimicrob Agents Chemother*. **48**: 3298-3304.

Marqué, S., L. Poirel, C. Héritier, S. Brisse, M. D. Blasco, R. Filip, G. Coman, T. Naas, and P. Nordmann. 2005. Regional occurrence of plasmid-mediated carbapenem-hydrolyzing oxacillinase OXA-58 in *Acinetobacter* spp. in Europe. *J Clin Microbiol*. **43**: 4885-4888.

Marti, S., F. Fernandez-Cuenca, A. Pascual, A. Ribera, J. Rodriguez-Bano, G. Bou, J. Miguel Cisneros, J. Pachon, L. Martinez-Martinez, and J. Vila. 2006. Prevalence of the *tetA* and *tetB* genes as mechanisms of resistance to tetracycline and minocycline in *Acinetobacter baumannii* clinical isolates. *Enferm Infecc Microbiol Clin*. **24**: 77-80.

Mazel, D. 2006. Integrons: agents of bacterial evolution. *Nat Rev Microbiol*. **4**: 608-620.

McClintock, B. 1950. The origin and behavior of mutable loci in maize. *Proc Natl Acad Sci U S A.* **36**: 344–355.

McDonald, L. C., S. N. Banerjee, and W. R. Jarvis. 1999. Seasonal variation of *Acinetobacter* infections: 1987–1996. Nosocomial Infections Surveillance System. *Clin. Infect. Dis*. **29**:1133–1137.

McGuire, J. M., P. L. Bunch, R. C. Anderson, H. E. Boaz, E.H. Flynn, E. H. Powell, J.W. Smith.1952. Ilotycin, a new antibiotic. *Antibiot Chemother*. **2**: 281-283

Meagher, A. K., P. G. Ambrose, T. H. Grasela and E. J. Ellis-Grosse. 2005. Pharmacokinetic/pharmacodynamic profile for tigecycline–a new glycylcycline antimicrobial agent. *Diagn Microbiol Infect Dis*. **52**: 165-171

Mendes, R. E., J. M. Bell, J. D. Turnidge, M. Castanheira, L. M. Deshpande, R. N. Jones. 2009. Codetection of *bla*$_{\text{OXA-23}}$-like gene *bla*$_{\text{OXA-133}}$ and *bla*$_{\text{OXA-58}}$ in *Acinetobacter radioresistens*: report from the SENTRY antimicrobial surveillance program. *Antimicrob Agents Chemother*. **53**: 843-844.

Merkier, A. K., and D. Centrón. 2006. *bla*$_{\text{OXA-51}}$-type beta-lactamase genes are ubiquitous and vary within a strain in *Acinetobacter baumannii*. *Int J Antimicrob Agents*. **28**: 110-113.

Merkier, A. K., M. Catalano, M. S. Ramírez, C. Quiroga, B. Orman, L. Ratier, A. Famiglietti, C. Vay, A. Di Martino, S. Kaufman, and D. Centrón. 2008. Polyclonal spread of *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-58}}$ in *Acinetobacter baumannii* isolates from Argentina. *J Infect Dev Ctries*. **2**: 235-240.

Metan, G., E. Alp, B. Aygen, and B. Sumerkan. 2007. Carbapenem-resistant *Acinetobacter baumannii*: an emerging threat for patients with post-neurosurgical meningitidis. *Int J Antimicrob Agents*. **29**: 112–116.

Miller J. 2005. *Acinetobacter* as a causative agent in preseptal cellulitis. *Optometry*.**76**: 176-80.

Moellering, R.C Jr. 1983. In vitro antibacterial activity of the aminoglycoside antibiotics. *Rev Infect Dis*. **5** (Suppl):S212-S232.

Mugnier, P. D., L. Poirel, and P. Nordmann. 2009. Functional analysis of insertion sequence IS*Aba1*, responsible for genomic plasticity of *Acinetobacter baumannii*. *J Bacteriol*. **191**: 2414-18.

Murray, C., P. T. Scott, K. A. Moran, and D. W. Craft. 2008. US Army Experience with *Acinetobacter* in Operation Iraqi Freedom. In Bergone-Bérézin, E., H. Friedman, and M. Bendinelli (eds). *Acinetobacter*: Biology and Pathogenesis. Springer science, New-York, Chap 12, pp. 197-209.

Mussi, M. A., A.S. Limansky, and A.M. Viale. 2005. Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of *Acinetobacter baumannii*: natural insertional inactivation of a gene encoding a member of a novel family of beta-barrel outer membrane proteins. *Antimicrob Agents Chemother.* **49:** 1432–40.

Naas, T., B. Coignard, A. Carbonne, K. Blanckaert, O. Bajolet, C. Bernet, X. Verdeil, P. Astagneau, J. C. Desenclos, and P. Nordmann. 2006a. VEB-1 extended-spectrum β-lactamase-producing *Acinetobacter baumannii*, France. *Emerg Infect Dis.* **12**: 1214-1222.

Naas, T., F. Namdari, H. Reglier-Poupet, C. Poyart, and P. Nordmann. 2007. Panresistant extended-spectrum β-lactamase SHV-5-producing *Acinetobacter baumannii* from New York City. *J Antimicrob Chemother*. **60**: 1174-1176

Naas, T., P. Bogaerts, C. Bauraing, Y. Degheldre, Y. glupczynski, and P. Nordmann. 2006b. Emergence of PER and VEB extended spectrum β-lactamases in *Acinetobacter baumannii* in Belgium. *J Antimicrob Chemother*. **58**: 178-182.

Naas, T., W. Sougakoff, A. Casetta, and P. Nordmann. 1998. Molecular characterization of OXA-20, a novel class D beta-lactamase, and its integron from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. **42**: 2074-83.

Nagano, N., Y. Nagano, C. Cordevant, N. Shibata, and Y. Arakawa. 2004. Nosocomial transmission of CTX-M-2 beta-lactamase-producing *Acinetobacter baumannii* in a neurosurgery ward. *J Clin Microbiol*. **42**: 3978–3984.

Naiemi, N. A., B. Duim, P. H. Savelkoul, L. Spanjaard, E. de Jonge, A. Bart, C. M. Vandenbroucke-Grauls, and M. D. de Jong. 2005. Widespread transfer of resistance genes between bacterial species in an intensive care unit: implications for hospital epidemiology. *J Clin Microbiol*. **43**: 4862–4864.

Navia, M. M., J. Ruiz, and J.Vila. 2002. Characterization of an Integron Carrying a New Class D β-Lactamase (OXA-37) in *Acinetobacter baumannii*. *Micro Drug Res*. **8**: 261-265.

Navon-Venezia, S., A. Leavitt, and Y. Carmeli. 2007. High tigecycline resistance in multidrug-resistant *Acinetobacter baumannii*. *J Antimicrob Chemother*. **59**: 772-774.

Neilands, J. B. 1995. Siderophores: Structure and function of microbial iron transport compounds. *J Biol Chem*. **270**: 26723–26726.

Nemec, A., L. Dijkshoorn, and P. Jezek. 2000. Recognition of two novel phenons of the genus Acinetobacter among non-glucose- acidifying isolates from human specimens. *J Clin Microbiol.* **38**: 3937-3941.

Nemec, A., L. Dolzani, S. Brisse, P. van den Broek, and L. Dijkshoorn. 2004. Diversity of aminoglycoside-resistance genes and their association with class 1 integrons among strains of pan-European *Acinetobacter baumannii* clones. *J Med Microbiol.* **53**: 1233-1240.

Nemec, A., L. Krizova., M. Maixnerova., T. J. K. van der Reijden., P. Deschaght., V. Passet., M. Vaneechoutte., S. Brisse, and L. Dijkshoorn. 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). *Res Microbiol.* **162**: 393-404

Nemec, A., M. Musilek., M. Maixnerova., T. De Baere., T. J. van der Reijden., M. Vaneechoutte., and L. Dijkshoorn. 2009. *Acinetobacter beijerinckii* sp. nov. and *Acinetobacter gyllenbergii* sp. nov.,haemolytic organisms isolated from humans. *Int J Syst Evol Microbiol,* **59**: 118-124.

Nemec, A., M. Musílek, O. Sedo, T. De Baere, M. Maixnerová, T. J. van der Reijden, Z. Zdráhal, M. Vaneechoutte, and L. Dijkshoorn. 2010. Acinetobacter bereziniae sp. nov. and Acinetobacter guillouiae sp. nov., to accommodate Acinetobacter genomic species 10 and 11, respectively. *Int J Syst Evol Microbiol*. **60**: 896-903.

Nicas, T. I., and R. E. Hancock. 1980. Outer membrane protein H1 of *Pseudomonas aeruginosa*: involvement in adaptive and mutational resistance to ethylenediamine-tetraacetate, polymyxin B, and gentamicin. *J Bacteriol*. **143**:872–878.

Nikaido, H. 2001. Preventing drug access to targets: cell surface permeability barriers and active efflux in bacteria. *Semin Cell Dev Biol* **12:** 215–223.

Nishimura, Y., T. Ino., and H. Iizuka. 1988. *Acinetobacter radioresistens* sp. nov isolated from cotton and soil. *Int J Syst Bacteriol*. **38**: 209-211.

Nishino, K., and A. Yamaguchi. 2002. EvgA of the two-component signal transduction system modulates production of the YhiUV multidrug transporter in *Escherichia coli*. *J Bacteriol*. **184**: 2319–2323.

Noppe-Leclercq, I., F. Wallet, S. Haentjens, R. Courcol, and M. Simonet. 1999. PCR detection of aminoglycoside resistance genes: a rapid molecular typing method for *Acinetobacter baumannii*. *Res Microbiol*. **150**: 317-322.

Noskin, G. A. 2005. Tigecycline: a new glycylcycline for treatment of serious infections. *Clin Infect Dis*. **41**: S303-314.

Obuekwe, C. O., Z. K. Al-Jadi, and E. S. Al-Saleh. 2009. Hydrocarbon degradation in relation to cell-surface hydrophobicity among bacterial hydrocarbon degraders from petroleum-contaminated Kuwait desert environment. *Int Biodeter Biodegr*. **63**: 273-279.

Ohtsubo, Y., H. Genka, H. Komatsu, Y. Nagata, and M. Tsuda. 2005. High-temperature-induced transposition of insertion elements in *Burkholderia multivorans* ATCC 17616. *Appl Environ Microbiol*. **71**: 1822-1828.

Ordonez, O. F., M. R. Flores, J. R. Dib, A. Paz, and M. E. Farias. 2009. Extremophile culture collection from Andean lakes: Extreme pristine environments that host a wide diversity of microorganisms with tolerance to UV radiation. *Microb Ecol*, In Press.

Pannek, S., P. G. Higgins, P. Steinke, D. Jonas, M. Akova, J. A. Bohnert, H. Seifert, and W. V. Kern. 2006 Multidrug efflux inhibition in *Acinetobacter baumannii*: comparison between 1-(1-naphthylmethyl)-piperazine and phenyl-arginine-β-naphthylamide. *J Antimicrob Chemother*. **57**: 970-974.

Pantophlet, R., J.A. Severin, A. Nemec, L. Brade, L. Dijkshoorn, and H. Brade. 2002. Identification of *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex with monoclonal antibodies specific for O antigens of their lipopolysaccharides. *Clin Diagn. Lab. Immunol*. **9**: 60–65.

Papanicolaou, G. A., A. A. Medeiros, and G. A. Jacoby. 1994. Novel plasmid-mediated beta-lactamase (MIR-1) conferring resistance to oxyimino- and alpha-methoxy beta-lactams in clinical isolates of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother*. **34**: 2200-2209.

Pasteran, F., M. Rapoport, A. Petroni, D. Faccone, A. Corso, M. Galas, M. Vazquez, A. Procopio, M. Tokumoto, and V. Cagnoni. 2006. Emergence of PER-2 and VEB-1a in *Acinetobacter baumannii* strains in the Americas. *Antimicrob. Agents Chemother*. **50**: 3222–3224.

Paton, R., R. S. Miles, J. Hood, and S. G. B. Amyes. 1993. ARI-1: β-lactamase mediated imipenem resistance in *Acinetobacter baumannii*. *Int J Antimicrob Agents*. **2**: 81–88.

Peix, A., E. Lang, S. Verbarg, C. Sproer, R. Rivas, I. Santa-Regina, P. F. Mateos, E. Martinez-Molina, C. Rodriguez-Barrueco, and E. Velazquez. 2009. *Acinetobacter* strains IH9 and OCI1, two rhizospheric phosphate solubilizing isolates able to promote plant growth, constitute a new genomovar of *Acinetobacter calcoaceticus*. *Syst Appl Microbiol*. **32**: 334-341.

Peleg, A. Y., C. Franklin, J. M. Bell, and D.W. Spelman. 2006. Emergence of carbapenem resistance in *Acinetobacter baumannii* recovered from blood cultures in Australia. *Infect Control Hosp Epidemiol*. **27**: 759-761.

Peleg, A. Y., H. Seifert, and D. L. Paterson. 2008. *Acinetobacter baumannii*: Emergence of a successful pathogen. *Clin Microbiol Rev*. **21**: 538-582

Peterson, A. A., S. W. Fesik, and E. J. McGroarty. 1987. Decreased binding of antibiotics to lipopolysaccharides from polymyxin-resistant strains of *Escherichia coli* and *Salmonella typhimurium*. *Antimicrob Agents Chemother*. **31**: 230-237.

Peterson, J. W. 1996. Bacterial pathogenesis. In Medical Microbiology, S. Baron (ed). Galveston: University of Texas Medical Branch.

Pfeifer, Y., W. Witte, M. Holfelder, J. Busch, P. Nordmann, and L. Poirel. 2011. NDM-1-producing *Escherichia coli* in Germany. *Antimicrob Agents Chemother*. **55**: 1318-1319.

Piddock, L. J. 2006. Multidrug-resistance efflux pumps – not just for resistance. *Nat Rev Microbiol*. **4**: 629-636.

Ploy, M. C., F. Denis, P. Courvalin, and T. Lambert. 2000. Molecular characterization of integrons in *Acinetobacter baumannii*: description of a hybrid class 2 integron. *Antimicrob Agents Chemother*. **44**: 2684-2688.

Poirel. L., L. Cabanne , H. Vahaboglu, and P. Nordmann. 2005a. Genetic environment and expression of the extended-spectrum β-lactamase bla_{PER-1} gene in Gram-negative bacteria. *Antimicrob Agents Chemother*. **49**: 1708–1713.

Poirel, L., and P. Nordmann. 2006a. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clin Microbiol Infect.* **12**: 826-836.

Poirel, L., and P. Nordmann. 2006b. Genetic structures at the origin of acquisition and expression of the carbapenem-hydrolyzing oxacillinase gene bla_{OXA-58} in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **50**: 1442-1448.

Poirel, L., and P. Nordmann. 2008. *Acinetobacter baumannii*: Mechanisms of resistance, multiple β-Lactamases. In Bergone-Bérézin, E., H. Friedman, and M. Bendinelli (eds). *Acinetobacter*: Biology and Pathogenesis. Springer science, New-York, Chap 7, pp. 129-143.

Poirel, L., S. Figueiredo, V. Cattoir, A. Carattoli, and P. Nordmann. 2008. *Acinetobacter radioresistens* as a silent source of carbapenem resistance for *Acinetobacter* spp. *Antimicrob Agents Chemother*. **52**: 1252-1256.

Poirel, L., S. Marqué, C. Héritier, C. Segonds, G. Chabanon, and P. Nordmann. 2005b. OXA-58, a novel class D β-lactamase involved in resistance to carbapenems in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **49**: 202-208.

Poirel, L., J. M. Rodríguez-Martínez, N. Al Naiemi, Y. J. Debets-Ossenkopp, and P. Nordmann. 2010a. Characterization of DIM-1, an integron-encoded metallo-beta-lactamase from a *Pseudomonas stutzeri* clinical isolate in the Netherlands. *Antimicrob Agents Chemother*. **54**: 2420-2424.

Poirel, L., T. Naas, and P. Nordmann. 2010b. Diversity, epidemiology, and genetics of class D β-lactamases. *Antimicrob Agents Chemother*. **54**: 24-38.

Pontiroli, A., A. Rizzi, P. Simonet, D. Daffonchio, T. M. Vogel and J.M. Monier. 2009. Visual evidence of horizontal gene transfer between plants and bacteria in the phytosphere of transplastomic tobacco. *Appl Environ Microbiol*. **75**: 3314-3322.

Projan, S. J. 2010. Francis Tally and the discovery and development of tigecycline: a personal reminiscence. *Clin Infect Dis*. **50**: S24-25

Quinteira, S., F. Grosso, H. Ramos, and L. Peixe. 2007. Molecular epidemiology of imipenem-resistant *Acinetobacter haemolyticus* and *Acinetobacter baumannii* isolates carrying plasmid-mediated OXA-40 from a Portuguese hospital. *Antimicrob Agents Chemother*. **51**: 3465-3466.

Rådström, P., O. Sköld, G. Swedberg, J. Flensburg, P. H. Roy, and L. Sundström. 1994. Transposon Tn*5090* of plasmid R751, which carries an integron, is related to Tn*7*, Mu, and the retroelements. *J Bacteriol*. **176**: 3257-3268.

Ravasi, P., A. S. Limansky, R. E. Rodriguez, A. M. Viale, and M. A. Mussi. 2011. IS*Aba825*, a functional insertion sequence modulating genomic plasticity and $bla_{\text{OXA-58}}$ expression in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **55**: 917-920.

Rello, J., E. Diaz, M. Roque, and J. Valles. 1999. Risk factors for developing pneumonia within 48 hours of intubation. *Am Jour Res & Crit Care Med.* **159**: 1742-1746.

Retailliau, H. F., A. W. Hightower, R. E. Dixon, and J. R. Allen. 1979. *Acinetobacter calcoaceticus*: a nosocomial pathogen with an unusual seasonal pattern. *J Infect Dis*. **139**: 371–375.

Ribera, A., J. Ruiz, and J. Vila. 2003a. Presence of the Tet M determinant in a clinical isolate of *Acinetobacter baumannii*. *Antimicrob. Agents Chemother*. **47**:2310–2312.
Ribera, A., J. Ruiz, M. T. Jiminez de Anta, and J. Vila. 2002. Effect of an efflux pump inhibitor on the MIC of nalidixic acid for *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* clinical isolates. *J Antimicrob Chemother*. **49**: 697-698.

Ribera, A., I. Roca, J. Ruiz, I. Gibert, and J. Vila. 2003b. Partil characterization of a transposon containing the *tet*(A) determinant in a clinical isolate of *Acinetobacter baumannii*. *J Antimicrob Chemother*. **52**: 477–480.

Riccio, M. L., N. Franceschini, L. Boschi, B. Caravelli, G. Cornaglia, R. Fontana, G. Amicosante, and G. M. Rossolini. 2000. Characterization of the metallo-beta-lactamase determinant of *Acinetobacter baumannii* AC-54/97 reveals the existence of bla_{IMP} allelic variants carried by gene cassettes of different phylogeny. *Antimicrob Agents Chemother*. **44**: 1229-1235

Roberts, M. C. 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiol Rev*. **19**: 1-24.

Roca, I., P. Espinal, S. Martí, and J. Vila. 2011. First identification and characterization of an AdeABC-like efflux pump in *Acinetobacter* genomospecies 13TU. *Antimicrob Agents Chemother*. **55**: 1285-1286.

Rodríguez-Martínez, J.-M., P. Nordmann, E. Ronco, and L. Poirel. 2010. Extended-spectrum cephalosporinase in *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **54**: 3484-3488.

Rossau, R., A. van Landschoot, M. Gillis, and J. de Ley. 1991. Taxonomy of *Moraxellaceae* fam. Nov., a new bacterial family to accommodate the genera *Moraxella*, *Acinetobacter*, and *Psychrobacter* and related organisms. *Int. J. Syst. Bacteriol*. **41**: 310-319

Ruiz, M., S. Marti, F. Fernandez-Cuenca, A. Pascual, and J. Vila. 2007. High prevalence of carbapenem-hydrolysing oxacillinases in epidemiologically related and unrelated *Acinetobacter baumannii* clinical isolates in Spain. *Clin Microbiol Infect* **13:** 1192-1198.

Ruiz, M., S. Marti, F. Fernandez-Cuenca, P. Alvaro, and J. Vila. 2007. Prevalence of IS*Aba1* in epidemiologically unrelated *Acinetobacter baumannii* clinical isolates. *FEMS Microbiol Lett*. 274: 63-66

Ruzin, A., D. Keeney, and P. A. Bradford. 2007. AdeABC multidrug efflux pump is associated with decreased susceptibility to tigecycline in *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex. *J Antimicrob Chemother*. **59**: 1001-1004

Rybak, M. J. 2004. Resistance to antimicrobial agents: an update. *Pharmacotherapy*. **24**: 203S-215S.

Schaub, I. G., and F. D. Hauber. 1948. A biochemical and serological study of a group of identical unidentifiable gram- negative bacilli from human sources. *J Bacteriol*. **56**: 379-385

Schneider, I., A. M. Queenan, and A. Bauernfeind. 2006. Novel carbapenem-hydrolyzing oxacillinase OXA-62 from *Pandoraea pnomenusa*. *Antimicrob Agents Chemother*. **50**: 1330-1335.

Schneiders, T., J. Findlay, and S. G. B Amyes. 2008. Efflux Pumps in *Acinetobacter baumannii.* In Bergone-Bérézin, E., H. Friedman, and M. Bendinelli (eds). *Acinetobacter*: Biology and Pathogenesis. Springer science, New-York, Chap 6, pp. 105-127.

Sechi, L. A., A. Karadenizli, A. Deriu, S. Zanetti, F. Kolayli, E. Balikci, and H. Vahaboglu. 2004. PER-1 type beta-lactamase production in *Acinetobacter baumannii* is related to cell adhesion. *Med Sci Monit*. **10**: 180–184.

Seifert, H., A. Strate, A. Schulze, and G. Pulverer. 1994. Bacteremia due to *Acinetobacter* species other than *Acinetobacter baumannii*. *Infection*. **22**: 379-385.

Seifert, H., L. Dijkshoorn, P. Grener-Smidt, N. Pelzer, I. Tjernberg, and M. Vaneechoutte. 1997. Distribution of *Acinetobacter* species on human skin: comparison of phenotypic and genotypic identification methods. *J Clin Microbiol*. **35**: 2819-2825

Seifert, H., and L. Dijkshoorn. 2008. Overview of the Microbial Characteristics, Taxonomy, and Epidemiology of *Acinetobacter*. In Bergogne-Bérézin E., H. Friedman., and M. Bendinelli (eds.) *Acinetobacter*: Biology and Pathogenesis. Springer Science, New-York, Chap 2, pp 25-29

Seifert, H., L. Dolzani, R. Bressan, T. van der Reijden, B. van Strijen, D. Stefanik, H. Heersma, and L. Dijkshoorn. 2005. Standardization and interlaboratory reproducibility assessment of pulsed-field gel electrophoresis-generated fingerprints of *Acinetobacter baumannii*. *J Clin Microbiol*. **43**: 4328–4335.

Sepulveda, M., M. Ruiz, H. Bello, M. Dominguez, M. A. 1artínez, M. E Pinto, G. Gonzalez, S. Mella, and R. Zemelman. 1998. Adherence of *Acinetobacter baumannii* to rat bladder tissue. *Microbios* **95**: 45–53.

Sevillano, E., E. Fernandez, Z. Bustamante, S. Zabalaga, I. Rosales, A. Umaran, and L. Gallego. 2012. Emergence and clonal dissemination of carbapenem-hydrolysing OXA-58-producing *Acinetobacter baumannii* isolates in Bolivia. *J Med Micro*. **61**: 80-84.

Sevillano, E., L. Gallego, and J. M. García-Lobo. 2009. First detection of the OXA-40 carbapenemase in *P. aeruginosa* isolates, located on a plasmid also found in *A. baumannii*. *Pathol Biol* (Paris). **57**: 493-495.

Seward, R. J., and K. J. Towner. 1999. Detection of integrons in worldwide nosocomial isolates of *Acinetobacter* spp. *Clin Microbiol Infect.* **5**: 308-318.

Shaw, K. J., P. N. Rather, R. S. Hare, and G. H. Miller. 1993. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Rev.* **57**: 138-163.

Siguier P, Perochon J, Lestrade L, Mahillon J, and M. Chandler. 2005. ISfinder: the reference centre for bacterial insertion sequences. *Nucl Acids Res*. **34**: D32-D36

Siroy, A., P. Cosette, D. Seyer, C. Lemaitre-Guillier, D. Vallenet, A. Van Dorsselaer, S. Boyer-Mariotte, T. Jouenne, and E. De. 2006. Global comparison of the membrane subproteomes between a multidrug-resistant *Acinetobacter baumannii* strain and a reference strain. *J Proteome Res*. **5**: 3385–98.

Siroy, A., V. Molle, C. Lemaitre-Guillier, D. Vallenet, M. Pestel-Caron, A. J. Cozzone, T. Jouenne, and E. De. 2005. Channel formation by CarO, the carbapenem resistance associated outer membrane protein of *Acinetobacter baumannii*. *Antimicrob Agents Chemother*. **49:** 4876–4883

Skerman, V. B. D., V. McGowan, and P. H. A Sneath (eds). 1980. Approved list of bacterial names. *Int J Syst Bacteriol*. **30**: 225-420

Smith, A. W., and K. E. Alpar. 1991. Immune response to *Acinetobacter calcoaceticus* infection in man. *J Med Microbiol*. **34**: 83–88.
Smith, A. W., S. Freeman, W. G. Minett, and P. A. Lambert. 1990. Characterisation of a siderophore from *Acinetobacter calcoaceticus*. *FEMS Microbiol Lett*. **70:** 29–32.

Smith, K., and J. J. Leyden. 2005. Safety of doxycycline and minocycline: a systematic review. *Clin Ther*. **27**: 1329-1342.

Smith, M. G., T. A. Gianoulis, S. Pukatzki, J. J. Mekalanos, L. N. Ornston, M. Gerstein, and M. Snyder. 2007. New insights into *Acinetobacter baumannii* pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. *Genes Dev*. **21**: 601–614.

Su, X. Z., J. Chen, T. Mizushima, T. Kuroda, and T. Tsuchiya. 2005. AbeM, an H-coupled *Acinetobacter baumannii* multidrug efflux pump belonging to the MATE family of transporters. *Antimicrob Agents Chemother*. **49**: 4362–4364.

Sundström, L., P. H. Roy, and O. Skold. 1991 Site-specific insertion of three structural gene cassettes in transposon Tn*7*. *J Bacteriol*. **173**: 3025-3028.

Szabó, D., F. Silveira, A. M. Hujer, R. A. Bonomo, K. M. Hujer, J. W. Marsh, C. R. Bethel, Y. Doi, K. Deeley, and D. L. Paterson. 2006. Outer membrane protein changes and efflux pump expression together may confer resistance to ertapenem in *Enterobacter cloacae*. *Antimicrob Agents Chemother*. **50**: 2833-2835.

Tenover, F. C., R. D. Arbeit, R. V. Goering, P. A. Mickelsen, B. E. Murray, D. H. Persing, and B. Swaminathan. 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol*. **33**: 2233–2239.

Thomson, K.S. 2010. Extended-Spectrum-β-Lactamase, AmpC, and Carbapenemase Issues. *J Clin Microbiol*. **48**: 1019–1025

Tian, G. B., J. M. Adams-Haduch, T. Bogdanovich, A. W. Pasculle, J. P. Quinn, H. N. Wang, and Y. Doi. 2011. Identification of diverse OXA-40 group carbapenemases, including a novel variant, OXA-160, from *Acinetobacter baumannii* in Pennsylvania. *Antimicrob Agents Chemother*. **55**: 429-432.

Timsit, J. F., S. Chevret, J. Valcke, B. Misset, B. Renaud, F.W. Goldstein, P. Vaury, and J. Carlet. (1996). Mortality of nosocomial pneumonia in ventilated patients: influence of diagnostic tools. *Am J Res and Cri Care Med*. **154**: 116-123.

Tjernberg, I., and J. Ursing. 1989. Clinical strains of *Acinetobacter* classified by DNA- DNA hybridization. *APIMS*. **97**: 595-605.

Toleman, M. A., A. M. Simm, T. A. Murphy, A. C. Gales, D. J. Biedenbach, R.N. Jones, and T. R. Walsh. 2002. Molecular characterization of SPM-1, a novel metallo-beta-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. *J Antimicrob Chemother*. **50**: 673-679.

Toleman, M. A., and T. R. Walsh. 2011. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiol Rev*. **35**: 912-935.

Tomaras, A. P., C.W. Dorsey, R. E. Edelmann, and L. A. Actis. 2003. Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. *Microbiology* **149**: 3473–3484.

Tomasz, A. 1979. The mechanism of the irreversible antimicrobial effects of penicillins: how the beta-lactam antibiotics kill and lyse bacteria. *Annu Rev Microbiol*. **33**: 113.

Touati, A., L. Brasme, S. Benallaoua, A. Gharout, J. Madoux, and C. De Champs. 2008. First report of *qnrB*-producing *Enterobacter cloacae* and *qnrA*-producing *Acinetobacter baumannii* recovered from Algerian hospitals. *Diagn Microbiol Infect Dis*. **60**: 287-290.

Trevors, J. T. 1986. Plasmid curing in bacteria. *FEMS Micro Rev*. **32**: 149-157

Trottier, V., P. G. Segura, N. Namias, D. King, L. R. Pizano, and C. I. Schulman. 2007. Outcomes of *Acinetobacter baumannii* infection in critically ill burned patients. *J. Burn Care Res*. **28**: 248–254.

Turton, J. F., M. E. Kaufmann, J. Glover, J. M. Coelho, M. Warner, R. Pike and T. L. Pitt. 2005. Detection and typing of integrons in epidemic strains of *Acinetobacter baumannii* found in the United Kingdom. *J Clin Microbiol*. **43**: 3074-3082.
Turton, J. F., M. E. Ward, N. Woodford, M. E. Kaufmann, R. Pike, D. M. Livermore, and T. L. Pitt. 2006a. The role of IS*Aba1* in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. *FEMS Microbiol Lett*. **258**: 72-77.

Turton, J. F., N. Woodford, J. Glover, S. Yarde, M. E. Kaufmann, and T. L. Pitt. 2006b. Identification of *Acinetobacter baumannii* by detection of the $bla_{\text{OXA-51-like}}$ carbapenemase gene intrinsic to this species. *J Clin Microbiol*. **44**: 2974-2976.

Vaara, M. 1992. Agents that increase the permeability of the outer membrane. *Microbiol Rev*. **56**: 395-411.

Vahaboglu. H., F. Budak, M. Kasap, G. Gacar, S. Torol, A. Karadenizli, F. Kolayli, and C. Eroglu. 2006. High prevalence of OXA-51-type class D β-lactamases among ceftazidime-resistant clinical isolates of *Acinetobacter* spp.: co-existence with OXA-58 in multiple centres. *J Antimicrob Chemother*. **58**: 537-542.

Valentine, S. C., D. Contreras, S. Tan, L. J. Real, S. Chu, and H. H Xu. 2008 Phenotypic and molecular characterization of *Acinetobacter baumannii* clinical isolates from nosocomial outbreaks in Los Angeles County, California. *Antimicrob Agents and Chemother*. **46**: 2499-2507.

Valero, C., M. C. Farinas, D. Garcia Palomo, J. C. Mazarrasa, and J. Gonzalez Macias. 1999. Endocarditis due to *Acinetobacter lwoffi* on native mitral valve. *Int. J. Cardiol*. **69**: 97–99.

Vaneechoutte, M., L. A. Devriese, L. Dijkshoorn, B. Lamote, P. Deprez, G. Verschraegen, and F. Haesebrouck. 2000. *Acinetobacter baumannii*-infected vascular catheters collected from horses in an equine clinic. *J Clin Microbiol*. **38**: 4280–4281.

Van Looveren, M., H. Goossens, and ARPAC Steering Group. 2004. Antimicrobial resistance of *Acinetobacter* spp. in Europe. *Clin Microbiol Infect*. **10**: 684-704.

Vila, J., and J. Pachón. 2008. Antimicrobial Resistance and Therapeutic Alternatives. In Bergone-Bérézin, E., H. Friedman, and M. Bendinelli (eds). *Acinetobacter*: Biology and Pathogenesis. Springer science, New-York, Chap 11, pp. 175-196.

Vila, J., S. Marti, and J. Sanchez-Cespedes. 2007. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. *J Antimicrob Chemother* **59**: 1210–1215.

Villegas, M. V., J. N. Kattan, A. Correa, K. Lolans, A. M. Guzman, N. Woodford, D. Livermore, and J. P. Quinn. 2007. Dissemination of *Acinetobacter baumannii* clones with OXA-23 Carbapenemase in Colombian hospitals. *Antimicrob Agents Chemother*. **51**: 2001-2004.

von Lingelshiem, W. 1908. Beitrage zur Epidemiologie der epidemischen Genickstarre nach den Ergebnissen der letzten Jahre. *Z Hyg Infektkrankh*. **59**: 457-460.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper, et al. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.* **23**: 4407-4414.

Waksman, S. A. 1947. What Is an Antibiotic or an Antibiotic Substance? *Mycologia*. 39: 565–569.

Walsh, T. R., J. Weeks, D.M. Livermore, and M.A. Toleman. 2011. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect Dis*. **11**: 355-362.

Walther-Rasmussen, J., and N. Høiby. 2006. OXA-type carbapenemases. *J Antimicrob Chemother*. **57**: 373-383.

Walzer, G., E. Rosenberg, and E. Z. Ron. 2006. The *Acinetobacter* outer membrane protein A (OmpA) is a secreted emulsifier. *Environ Microbiol*. **8**: 1026-1032.

Wang, H., P. Guo, H. Sun, H. Wang, Q. Yang, M. Chen, Y. Xu, and Y. Zhu. 2007. Molecular epidemiology of clinical isolates of carbapenem-resistant *Acinetobacter* spp. from Chinese hospitals. *Antimicrob Agents Chemother* **51**: 4022–4028

Wang, X., Z. Zong, X. Lü. 2011. Tn2008 is a major vehicle carrying *bla*$_{OXA-23}$ in *Acinetobacter baumannii* from China. *Diagn Microbiol Infect Dis*. **69**: 218-22.

Weill, F. X., M. Demartin, D. Tandé, E. Espié, I. Rakotoarivony, and P. A. D. Grimont. 2004. Extended-spectrum-ß-lactamase (SHV-12 like)-producing strains of *Salmonella enterica* serotypes Babelsberg and Enteritidis isolated in France among infants adopted from Mali. *J Clin Microbiol*. **42**: 2432-2437.

Willmott, C. J., and A. Maxwell. 1993. A single point mutation in the DNA gyrase A protein greatly reduces binding of fluoroquinolones to the gyrase-DNA complex. *Antimicrob Agents Chemother*. **37**: 126-127.

Wise, R. 1990. The pharmacokinetics of the oral cephalosporins--a review. *J Antimicrob Chemother*. **26**(suppl E):13-20.

Woodford, N., E. J. Fagan and M. J. Ellington. 2005. Multiplex PCR for rapid detection of genes encoding CTX-M extended-spectrum β-lactamases. *J Antimicrob Chemother*. **57**: 154-155

Woodford, N., J. F. Turton and D. M. Livermore. 2011. MultiresistantGram-negative bacteria: the role of high-risk clones in the dissemination ofantibiotic resistance. *FEMS Microbiol Rev*. **35**: 736-755

Woodford, N., M. J. Ellington, J. M. Coelho, J. F. Turton, M. E. Ward, S. Brown, S. G. Amyes and D. M. Livermore. 2006. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter spp. Int J Antimicrob Agents*. **27**: 351–353.

Woods, D.D. 1940 Relation of p-aminobenzoic acid to mechanism of action of sulphanilamide. *Br J Exp Pathol*. **21**: 74-90

Yamamoto, S., N. Okujo, and Y. Sakakibara. 1994. Isolation and structure elucidation of acinetobactin, a novel siderophore from *Acinetobacter baumannii*. *Arch. Microbiol*. **162**: 249–254.

Yamane, K., J. Wachino, S. Suzuki, K. Kimura, N. Shibata, H. Kato, K. Shibayama, T. Konda, and Y. Arakawa. 2007. New plasmid-mediated fluoroquinolone efflux pump, QepA, found in an *Escherichia coli* clinical isolate. *Antimicrob. Agents Chemother*. **51**: 3354–3360.

Yamane, K., J. Wachino, Y. Doi, H. Kurokawa, and Y. Arakawa. 2005. Global spread of multiple aminoglycoside resistance genes. *Emerg Infect Dis*. **11**: 951-953.

Yokoyama, K., Y. Doi, K. Yamane, H. Kurokawa, N. Shibata, K. Shibayama, T. Yagi, H. Kato, and Y. Arakawa Y. 2003. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. *Lancet*. **362**: 1888-1893.

Yong, D., K. Lee, J. H. Yum, H. B. Shin, G. M. Rossolini, and Y. Chong. 2002. Imipenem-EDTA disk method for differentiation of metallo- β -lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *J Clin Microbiol*. **40**: 3798–3801.

Yong, D., M. A. Toleman, C. G. Giske, H. S. Cho, K.Sundman, K. Lee, and T. R. Walsh. 2009. Characterization of a New Metallo- β -Lactamase Gene, *bla*$_{NDM-1}$, and a Novel Erythromycin Esterase Gene Carried on a Unique Genetic Structure in *Klebsiella pneumoniae* Sequence Type 14 from India. *Antimicrob Agents Chemother.* **53**: 5046-5054.

Yu, Y. S., H. Zhou, Q. Yang, Y. G. Chen, L. J. Li. 2007. Widespread occurrence of aminoglycoside resistance due to ArmA methylase in imipenem-resistant *Acinetobacter baumannii* isolates in China. *J Antimicrob Chemother*. **60**: 454-455.

Yu, Y. S., Q. Yang , X. W. Xu, H.S. Kong, G. Y. Xu, and B. Y. Zhong. 2004. Typing and characterization of carbapenem-resistant *Acinetobacter calcoaceticus-baumannii* complex in a Chinese hospital. *J Med Microbiol*. **53**: 653-656.

Yuan, Y. W., and S. R. Wessler. 2011. The catalytic domain of all eukaryotic cut-and-paste transposase superfamilies. *Proc Natl Acad Sci USA*. **108**: 7884–9.

Yum, J. H., K. Yi, H. Lee, D. Yong, K. Lee, J. M. Kim, G. M. Rossolini, and Y. Chong. 2002. Molecular characterization of metallo-beta-lactamase-producing *Acinetobacter baumannii* and *Acinetobacter genomospecies 3* from Korea: identification of two new integrons carrying the *bla*$_{VIM-2}$ gene cassettes. *J Antimicrob Chemother*. **49**: 837-840.

Valentine, S. C., D. Contreras, S. Tan, L. J. Real, S. Chu, and H. H. Xu. 2008. Phenotypic and molecular characterization of *Acinetobacter baumannii* clinical isolates from nosocomial outbreaks in Los Angeles County, California. *Antimicrob Agents and Chemother*. **46**: 2499-2507.

Zarrilli, R., D. Vitale, A. Di Popolo, M. Bagattini, Z. Daoud, A. U. Khan, C. Afif, and M. Triassi. 2008. A plasmid-borne *bla*$_{OXA-58}$ gene confers imipenem resistance to *Acinetobacter baumannii* isolates from a Lebanese hospital. *Antimicrob Agents Chemother*. **52**: 4115-4120.

APPENDIX A:

Published papers

Journal of Medical Microbiology (2011), 60, 211–215 DOI 10.1099/jmm.0.025544-0

Effect of frameshift mutagen acriflavine on control of resistance genes in *Acinetobacter baumannii*

B. S. Lopes, A. Hamouda, J. Findlay and S. G. B. Amyes

Centre for Infectious Diseases, University of Edinburgh, Edinburgh, UK

Correspondence
S. G. B. Amyes
s.g.b.amyes@ed.ac.uk

Acinetobacter baumannii is a Gram-negative pathogenic bacterium that often exhibits a multidrug-resistant phenotype causing infections at various sites of the body and increasingly leading to septicaemic shock. This study evaluated the role of acriflavine, a frameshift mutagen, on the movement of insertion sequence IS*Aba1* in clinical isolates of *A. baumannii*, with the focus on changes in expression levels of the bla_{ADC} and $bla_{OXA\text{-}51\text{-}like}$ genes. Resistance profiles were assessed with consideration of IS*Aba1* acting as a promoter upstream of the bla_{ADC} or $bla_{OXA\text{-}51\text{-}like}$ gene. IS*Aba1* movement was observed in the acriflavine mutants Ab153M and Ab1225M. Ab153M exhibited an increase in the MIC values of carbapenems and ceftazidime, with IS*Aba1* gained upstream of the bla_{ADC} and $bla_{OXA\text{-}51\text{-}like}$ genes, correlating with an increase in gene expression. Reduced expression of the 17, 23 and 25 kDa outer-membrane proteins (OMPs) was also observed in Ab153M. There was a significant decrease in MIC values of carbapenems with the loss of IS*Aba1* upstream of the bla_{ADC} and $bla_{OXA\text{-}51\text{-}like}$ genes in strain Ab1225M, and a significant decrease in $bla_{OXA\text{-}51\text{-}like}$ gene expression and, to a lesser extent, in bla_{ADC} expression. Ab1225M and a serially subcultured Ab1225 strain (Ab1225s) exhibited overexpression of the 17, 23, 25 and 27 kDa OMPs. There was a decrease in MIC values of the carbapenems and piperacillin/tazobactam but not of ceftazidime in Ab1225s, which had IS*Aba1* upstream of the bla_{ADC} and $bla_{OXA\text{-}51\text{-}like}$ genes. A significant decrease in $bla_{OXA\text{-}51\text{-}like}$ expression was observed in Ab1225s, whereas the expression of bla_{ADC} was similar to that in the Ab1225 parental strain. The attenuation in this strain may be due to overexpression of OMPs and it is clear that, even if IS*Aba1* is present upstream of an antibiotic resistance gene, it may not necessarily contribute towards the overexpression of antibiotic resistance genes ($bla_{OXA\text{-}51\text{-}like}$ in Ab1225s). Movement of the IS element within the *A. baumannii* chromosome may be an important regulatory mechanism employed by the bacterium under particular stress conditions, and the ability to upregulate the expression of antibiotic resistance genes is likely to be an important factor in the pathogenicity of this bacterium.

Received 24 August 2010
Accepted 15 October 2010

INTRODUCTION

Acinetobacter baumannii is one of the most difficult noscocomial pathogens to treat, similar to other bacterial pathogens such as meticillin-resistant *Staphylococcus aureus*, *Clostridium difficile* and *Pseudomonas aeruginosa*. Hospital-acquired pneumonia is the most common infection caused by this organism (Peleg *et al.*, 2008).

Nosocomial infections may be transmitted via aerosols, staff or even by inadequately disinfected medical devices (Spelman, 2002). Other common infections caused by this bacterium are ventilator-associated pneumonia, urinary tract infections and bacteraemia (Perez *et al.*, 2007). It can also cause suppurative infections of the skin, and abdominal and central nervous system infections (Fournier & Richet, 2006). The bacterium has developed resistance to different types of β-lactam antibiotics by the production of β-lactamases, which include plasmid-encoded class A (TEM/PER-1/VEB/SHV/CTX-M), class B (metallo-β-lactamases), class C (*Acinetobacter* derived cephalosporinases AmpC/bla_{ADC}) and class D (the oxacillinases: OXA-23-like, OXA-40-like, OXA-58-like and OXA-51-like, the latter being an intrinsic β-lactamase of *A. baumannii*) β-lactamases (Brown & Amyes, 2006; Perez *et al.*, 2007).

Outer membranes, like other biological membranes, are built as lipid bilayers that are permeable to hydrophobic molecules and are involved in the influx of nutrients and the efflux of toxic substrates or antibiotics which can hamper normal cell function (Nikaido, 2003). Three outer-membrane proteins (OMPs) have been reported to be missing in the imipenem-resistant strains of *A. baumannii*: the first is a 33–36 kDa protein, the second is a 29 kDa protein designated CarO and the last is a 43 kDa protein

Abbreviation: OMP, outer-membrane protein.

that shows significant peptide homology with OprD from *P. aeruginosa* (Vila *et al.*, 2007).

Transposable elements are important in providing genetic variability and are generally tightly regulated (Mugnier *et al.*, 2009). Insertion sequences are capable of independent transposition in the microbial genome and are also responsible for the spread of resistance and virulence determinants within the species (Mugnier *et al.*, 2009). IS*Aba1* has been associated with the expression of several antibiotic resistance genes including $bla_{\text{OXA-51-like}}$ and bla_{ADC} (Corvec *et al.*, 2003, 2007). It is also known that frameshift products are involved in transposition of IS*629*, a member of the IS*3* family (Chen & Hu, 2006; Mahillon & Chandler, 1998). IS*Aba1* belongs to the IS*4* family, and it has been reported recently that it is capable of transposition and that the transposase gene is downregulated by translational frameshifting (Mugnier *et al.*, 2009). It has also been reported that IS*4Bsu1*, a member of the IS*4* family in *Bacillus subtilis*, is responsible for the genetic instability of poly-γ-glutamic acid production and that IS*1999*, a member of the same family, is responsible for expression of β-lactam resistance genes (Aubert *et al.*, 2006; Nagai *et al.*, 2000).

Acriflavine is known to cause frameshift mutations by intercalation in DNA (Kornberg, 1980). In this study, we evaluated the role of OMPs, other than the major OMPs, that might be involved in the transport of nutrients and influx of antibiotics, particularly imipenem, meropenem, ceftazidime and piperacillin/tazobactam, by comparing a parental strain, an acriflavine-resistant mutant and a strain serially subcultured on MacConkey agar. The role of acriflavine as a frameshift mutagen was evaluated with respect to its effect on the control of $bla_{\text{OXA-51-like}}$ and bla_{ADC} as a result of the transposition of IS*Aba1*. This study also aimed to identify the changes in levels of expression of antibiotic resistance genes due to the movement of IS*Aba1*.

METHODS

The bacterial strains used this study were *A. baumannii* 153 (Ab153) (Nottingham, UK), *A. baumannii* 153 acriflavine mutant (Ab153M), *A. baumannii* 1225 (Ab1225) (Wroclaw, Poland), *A. baumannii* 1225 acriflavine mutant (Ab1225M) and *A. baumannii* 1225 serially subcultured strain (Ab1225s) (Wroclaw, Poland). All parental strains were kindly supplied by Dr K. J. Towner (Queens Medical Centre, Nottingham, UK).

Identification and typing. Isolates were screened for the conserved 16S–23S rRNA intergenic spacer sequence using primer 1 (5′-TTGTACACACCGCCCGTCA-3′) and primer 2 (5′-GGTACTTAGATGTTTCAGTTC-3′). The expected product size was 975 bp. Restriction of the product by the enzyme *Alu*I produced fragments of 50, 125, 135, 165, 175 and 330 bp specific for *A. baumannii*. Restriction of the same product with *Nde*II produced fragments of 50, 110, 145, 330 and 360 bp specific for *A. baumannii* (Dolzani *et al.*, 1995).

The strains were further identified as *A. baumannii* by amplification of the intrinsic $bla_{\text{OXA-51-like}}$ gene by PCR with the primers oxa-69A (5′-CTAATAATTGATCTACTCAAG-3′) and oxa-69B (5′-CCAGTGGATGGATAGATTATC-3′) at an annealing temperature of 48 °C. The expected product sizes were 975 bp for strains without IS*Aba1* present upstream of the $bla_{\text{OXA-51-like}}$ gene and 2155 bp for strains with IS*Aba1* present upstream of the $bla_{\text{OXA-51-like}}$ gene (Héritier *et al.*, 2005).

The strains were screened for the bla_{ADC} gene with primers ISADC1 (5′-GTTGCACTTGGTCGAATGAAAA-3′) and ISADC2 (5′-ACGTCGCGAGTTGAAGTAAGTT-3′) with an annealing temperature of 51 °C. The expected product size was 751 bp if IS*Aba1* was located upstream of the bla_{ADC} gene (Ruiz *et al.*, 2007).

Mutational analysis and strain subculture. Strains Ab153 and Ab1225 were subjected to treatment with acriflavine after overnight growth in nutrient broth. Mutants were isolated using a gradient plate technique as described previously with a concentration range between 0.03 and 0.3 % (Hunt & Sandham, 1969). A single colony at the highest concentration was selected and tested for further analysis. Strain Ab1225s was serially subcultured daily on MacConkey agar for more than 150 days in order to check for OMP changes and the stability of IS*Aba1*.

Antimicrobial susceptibility testing. The isolates were tested for their susceptibility to imipenem, meropenem, ceftazidime and piperacillin/tazobactam. MICs were determined by the agar double-dilution method according to British Society for Antimicrobial Chemotherapy (BSAC) methodology (Anonymous, 1991). The results were interpreted according to BSAC guidelines. The reference strains used for MIC testing were *A. baumannii* ATCC 19606, *Escherichia coli* NCTC 10418, *P. aeruginosa* NCTC 10662 and *S. aureus* NCTC 6571 (Andrews, 2007).

Analysis of gene expression. Expression of the $bla_{\text{OXA-51-like}}$ and bla_{ADC} genes was analysed by RT-PCR. The primers used for bla_{ADC} were ADC1 (5′-CCGCGACAGCAGGTGGATA-3′) and ADC2 (5′-TCGGCTGATTTTCTTGGTT-3′) with an annealing temperature of 51 °C, producing a product of 451 bp (Ruiz *et al.*, 2007). The primers used for $bla_{\text{OXA-51-like}}$ were 51F (5′-TTTCAGCCTGCTCACCTT-3′) and 51R (5′-TTCCCTTGAGGCTGAACAAC-3′) with an annealing temperature of 56 °C, producing a product of 679 bp (this study). Total RNA was extracted from isolates in the exponential growth phase using a RiboPure Bacteria kit (Ambion) and treated with the DNase I provided in the kit. cDNA was synthesized from 250 ng RNA using an Access Quick RT-PCR System kit (Promega). PCR products were run on an agarose gel and stained with GelRed (Cambridge Bioscience) for visualization. PCR products were quantified using Quantity One Software version 4.6.1 (Bio-Rad). The 16S rRNA gene was amplified as an internal control using primers 16S-F (5′-GACGTACTCGCAGAATAAGC-3′) and 16S-R (5′-TTAGTCTTGCGACCGTACTC-3′) at an annealing temperature of 56 °C (Lin *et al.*, 2009). The product size was 426 bp.

Analysis of OMP profiles. Strains were grown overnight in nutrient broth and OMP extraction was performed using a method described previously (Bossi & Bossi-Figueroa, 2007). The protein profiles were studied by 10 % SDS-PAGE with Molecular Weight Standards, Broad Range (New England Biolabs) used as a size marker. The protein concentration was estimated after reconstitution in buffer and approximately 45 µg protein was loaded into each well. After electrophoresis, the gels were stained with Coomassie Blue R-250 (Sigma).

RESULTS

Identification and typing

The isolates were screened for the conserved 16S–23S rRNA intergenic spacer sequences using primer 1 and

primer 2 as described above, amplifying a product of 975 bp, specific to *A baumannii*.

A fragment of approximately 2155 bp was obtained for strains Ab1225 and Ab1225s after amplification with the oxa-69A and oxa-69B primers, suggesting that the IS*Aba1* element was present upstream of the $bla_{\text{OXA-51-like}}$ gene. If the IS*Aba1* element was not present upstream, the fragment size was 975 bp, which was the size obtained for strain Ab153. IS*Aba1* was found upstream of the bla_{ADC} gene in strains Ab1225 and Ab1225s, giving a product of 751 bp. To check for overexpression of the $bla_{\text{OXA-51-like}}$ gene and transposition of the IS*Aba1* element, mutational analysis was performed using the dye acriflavine as a frameshift mutagen.

Sequencing of the $bla_{\text{OXA-51-like}}$ gene revealed that $bla_{\text{OXA-66}}$ was present in strain Ab153 and $bla_{\text{OXA-110}}$ in strain Ab1225.

Mutational analysis

IS*Aba1* movement was recorded in Ab1225M and Ab153M after acriflavine treatment had been performed. It was observed that strain Ab1225M lost its IS*Aba1* element, previously present upstream of the $bla_{\text{OXA-51-like}}$ gene, giving a product size of 975 bp, whereas strain Ab153M gained the IS*Aba1* element upstream of the $bla_{\text{OXA-51-like}}$ gene with a product size of 2155 bp.

IS*Aba1* movement was seen upstream of the bla_{ADC} gene in the acriflavine-treated Ab153M strain, but its loss was observed in the similarly treated strain Ab1225M. IS*Aba1* movement was not seen in the subcultured strain Ab1225s and its features remained identical with respect to the parental strain Ab1225. The IS*Aba1* sequence of strains Ab153M, parental Ab1225 and Ab1225s did not show any mutations or nucleotide substitutions.

Antimicrobial susceptibility testing

Table 1 shows the changes in MICs in the *A. baumannii* parental, mutant and serially subcultured strains. An eightfold rise in MICs for imipenem was seen in Ab153M and an eightfold decrease was seen in Ab1225M with respect to the parental strains Ab153 and Ab1225. A fourfold and twofold decrease in MIC for imipenem and meropenem was seen in Ab1225s with respect to Ab1225. There was negligible difference seen in MIC values for ceftazidime. No major difference was seen in the MIC values of piperacillin/tazobactam except for a fourfold decrease observed in strain Ab1225s in comparison with the Ab1225 parental strain. As no IS*Aba1* change was observed in Ab1225s, it was important to see whether there were any significant changes related to the OMPs of this strain, as it had a fourfold and twofold decrease in MIC for imipenem and meropenem compared with its parent, Ab1225.

Analysis of gene expression

Expression of the $bla_{\text{OXA-51-like}}$ and bla_{ADC} genes was analysed by RT-PCR and the products obtained were quantified using Quantity One software version 4.6.1. Product analysis determined that there was 2.7-fold increase in expression of the bla_{ADC} gene for Ab153M compared with Ab153. There was a small decrease (1.2-fold) seen in expression of the bla_{ADC} gene for Ab1225M compared with Ab1225. A negligible difference was seen in expression of the bla_{ADC} gene between Ab1225 and Ab1225s. It was observed that there was a 2.5-fold increase in expression of the $bla_{\text{OXA-51-like}}$ gene in Ab153M compared with Ab153. There was a fivefold decrease in expression of $bla_{\text{OXA-51-like}}$ in Ab1225M compared with Ab1225, and a fourfold decrease in expression of $bla_{\text{OXA-51-like}}$ in Ab1225s compared with Ab1225. This was confirmed three times and the results recorded were based on the mean increase or decrease of individual strains.

Analysis of OMP profiles

Fig. 1 shows the OMP profiles obtained for the *A. baumannii* parental, mutant and serially subcultured strains. Analysis of the OMP profiles revealed significant differences between the Ab153 parental strain and Ab153M. There was reduced expression of the 17, 23 and 25 kDa OMPs in the Ab153M strain with an increase in

Table 1. MICs of various antibiotics in the *A. baumannii* strains

Strain	MIC (mg l^{-1})				Expression of $bla_{\text{OXA-51-like}}$ gene*	Expression of bla_{ADC} gene*
	Imipenem	Meropenem	Piperacillin/tazobactam	Ceftazidime		
Ab153	0.25	1	32	32	0	0
Ab153M	2	4	64	128	+2.5	+2.7
Ab1225	2	4	64	128	0	0
Ab1225M	0.25	1	64	64	−5	−1.2
Ab1225s	0.5	2	16	128	−4	0

*Fold increase/decrease.

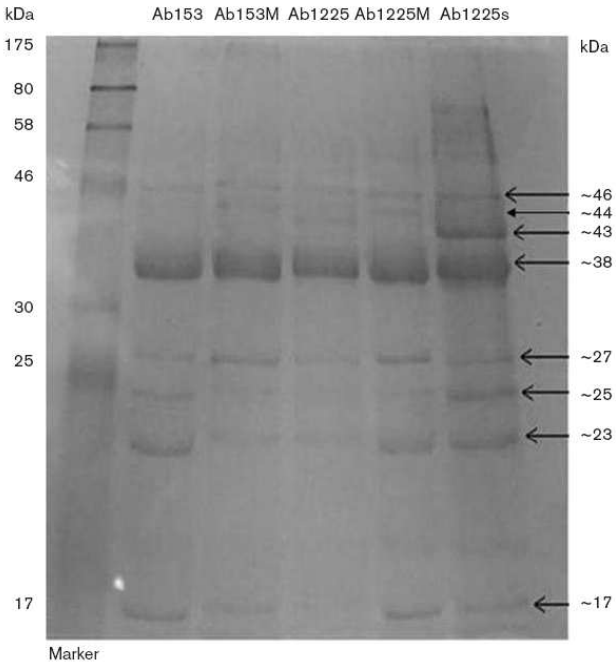


Fig. 1. OMP profiles of *A. baumannii* isolates. Ab153, parental strain; Ab153M, acriflavine mutant strain; Ab1225, parental strain; Ab1225M, acriflavine mutant strain; Ab1225s, MacConkey agar-subcultured strain.

MIC values. Ab1225M and Ab1225s exhibited an increase in expression of the 17, 23, 25 and 27 kDa OMPs with a decrease in MICs, rendering the strain more susceptible to some antibiotics. The OMP pattern produced by Ab1225s showed significant overexpression of a 43 kDa OMP.

DISCUSSION

All the *A. baumannii* strains were shown to exhibit different resistance profiles, which correlated with the movement of IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ or bla_{ADC} gene. The MIC values for the Ab153 and Ab1225 parental and mutant strains suggested that IS*Aba1* may act as a promoter for the $bla_{\text{OXA-51-like}}$ or bla_{ADC} gene. This is dependent upon IS*Aba1* acting as a promoter rather than just being present upstream of the antibiotic resistance gene. This also addressed the fact that IS*Aba1* may have a preferential role with respect to specific antibiotic resistance genes.

Strain Ab1225s had negligible effect on expression of the bla_{ADC} gene with respect to its Ab1225 parent, both of which had IS*Aba1* present upstream of the bla_{ADC} gene. This is supported by the fact that resistance to oxyiminocephalosporins is mainly due to hyperproduction of AmpC-type β-lactamases (Rodríguez-Martínez *et al.*, 2010). Ab1225s showed a fourfold decrease in $bla_{\text{OXA-51-like}}$ gene expression concurring with MICs to the carbapenems. There was increased expression of the 17, 23, 25, 27 and 43 kDa OMPs in Ab1225s, which led to a fourfold decrease in the MIC for imipenem, a twofold decrease in the MIC for meropenem and fourfold drop in the MIC for piperacillin/tazobactam. It was concluded that OMPs play a role in antibiotic resistance depending on the stress load of the compound and that they might be important for transport of the compound across the cell wall in either direction. The attenuation in Ab1225 may be due to OMP overexpression.

It was observed that all the *A. baumannii* isolates were resistant to ceftazidime, a fourth-generation cephalosporin, and thus IS*Aba1* may play a preferential role in increased expression of the $bla_{\text{OXA-51-like}}$ or bla_{ADC} gene. This concurs with the fact that genetic organization of genes and their control play a crucial role in antibiotic resistance.

It was also observed that there was overexpression of OMPs in Ab1225M, which correlated with a decrease in MIC values for different antibiotics. Strains Ab153 and Ab153M had changes with respect to their OMP profiles. As an eightfold increase in resistance to imipenem and fourfold increase to meropenem was seen in Ab153M, it can be deduced that the resistance developed in Ab153M may be due not only to the movement of IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ gene but also to a contribution by the reduced expression of three OMPs corresponding to the 17, 23 and 25 kDa proteins, all of which were poorly expressed in the mutant strain.

In contrast, overexpression of the 17, 23, 25 and 27 kDa OMPs could be seen in Ab1225M with a decrease in MIC

values, suggesting that these proteins may have some implications with respect to the increased susceptibility in this strain, which had lost IS*Aba1* upstream of the $bla_{\text{OXA-51-like}}$ and bla_{ADC} genes. The MICs of ceftazidime and piperacillin/tazobactam in Ab153 and Ab1225 parent and mutant strains coupled with the changes in β-lactamase expression may indicate the contributions made by the individual enzymes to the level of resistance; for example, changes in expression of the bla_{ADC} gene mirrored changes in the level of ceftazidime resistance.

It is clear from this study that the movement of IS*Aba1* is a strong factor for conferring resistance provided it is crucial for the gene action; if there is overexpression of primary surface structures of the bacterium, it can render the organism sensitive to a particular drug or poison.

Switching on the gene may be advantageous to this bacterium when it is required, as OMP changes can act synergistically; this was seen in Ab1225s and other strains and this depends on the action of promoters present for specific genes. This undoubtedly contributes to the organism's plasticity, making it resistant to different classes of antibiotics. Further studies to decipher the role of various factors involved in the pathogenesis of *A. baumannii* are necessary in order to understand its emergence as a multidrug-resistant pathogen.

ACKNOWLEDGEMENTS

We are grateful to the University of Edinburgh for the Overseas Research Scholarship and the College of Medicine and Veterinary Medicine bursary to B. S. L. We are grateful to the Medical Research Council for grant number RA0119, which funded part of this work.

REFERENCES

Andrews, J. M. (2007). BSAC standardized disc susceptibility testing method (version 6). *J Antimicrob Chemother* **60**, 20–41.

Anonymous (1991). A guide to sensitivity testing. Report of the Working Party on Antibiotic Sensitivity Testing of the British Society for Antimicrobial Chemotherapy. *J Antimicrob Chemother* **27** (Suppl. D), 1–50.

Aubert, D., Naas, T., Héritier, C., Poirel, L. & Nordmann, P. (2006). Functional characterization of IS*1999*, an IS*4* family element involved in mobilization and expression of β-lactam resistance genes. *J Bacteriol* **188**, 6506–6514.

Bossi, L. & Bossi-Figueroa, N. (2007). A small RNA downregulates LamB maltoprotein in *Salmonella*. *Mol Microbiol* **65**, 799–810.

Brown, S. & Amyes, S. G. B. (2006). OXA β-lactamases in *Acinetobacter*: the story so far. *J Antimicrob Chemother* **57**, 1–3.

Chen, C.-C. & Hu, S.-T. (2006). Two frameshift products involved in the transposition of bacterial insertion sequence IS*629*. *J Biol Chem* **281**, 21617–21628.

Corvec, S., Caroff, N., Espaze, E., Giraudeau, C., Drugeon, H. & Reynaud, A. (2003). AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains. *J Antimicrob Chemother* **52**, 629–635.

Corvec, S., Poirel, L., Naas, T., Drugeon, H. & Nordmann, P. (2007). Genetics and expression of the carbapenem-hydrolyzing oxacillinase gene $bla_{\text{OXA-23}}$ in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **51**, 1530–1533.

Dolzani, L., Tonin, E., Lagatolla, C., Prandin, L. & Monti-Bragadin, C. (1995). Identification of *Acinetobacter* isolates in the *A. calcoaceticus–A. baumannii* complex by restriction analysis of the 16S–23S rRNA intergenic spacer sequences. *J Clin Microbiol* **33**, 1108–1113.

Fournier, P. E. & Richet, H. (2006). The epidemiology and control of *Acinetobacter baumannii* in health care facilities. *Clin Infect Dis* **42**, 692–699.

Héritier, C., Poirel, L., Fournier, P.-E., Claverie, J.-M., Raoult, D. & Nordmann, P. (2005). Characterization of the naturally occurring oxacillinase of *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **49**, 4174–4179.

Hunt, D. E. & Sandham, H. J. (1969). Improved agar gradient-plate technique. *Appl Microbiol* **17**, 329–330.

Kornberg, A. (1980). *DNA Replication*. San Francisco, USA: W. H. Freeman and Co.

Lin, L., Ling, B.-D. & Li, X.-Z. (2009). Distribution of the multidrug efflux pump genes, *adeABC*, *adeDE* and *adeIJK*, and class 1 integron genes in multiple-antimicrobial-resistant clinical isolates of *Acinetobacter baumannii–Acinetobacter calcoaceticus* complex. *Int J Antimicrob Agents* **33**, 27–32.

Mahillon, J. & Chandler, M. (1998). Insertion sequences. *Microbiol Mol Biol Rev* **62**, 725–774.

Mugnier, P. D., Poirel, L. & Nordmann, P. (2009). Functional analysis of insertion sequence IS*Aba1*, responsible for genomic plasticity of *Acinetobacter baumannii*. *J Bacteriol* **191**, 2414–2418.

Nagai, T., Phan Tran, L. S., Inatsu, Y. & Itoh, Y. (2000). A new IS*4* family insertion sequence, IS*4Bsu1*, responsible for genetic instability of poly-γ-glutamic acid production in *Bacillus subtilis*. *J Bacteriol* **182**, 2387–2392.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* **67**, 593–656.

Peleg, A. Y., Seifert, H. & Paterson, D. L. (2008). *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin Microbiol Rev* **21**, 538–582.

Perez, F., Hujer, A. M., Hujer, K. M., Decker, B. K., Rather, P. N. & Bonomo, R. A. (2007). Global challenge of multidrug-resistant *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **51**, 3471–3484.

Rodriguez-Martinez, J.-M., Nordmann, P., Ronco, E. & Poirel, L. (2010). Extended-spectrum cephalosporinase in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* **54**, 3484–3488.

Ruiz, M., Marti, S., Fernandez-Cuenca, F., Alvaro, P. & Vila, J. (2007). Prevalence of IS*Aba1* in epidemiologically unrelated *Acinetobacter baumannii* clinical isolates. *FEMS Microbiol Lett* **274**, 63–66.

Spelman, D. W. (2002). Hospital-acquired infections. *Med J Aust* **176**, 286–291.

Vila, J., Martí, S. & Sánchez-Céspedes, J. (2007). Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. *J Antimicrob Chemother* **59**, 1210–1215.

Journal of Antimicrobial Chemotherapy Advance Access published October 5, 2011

J Antimicrob Chemother
doi:10.1093/jac/dkr415

Journal of Antimicrobial Chemotherapy

Disruption of the $bla_{OXA-51-like}$ gene by IS*Aba16* and activation of the bla_{OXA-58} gene leading to carbapenem resistance in *Acinetobacter baumannii* Ab244

Bruno S. Lopes[1], Benjamin A. Evans[2] and Sebastian G. B. Amyes[1]*

[1]*Centre for Infectious Diseases, The University of Edinburgh, 49 Little France Crescent, Edinburgh EH16 4SB, UK;* [2]*Faculty of Life Sciences, The University of Manchester, Michael Smith Building, Oxford Road, Manchester M13 9PT, UK*

*Corresponding author. Tel: +44-0131-242-6652; Fax: +44-0131-242-6611; E-mail: s.g.b.amyes@ed.ac.uk

Received 15 July 2011; returned 13 August 2011; revised 26 August 2011; accepted 6 September 2011

Objectives: This study examines the mechanism of carbapenem resistance in *Acinetobacter baumannii* isolate Ab244.

Methods: A multiplex PCR for the detection of the $bla_{OXA-23-like}$, $bla_{OXA-40-like}$, $bla_{OXA-51-like}$ and $bla_{OXA-58-like}$ families was performed. MICs of imipenem and meropenem were determined by the agar dilution method. The sequence surrounding the $bla_{OXA-132}$ gene was determined by amplification with primer pairs encompassing a part of *fxsA* and an acetyltransferase gene (*GNAT*). The sequence upstream of the bla_{OXA-58} gene was determined by sequencing. SDS–PAGE and *carO* PCR were performed to check the integrity of the outer membrane proteins. RT–PCRs for the expression of the $bla_{OXA-132}$ gene and the bla_{OXA-58} gene were performed.

Results: Isolate Ab244 harboured $bla_{OXA-132}$ belonging to the $bla_{OXA-51-like}$ gene cluster and a bla_{OXA-58} gene. The 4239 bp region between *fxsA* and *GNAT* showed an insert of IS*Aba16* (where IS stands for insertion sequence) after the first 15 nucleotides of the $bla_{OXA-132}$ gene, with an 8 bp target site duplication at the 5′ and 3′ ends of IS*Aba16*. The sequence oriented in the 5′→3′ direction caused insertional inactivation of the $bla_{OXA-132}$ gene. The bla_{OXA-58} gene was highly expressed by the promoters provided by an IS*Aba3*-like structure found upstream of the gene. The isolate was resistant to meropenem and had intermediate resistance to imipenem, and was also positive for IS*Aba1*.

Conclusions: This is the first report showing IS*Aba16*-mediated inactivation of the $bla_{OXA-132}$ gene in strain Ab244. The resistance to carbapenems in strain Ab244 is related to the acquisition of the bla_{OXA-58} gene, here governed by an IS*Aba3*-like element.

Keywords: *A. baumannii*, insertion sequences, β-lactamases, gene environment

Downloaded from jac.oxfordjournals.org at University of Edinburgh on October 9, 2011

Introduction

Acinetobacter baumannii is a pathogenic bacterium responsible for a wide range of infections, such as septicaemia, meningitis, pneumonia and urinary tract infections, and is currently one of the most important Gram-negative pathogens causing infections in immunocompromised patients.[1] β-Lactam antibiotics, mainly carbapenems, are the first choice against these microorganisms. However, in the last decade, resistance to these antimicrobial agents has appeared in hospitals worldwide, owing to the production of β-lactamases, changes in permeability, an increase in efflux and modification of the affinity of penicillin-binding proteins.[2,3] *A. baumannii* has been considered the paradigm of multiresistant bacteria, because of emerging multidrug resistance to various antimicrobial agents.[3] Transposable elements play an important role in the regulation of resistance genes in this bacterium.[4] Insertion sequences (ISs), such as IS*Aba1*, have been shown to govern the expression of the $bla_{OXA-51-like}$, $bla_{OXA-23-like}$ and bla_{ADC} genes.[5] ISs, such as IS*Aba2*, IS*Aba3* and IS*18*, have been reported to be present upstream of the $bla_{OXA-58-like}$ gene, causing its overexpression by providing strong promoters.[6] It can be shown by aligning the genomic sequences that the chromosomal regions in *A. baumannii* isolates have an *fxsA* gene and an intergenic spacer of 399 bp upstream of the $bla_{OXA-51-like}$ gene in susceptible strains. In some carbapenem-resistant isolates, an insertion element, such as IS*Aba1*, may be seen downstream of the intergenic spacer sequence but upstream of the $bla_{OXA-51-like}$ gene, and this usually provides a strong promoter to drive the expression of the $bla_{OXA-51-like}$ gene, as proven by others.[5,6] This study

© *The Author 2011. Published by Oxford University Press on behalf of the British Society for Antimicrobial Chemotherapy. All rights reserved. For Permissions, please e-mail: journals.permissions@oup.com*

aims to investigate the underlying carbapenem resistance mechanisms in the isolate Ab244, by mapping the regions upstream and downstream of the $bla_{OXA-51-like}$ gene and screening for other carbapenemases.

Materials and methods

A. baumannii isolate Ab244 was isolated from a patient from Carnaxide, Portugal. The preliminary method employed for identification was by restriction of amplified DNA fragments of the conserved 16S–23S rRNA spacer region by the enzymes AluI and NdeII, as described by Dolzani *et al.*[7] *A. baumannii* strain Ab2 was used as a control. The strains were kindly supplied by Dr Kevin Towner (Queens Medical Centre, Nottingham, UK).

Antimicrobial susceptibility testing

Ab244 was tested for its susceptibility to imipenem and meropenem. MICs were determined by the agar double dilution method according to BSAC methodology.[8] The results were interpreted according to BSAC guidelines.[8] The reference strains used for MIC testing were *Escherichia coli* NCTC 10418, *Pseudomonas aeruginosa* ATCC 10662 and *Staphylococcus aureus* NCTC 6571.

Screening for antimicrobial resistance determinants

The primers used in this study are listed in Table 1. Isolate Ab244 was screened by PCR for the presence of genes of the $bla_{OXA-23-like}$, $bla_{OXA-40-like}$, $bla_{OXA-51-like}$ and $bla_{OXA-58-like}$ families using multiplex primers, as described previously by Woodford *et al.*[9]

Primers FxOxa_F and FxOxa_R were designed to screen for the presence of an insertion element upstream of the $bla_{OXA-51-like}$ gene. They were also used for sequencing purposes. The internal primers used for walking were ISAba16_F1, ISAba16_R1, ISAba16_F2 and ISAba16_R2.

The primers 51F and IntaceR were used for mapping the $bla_{OXA-51-like}$ gene by screening for an insertion, if any, present downstream of the $bla_{OXA-51-like}$ gene.

The primers previously described by Héritier *et al.*[10] were used for the amplification of a 934 bp external fragment of the $bla_{OXA-58-like}$ gene. The primer SM2 described earlier by Poirel and Nordmann[6] was used in combination with the primer walk-58-R for the amplification of the region upstream of the $bla_{OXA-58-like}$ gene and for sequencing purposes.

The primers ISAba1A and ISAba1B were used for the identification of IS*Aba1*, as described previously by Poirel and Nordmann.[6]

The PCR conditions for the primers FxOxa_F and FxOxa_R were: initial denaturation at 95°C for 7 min; followed by 30 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 2 min; and a final extension at 72°C for 7 min. The PCR conditions for the rest of the primers were: initial denaturation at 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 55°C for 40 s and 72°C for 50 s; and a final extension at 72°C for 6 min.

Analysis of outer membrane proteins (OMPs)

The strains were grown overnight in nutrient broth and OMP extraction was performed as described previously.[11] The profiles were studied by SDS–PAGE using a 15% polyacrylamide gel and strain Ab2 as a comparison. The gels were stained with Coomassie blue R-250 (Sigma, UK). The protein concentration was estimated using a NanoDrop spectrophotometer (ND-1000). A concentration of 10 mg/mL of proteins reconstituted in buffer was loaded in each well, providing 100 μg of protein.

Insertion causing disruption of the *carO* gene (29 kDa OMP) was checked by using the established primers described earlier by Mussi *et al.*[12]

Analysis of gene expression

Expression of the $bla_{OXA-51-like}$ and $bla_{OXA-58-like}$ genes was studied by RT–PCR. Ab2, which was used as a control strain, possessed the $bla_{OXA-58-like}$ gene without an insertion upstream of the $bla_{OXA-58-like}$ or the $bla_{OXA-51-like}$ gene. The internal gene primers for expression of the $bla_{OXA-51-like}$ gene were those described earlier by Lopes *et al.*[13] Those for the $bla_{OXA-58-like}$ gene were as described earlier by Woodford *et al.*[9] Total RNA was extracted from isolates in exponential-phase growth using the RiboPure Bacteria kit (Ambion, UK) and treated with the DNase I provided with the kit. cDNA was synthesized from 100 ng of RNA using the Access quick RT–PCR system kit (Promega). PCR products were quantified using Bio-Rad Quantity One Software 4.6.1 (Bio-Rad). Normalization of DNA was done with the 16S rRNA primers described earlier by Lin *et al.*,[14]

Table 1. Primers used for PCR and genome walking

Primer name	Sequence (5′→3′)	Purpose	No. in Figure 1	No. in Figure 2
FxOxa_F	GAT ACC AGA CCT GGC AAC AT	W and C	1	
FxOxa_R	GCA CGA GCA AGA TCA TTA CC	W and C	2	
ISAba16_F1	ATC CGG CTA ACA CAG CCT TA	W	3	
ISAba16_R1	GAC CTG GCC TGA TTT AAA GC	W	4	
ISAba16_F2	AGG CAC TGA TCC AAG GTT TG	W	5	
ISAba16_R2	TAG TGC GAG TTC ATG CTC GT	W	6	
51F	TTT CAG CCT GCT CAC CTT	W and C	7	
IntaceR	GTT TTA CCC ACG CTG GTA CT	W and C	8	
SM2[a]	AAG TGT CTA TAT TCT CAC C	W and C		9
walk-58-R	CAG CAC AAG CCC CAA TAC TT	W and C		10
ISAba1A[a]	GTG CTT TGC GCT CAT CAT GC	C		
ISAba1B[a]	CAT GTA AAC CAA TGC TCA CC	C		
preOXA-58prom+[b]	TTA TCA AAA TCC AAT CGG C	W and C		11
preOXA-58B[b]	TAA CCT CAA ACT TCT AAT TC	W and C		12

W, chromosome walking; C, confirmatory PCR.
[a]Primers designed by Poirel and Nordmann.[6]
[b]Primers designed by Héritier *et al.*[10]

Downloaded from jac.oxfordjournals.org at University of Edinburgh on October 9, 2011

Table 2. MICs and the level of expression of bla_{OXA} genes

Strain	MIC (mg/L)		Quantification of gene expression							
	imipenem	meropenem	expression of the bla_{OXA-51} gene				expression of the bla_{OXA-58} gene			
Ab244	4	16	16S rRNA	209	OXA-51	0	16S rRNA	155	OXA-58	154
Ab2	0.25	1	16S rRNA	198	OXA-51	95.8	16S rRNA	160	OXA-58	69.1

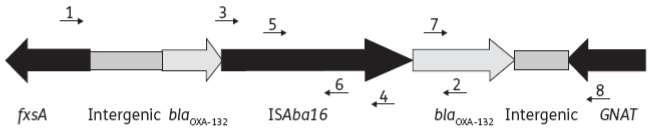


Figure 1. Genetic arrangement of the disrupted $bla_{OXA-132}$ gene due to the insertion of IS*Aba16*. The numbered arrows represent the primers described in Table 1. Figure is not to scale.

with the appropriate amounts of serially diluted RNA used for cDNA synthesis. A total of 15 cycles were employed for the amplification of the 16S rRNA gene and 25 cycles were used for the amplification of the antibiotic target genes. The RT–PCR assays were repeated thrice for confirmation purposes under the same optimal conditions.

Results

Identification and typing

The isolate Ab244 was confirmed as *A. baumannii* by restriction of the conserved 16S–23S rRNA intergenic spacer sequences, as described previously by Dolzani *et al.*[7]

Antimicrobial susceptibility testing

Isolate Ab244 had an imipenem MIC of 4 mg/L and a meropenem MIC of 16 mg/L (Table 2). The strain was classified as resistant to meropenem and intermediately resistant to imipenem.

PCR analysis and sequencing

The multiplex PCR for the OXA group of enzymes showed a positive result for the $bla_{OXA-51-like}$ and $bla_{OXA-58-like}$ genes, but was negative for the $bla_{OXA-23-like}$ and $bla_{OXA-40-like}$ genes. The expected product size was 889 bp without any insertion present upstream of the $bla_{OXA-51-like}$ gene, but the primers FxOxa_F and FxOxa_R amplified a product of 3449 bp.

Sequencing revealed that the chromosomal region, upstream of the $bla_{OXA-51-like}$ gene, had the conserved gene encoding a putative suppressor of F exclusion of phage T7 (*fxsA*), as observed earlier by Chen *et al.*,[15] followed by the 399 bp intergenic fragment, followed by a new IS named IS*Aba16* located following the first 15 bp and disrupting the $bla_{OXA-51-like}$ gene. An 8 bp target site duplication of the $bla_{OXA-51-like}$ gene encoding the sequence CTCTTACT was seen at the 5′ and 3′ ends of IS*Aba16* causing insertional inactivation of the $bla_{OXA-51-like}$ gene (Figure 1). Analysis of the IS revealed that it had the left inverted repeat 5′-GTAAGCATCCGGCTAA-3′ and the right inverted repeat 5′-TTCAGCGGACGCTTAC-3′. The IS*Aba16* sequence comprised three open reading frames (ORFs) encoding transposases A, B and C, respectively.

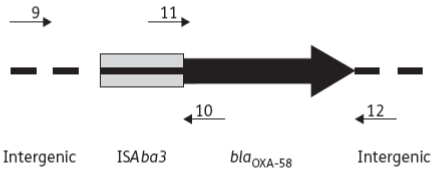


Figure 2. Genetic arrangement of the bla_{OXA-58} gene in Ab244. The numbered arrows represent the primers described in Table 1. Figure is not to scale.

The primers 51F and IntaceR amplified a product of 966 bp, and sequencing confirmed that the $bla_{OXA-51-like}$ gene was identical to $bla_{OXA-132}$. There was no insertion present downstream of the $bla_{OXA-132}$ gene, just the expected intergenic region and the phosphothricin acetyltransferase (GNAT) gene.

The primers preOXA-58prom+ and preOXA-58B, previously described by Héritier *et al.*[10] (Figure 2), amplified a 934 bp product of the $bla_{OXA-58-like}$ gene. The bla_{OXA-58} gene had no nucleotide substitutions and this was confirmed by sequencing.

The primers SM2 and walk-58-R (Figure 2) amplified a region of 575 bp, present upstream of the bla_{OXA-58} gene, the sequencing of which revealed an IS*Aba3*-like structure. A putative promoter with −35 (TTTATC) and −10 (TTTCTT) motifs was present 44 bp upstream of the $bla_{OXA-58-like}$ gene, as described earlier by Poirel and Nordmann.[6] The IS*Aba3*-like structure had a codon change at position 25 (GAT to TAT), causing the amino acid change D25Y, and a second codon change at position 110 (AAT to AGT), leading to the change N110S. The primers ISAba1A and ISAba1B detected the presence of IS*Aba1* in Ab244.

Analysis of OMPs

No change was seen in the OMPs after extraction and isolation on SDS–PAGE in comparison with OMPs of *A. baumannii* strain

Downloaded from jac.oxfordjournals.org at University of Edinburgh on October 9, 2011

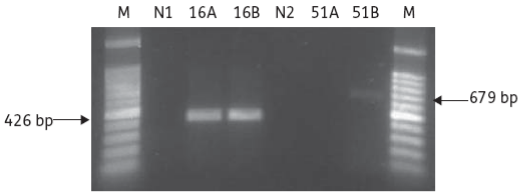


Figure 3. Expression of the $bla_{OXA-132}$ gene. N1, 16S rRNA negative control; 16A, expression of the 16S rRNA gene in Ab244; 16B, expression of the 16S rRNA gene in the control strain Ab2; N2, $bla_{OXA-51-like}$ negative control; 51A, expression of the $bla_{OXA-51-like}$ gene in Ab244; and 51B, expression of the $bla_{OXA-51-like}$ gene in Ab2. The $bla_{OXA-51-like}$ gene is shown at 679 bp and the 16S rRNA gene is shown at 426 bp.

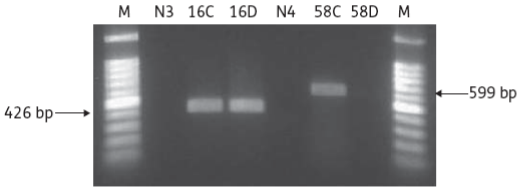


Figure 4. Expression of the bla_{OXA-58} gene. N3, 16S rRNA negative control; 16C, expression of the 16S rRNA gene in Ab244; 16D, expression of the 16S rRNA gene in the control strain Ab2; N4, $bla_{OXA-58-like}$ negative control; 58C, expression of the $bla_{OXA-58-like}$ gene in Ab244; and 58D, expression of the $bla_{OXA-58-like}$ gene in Ab2. The bla_{OXA-58} gene is shown at 599 bp and the 16S rRNA gene is shown at 426 bp.

Ab2. The primers described by Mussi *et al.*[12] for the *carO* gene did not identify any insert disrupting the *carO* gene.

Analysis of gene expression

Studies of $bla_{OXA-51-like}$ gene expression revealed that the gene was not expressed in Ab244 in comparison with strain Ab2, which had no insertion upstream of the $bla_{OXA-132}$ gene (Figure 3 and Table 2). In contrast, the $bla_{OXA-58-like}$ gene was highly expressed in Ab244 in comparison with the control strain Ab2, which had no insertion upstream of the $bla_{OXA-58-like}$ gene (Figure 4 and Table 2).

Discussion

In *A. baumannii* isolate Ab244, the conserved F-like structure gene (*fxsA*) was present on the chromosome with the intergenic fragment followed downstream by IS*Aba16*, which was inserted within the $bla_{OXA-51-like}$ gene causing insertional inactivation of the gene. Sequencing of the region between *fxsA* and the phosphinothricin acetyltransferase (*GNAT*) gene revealed that there was no insert present in the intergenic region downstream of the $bla_{OXA-51-like}$ gene. The region mapped in this study was 4239 bp, encompassing a part of the *fxsA* gene, the intergenic fragment upstream of the $bla_{OXA-51-like}$ gene, the $bla_{OXA-51-like}$ gene, the intergenic fragment downstream of *GNAT* and a part of *GNAT*. The inactivated $bla_{OXA-51-like}$ gene was identified as the $bla_{OXA-132}$ gene. Sequencing studies showed that IS*Aba16* inserted after the first 15 nucleotides of the $bla_{OXA-132}$ gene, which encode the Met-Asn-Ile-Lys-Thr amino acid sequence. A target site duplication of 8 bp having the sequence CTCTTACT was observed upstream and downstream of the IS*Aba16* element. RT–PCR showed no expression of the $bla_{OXA-132}$ gene. This was because of the interruption caused by IS*Aba16* being located following the first 15 bp of the $bla_{OXA-132}$ gene.

Analysis reveals that the insertion element IS*Aba16* belongs to the IS*66* family. It has been reported that the IS*66*-family elements may comprise three or more ORFs.[16] IS*Aba16* has the ability to transpose and to duplicate an 8 bp target site sequence on its transposition, as observed by the target site duplications present at the 5′ and 3′ ends of the insertion element. Transposases encoded by many IS elements belonging to IS families other than the IS*66* family have a DNA-binding domain with an α-helix–turn–α-helix DNA-binding motif and a catalytic domain with a DDE motif.[17] The insertion element IS*Aba16* shows the presence of three ORFs where the TnpA protein has an α-helix–turn–α-helix DNA-binding motif, and the TnpC protein has a potential DDE motif located at the catalytic core domain. The transposases from several superfamilies possess a protein domain containing an acidic amino acid triad (DDE or DDD) that catalyses the 'cut and paste' transposition reaction.[18]

The TnpB proteins, however, seem to have no homology to any of the motifs identified in the transposases encoded by the IS elements of different IS families.[17] Previous studies show that the TnpA, TnpB and TnpC proteins may be produced independently in appropriate amounts to form a complex, which acts as a transposase promoting the transposition of IS*66*-family elements, such as IS*Aba16* in strain Ab244.

As the multiplex PCR was positive for the $bla_{OXA-58-like}$ gene alongside the $bla_{OXA-132}$ gene, it was essential to map the sequence of the $bla_{OXA-58-like}$ gene; hence, a PCR was performed by using the primers described earlier by Héritier *et al.*[10] and sequencing of the PCR product revealed similarity to the bla_{OXA-58} gene without any nucleotide substitutions. The primers SM2 and walk-58-R amplified a 575 bp region, the sequencing of which revealed an IS*Aba3*-like structure. A putative promoter with −35 (TTTATC) and −10 (TTTCTT) motifs was present 44 bp upstream of the $bla_{OXA-58-like}$ gene. The promoters had been previously identified by Poirel and Nordmann.[6] RT–PCR proved that the $bla_{OXA-58-like}$ gene was highly expressed. The frequent association between the bla_{OXA-58} genes and ISs could be connected with the low levels of $bla_{OXA-51-like}$ gene expression in a wild-type strain having ISs dispersed in the genome. IS activation may depend on activation complexes formed by repressor-inhibitory mechanisms under antibiotic stress conditions.[19] Selection for ISs that insert into sites that allow the bacterium to survive antibiotic stress, such as upstream of β-lactamase genes where the IS elements provide strong promoters for β-lactamase gene expression, has provided the bacterium with a mechanism for transiently switching on resistance genes in response to the stress.[20]

Ab244 was resistant to meropenem and intermediately resistant to imipenem, although no change in the OMP pattern was seen. Furthermore, no insertional activation of the *carO* gene was identified.

Downloaded from jac.oxfordjournals.org at University of Edinburgh on October 9, 2011

The results obtained in this study portray Ab244 as a carbapenem-resistant pathogen that can employ selective mechanisms of resistance through the expression of specific antibiotic resistance genes. The study shows a genetic structure with IS*Aba16* inactivating the $bla_{OXA-132}$ gene and this could be due to the bacterium not requiring a mechanism of resistance when it was not under clinical conditions. The acquisition of the bla_{OXA-58} gene in the clinical setting facilitates the survival of the bacterium by helping it thrive better under antibiotic stress. This clearly demonstrates the acclimatization of Ab244 to various drug environments by the acquisition of carbapenemases, such as the bla_{OXA-58} gene governed by IS*Aba3*-like elements.

This is the first report describing the insertional inactivation of the $bla_{OXA-132}$ gene by IS*Aba16* and the employment of an alternate resistance mechanism by the overexpression of the bla_{OXA-58} gene leading to carbapenem resistance in strain Ab244.

Nucleotide sequence accession number

The nucleotide sequence described here has been submitted to the GenBank database under accession number JN415682.

Funding

We are grateful to the University of Edinburgh for the Overseas Research Scholarship and the College of Medicine and Veterinary Medicine bursary to B. S. L. We are grateful to the Medical Research Council for grant number RA0119, which funded part of this work.

Transparency declarations

None to declare.

References

1 Bergogne-Bérèzin E, Towner KJ. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. *Clin Microbiol Rev* 1996; **9**: 148–65.

2 Poirel L, Nordmann P. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clin Microbiol Infect* 2006; **12**: 826–36.

3 Vila J, Martí S, Sánchez-Céspedes J. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. *J Antimicrob Chemother* 2007; **59**: 1210–5.

4 Mugnier PD, Poirel L, Nordmann P. Functional analysis of insertion sequence IS*Aba1*, responsible for genomic plasticity of *Acinetobacter baumannii*. *J Bacteriol* 2009; **191**: 2414–8.

5 Turton JF, Ward ME, Woodford N *et al*. The role of IS*Aba1* in expression of OXA carbapenemase genes in *Acinetobacter baumannii*. *FEMS Microbiol Lett* 2006; **258**: 72–7.

6 Poirel L, Nordmann P. Genetic structures at the origin of acquisition and expression of the carbapenem-hydrolyzing oxacillinase gene bla_{OXA-58} in *Acinetobacter baumannii*. *Antimicrob Agents Chemother* 2006; **50**: 1442–8.

7 Dolzani L, Tonin E, Lagatolla C. Identification of *Acinetobacter* isolates in the *A. calcoaceticus-A. baumannii* complex by restriction analysis of the 16S-23S rRNA intergenic spacer sequences. *J Clin Microbiol* 1995; **33**: 1108–13.

8 BSAC. *Methods for Antimicrobial Susceptibility Testing—Version 6.1*. http://www.bsac.org.uk/Resources/BSAC/version_6.1.pdf (27 September 2011, date last accessed).

9 Woodford N, Ellington MJ, Coelho JM *et al*. Multiplex PCR for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. *Int J Antimicrob Agents* 2006; **27**: 351–3.

10 Héritier C, Dubouix A, Poirel L *et al*. A nosocomial outbreak of *Acinetobacter baumannii* isolates expressing the carbapenem-hydrolysing oxacillinase OXA-58. *J Antimicrob Chemother* 2005; **55**: 115–8.

11 Bossi L, Figueroa-Bossi N. A small RNA downregulates LamB maltoprotein in *Salmonella*. *Mol Microbiol* 2007; **65**: 799–10.

12 Mussi MA, Limansky AS, Viale AM. Acquisition of resistance to carbapenems in multidrug-resistant clinical strains of *Acinetobacter baumannii*: natural insertional inactivation of a gene encoding a member of a novel family of β-barrel outer membrane proteins. *Antimicrob Agents Chemother* 2005; **49**: 1432–40.

13 Lopes BS, Hamouda A, Findlay J *et al*. Effect of frame-shift mutagen acriflavine on control of resistance genes in *Acinetobacter baumannii*. *J Med Microbiol* 2010; **60**: 211–5.

14 Lin L, Ling BD, Li XZ. Distribution of the multidrug efflux pump genes, *adeABC*, *adeDE* and *adeIJK*, and class 1 integron genes in multiple-antimicrobial-resistant clinical isolates of *Acinetobacter baumannii–Acinetobacter calcoaceticus* complex. *Int J Antimicrob Agents* 2009; **33**: 27–32.

15 Chen TL, Lee YT, Kuo SC *et al*. Emergence and distribution of plasmids bearing the $bla_{OXA-51-like}$ gene with an upstream ISAba1 in carbapenem-resistant *Acinetobacter baumannii* isolates in Taiwan. *Antimicrob Agents Chemother* 2010; **54**: 4575–81.

16 Mahillon J, Chandler M. Insertion sequences. *Microbiol Mol Biol Rev* 1998; **62**: 725–74.

17 Han CG, Shiga Y, Tobe T *et al*. Structural and functional characterization of IS*679* and IS*66*-family elements. *Antimicrob Agents Chemother* 2001; **183**: 4296–304.

18 Yuan YW, Wessler SR. The catalytic domain of all eukaryotic cut-and-paste transposase superfamilies. *Proc Natl Acad Sci USA* 2011; **108**: 7884–9.

19 Escoubas JM, Prère MF, Fayet O *et al*. Translational control of transposition activity of the bacterial insertion sequence IS*1*. *EMBO J* 1991; **10**: 705–12.

20 Toleman MA, Walsh TR. Combinatorial events of insertion sequences and ICE in Gram-negative bacteria. *FEMS Microbiol Rev* 2011; **35**: 912–35.

Downloaded from jac.oxfordjournals.org at University of Edinburgh on October 9, 2011

G Model
ANTAGE-3761; No. of Pages 2
ARTICLE IN PRESS
International Journal of Antimicrobial Agents xxx (2012) xxx–xxx
Contents lists available at SciVerse ScienceDirect
International Journal of Antimicrobial Agents
journal homepage: http://www.elsevier.com/locate/ijantimicag

Letter to the Editor

The resistance profile of *Acinetobacter baumannii* strains isolated from the Aberdeen Royal Infirmary

Sir,

The increase in carbapenem resistance in *Acinetobacter baumannii* is largely attributable to the Ambler class D β-lactamases, in particular enzymes related to OXA-23 and OXA-58. The purpose of this study was to analyse the resistance in *A. baumannii* strains isolated from Aberdeen Royal Infirmary (Aberdeen, UK) from 2006 to 2010.

Nine non-repetitive *A. baumannii* strains were chosen for this study. The strains were identified by polymerase chain reaction (PCR) of the $bla_{\text{OXA-51-like}}$ gene and by sequencing of the *rpoB* gene. Minimum inhibitory concentrations (MICs) were determined according to the guidelines of the British Society for Antimicrobial Chemotherapy (BSAC). The $bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$, $bla_{\text{OXA-51-like}}$, $bla_{\text{OXA-58-like}}$ and $bla_{\text{OXA-143-like}}$ gene families were screened by multiplex PCR [1]. ISADC1 and OXA-23R primers were used for the detection of IS*Aba1* upstream of the $bla_{\text{OXA-23-like}}$ gene [1,2]. Aminoglycoside resistance genes were identified by multiplex PCR [3]. Primers for amplification of the bla_{ADC} gene have been described previously [2], and primers FU (5′-GCG CCG TGA ATT CTT AAG TG-3′) and RU (5′-AGC CAT ACC TGG CAC ATC AT-3′) were used to amplify the intergenic region upstream of the bla_{ADC} gene. PCR was performed for amplification of the quinolone resistance-determining regions (QRDRs) of the *gyrA* and *parC* genes [4], and gene fragments were sequenced for determining specific amino acid changes. PCR for detection of the class 1 integrase gene was performed as described previously [4].

A macrorestriction assay followed by pulsed-field gel electrophoresis (PFGE) was performed on all *A. baumannii* strains. Cluster analysis was performed by the unweighted pair-group method with mathematical averaging (UPGMA), and DNA relatedness was calculated using the band-based Dice coefficient with a tolerance setting of 1.5% band tolerance and 1.5% optimisation setting for the whole profile. Gel analysis was performed using BioNumerics v2.5 software (Applied Maths, Sint-Martens-Latem, Belgium). A value of ≥80% was chosen as the threshold for the establishment of clonal relatedness of the isolates.

S1 nuclease (Promega, Southampton, UK) digestion using 10 U of enzyme with incubation at 37 °C for 45 min was performed for the PFGE plugs according to the manufacturer's instructions. Plasmid curing was performed using acriflavine and with an elevated temperature of incubation. The strains were serially subcultured for 14 days at 47 °C.

Two novel variants of the $bla_{\text{OXA-51-like}}$ gene were found (Table 1): strain 14 had serine-14 (TCT) of the $bla_{\text{OXA-180}}$ gene replaced by phenylalanine (TTT) and is now designated $bla_{\text{OXA-216}}$; and strain 6n had threonine-255 (ACA) of the $bla_{\text{OXA-78}}$ gene substituted by isoleucine (ATA) and is now designated $bla_{\text{OXA-217}}$. Isolates 10 and 10n had variants of $bla_{\text{OXA-65}}$ gene with synonymous mutations.

Strains 3 and 12 possessed the *aac(3′)-Ia* gene conferring gentamicin resistance and had high ceftazidime MICs owing to the presence of IS*Aba1* upstream of the bla_{ADC} gene. All of the remaining strains (except 10) had the bla_{ADC} gene without IS*Aba1* upstream and thus were susceptible to ceftazidime (Table 1). Strain 10 completely lacked the bla_{ADC} gene. All of the isolates were susceptible to colistin.

Strains 3 and 12 had amino acid changes at position 83 of GyrA (serine83 → leucine) and position 80 of ParC (serine80 → leucine) conferring ciprofloxacin resistance. Both strains possessed integrase genes and sequencing confirmed the presence of putative glucose dehydrogenase precursor that could be responsible for catabolism of glucose by oxidation.

The PFGE profiles of the strains showed that most were not clonally related as they had <80% similarity (data not shown). PCR for insertions causing disruption of *carO* (29 kDa outer membrane protein) was not detected for any of the strains. PFGE analysis revealed that strains 3 and 12 had 83% similarity; strain 3, isolated in the year 2006, was negative for the IS*Aba1*–$bla_{\text{OXA-23}}$ gene, whereas strain 12, isolated in the year 2008, was positive and was resistant to imipenem and meropenem (Table 1). Strains 3 and 12

Table 1
Resistance profiles of *Acinetobacter baumannii* clinical strains.

Strain no.	Source of isolation	Date of isolation	MIC (mg/L)						*intI*	bla_{ADC}	IS*Aba1*–bla_{ADC}	$bla_{\text{OXA-51-like}}$ gene
			MEM	IPM	CAZ	GEN	CIP	COL				
3	Blood	09/09/2006	1	0.5	64	256	32	0.25	+	+	+	66
10	Blood	08/06/2008	0.06	0.06	1	0.12	0.12	0.12	–	–	–	65 (variant)
12	Blood	25/08/2008	16	16	64	8	32	0.5	+	+	+	66
14	Blood	21/09/2009	0.06	0.06	4	0.03	0.12	0.03	–	+	–	216
16	Sputum	20/02/2010	0.5	0.5	4	0.12	0.12	0.12	–	+	–	51
3n	Blood	27/04/2006	0.5	0.25	2	0.03	0.12	0.12	–	+	–	64
6n	Blood	17/07/2007	1	0.5	8	0.12	0.5	0.5	–	+	–	217
10n	Blood	22/10/2008	0.06	0.06	1	0.03	0.12	0.12	–	+	–	65 (variant)
14n	Blood	14/09/2009	0.5	0.25	8	0.12	0.5	0.12	–	+	–	89

MIC, minimum inhibitory concentration; MEM, meropenem; IPM, imipenem; CAZ, ceftazidime; GEN, gentamicin; CIP, ciprofloxacin; COL, colistin.

0924-8579/$ – see front matter © 2012 Elsevier B.V. and the International Society of Chemotherapy. All rights reserved.
doi:10.1016/j.ijantimicag.2011.12.004

Please cite this article in press as: Lopes BS, et al. The resistance profile of *Acinetobacter baumannii* strains isolated from the Aberdeen Royal Infirmary. Int J Antimicrob Agents (2012), doi:10.1016/j.ijantimicag.2011.12.004

had an identical $bla_{OXA-51-like}$ allele 1 corresponding to sequence group 1. In addition, strain 12 had the bla_{OXA-23} clone 1 allele, which belongs to European clone II. This indicates the *A. baumannii* is actively acquiring resistance genes, probably through plasmid transfer, although *S1* nuclease digestion and plasmid extraction procedures did not detect any plasmids, and elimination studies with acriflavine did not remove the resistance determinants. This suggests that if bla_{OXA-23} was plasmid borne, it is now integrated in the host chromosome of strain 12 endowing it with a stable mechanism of carbapenem resistance.

These results show that the clinical situation in the hospital in Aberdeen is in a state of flux. New variant strains are emerging and, most importantly, a carbapenem-sensitive strain has become resistant through acquisition of the bla_{OXA-23} gene with an IS*Aba1* element upstream that carries a promoter allowing expression of the β-lactamase. The bla_{OXA-23} gene was first found in Scotland more than 20 years ago and it has remained the sole mechanism of carbapenem resistance until this point [5].

Nucleotide accession numbers

The $bla_{OXA-216}$ and $bla_{OXA-217}$ genes have been deposited in GenBank under the accession nos. FR865168 and JN603240, respectively.

Funding: University of Edinburgh Overseas Research Scholarship to BSL; MRC grant number RA0119.

Competing interests: None declared.

Ethical approval: Not required.

References

[1] Higgins PG, Lehmann M, Seifert H. Inclusion of OXA-143 primers in a multiplex polymerase chain reaction (PCR) for genes encoding prevalent OXA carbapenemases in *Acinetobacter* spp. Int J Antimicrob Agents 2010;35:305.

[2] Ruiz M, Marti S, Fernandez-Cuenca F, Pascual A, Vila J. Prevalence of IS*Aba1* in epidemiologically unrelated *Acinetobacter baumannii* clinical isolates. FEMS Microbiol Lett 2007;274:63–6.

[3] Noppe-Leclercq I, Wallet F, Haentjens S, Courcol R, Simonet M. PCR detection of aminoglycoside resistance genes: a rapid molecular typing method for *Acinetobacter baumannii*. Res Microbiol 1999;150:317–22.

[4] Golanbar GD, Lam CK, Chu YM, Cueva C, Tan SW, Silva I, et al. Phenotypic and molecular characterization of *Acinetobacter* clinical isolates obtained from inmates of California correctional facilities. J Clin Microbiol 2011;49:2121–31.

[5] Donald HM, Scaife W, Amyes SG, Young HK. Sequence analysis of ARI-1, a novel OXA β-lactamase, responsible for imipenem resistance in *Acinetobacter baumannii* 6B92. Antimicrob Agents Chemother 2000;44:196–9.

B.S. Lopes
Centre for Infectious Diseases, The University of Edinburgh, Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK

I.M. Gould
Medical Microbiology, Aberdeen Royal Infirmary, Foresterhill, Aberdeen AB25 2ZN, UK

A.F. Opazo
S.G.B. Amyes*
Centre for Infectious Diseases, The University of Edinburgh, Chancellor's Building, 49 Little France Crescent, Edinburgh EH16 4SB, UK

*Corresponding author. Tel.: +44 131 242 6652; fax: +44 131 242 6611.
E-mail address: s.g.b.amyes@ed.ac.uk (S.G.B. Amyes)

21 October 2011

Please cite this article in press as: Lopes BS, et al. The resistance profile of *Acinetobacter baumannii* strains isolated from the Aberdeen Royal Infirmary. Int J Antimicrob Agents (2012), doi:10.1016/j.ijantimicag.2011.12.004

APPENDIX B:

Abstracts of conference posters and presentations

Role of IS*Aba825* in the expression of the *bla*$_{\text{OXA-51-like}}$ gene in clinical strains of *Acinetobacter baumannii*

B. S. Lopes and S.G.B Amyes

University of Edinburgh, UK

Objective:

The number of nosocomial *Acinetobacter* infections has risen in recent years. These gram-negative coccobacilli are now major opportunistic pathogens, predominantly causing infections of the respiratory and urinary tracts and of the skin, catheter-related infections and bacteremias. The aim of this study was to investigate the role of insertion sequences involved in the expression of *bla*$_{\text{OXA-51-like}}$ and *bla*$_{\text{ADC}}$ gene present in *A. baumannii.*

Methods:

7 multi drug resistant strains isolated in the United States were screened for the presence of insertion sequences and antibiotic resistant genes. The isolates were confirmed as *A. baumannii* by *bla*$_{\text{OXA-51-like}}$ PCR and by restriction analysis using the enzymes *Alu*I and *Nde*II. The isolates were screened for the presence of insertion sequence upstream and downstream of *bla*$_{\text{OXA-51-like}}$ and *bla*$_{\text{ADC}}$ gene. The MICs of imipenem, meropenem and ceftazidime were determined by the BSAC guidelines. Multiplex PCR for oxacillinases was performed as described previously.

Results:

All the strains were positive for the presence of *bla*$_{\text{OXA-51-like}}$ gene and *bla*$_{\text{OXA-58}}$ gene; 4 isolates showed the presence of insertion sequence IS*Aba825* upstream of the *bla*$_{\text{OXA-51-like}}$ gene and 3 had an insert of IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene. The MIC of IPM strains having IS*Aba825* was 16mg/L and that of meropenem was 4 mg/L. The chromosomal *bla*$_{\text{OXA-51-like}}$ genes of these 4 strains were *bla*$_{\text{OXA-65}}$.

The MIC of IPM ranged from 4-8 and for MEM ranged from 4-16 for the 3 strains having IS*Aba1* upstream of the *bla*$_{\text{OXA-51-like}}$ gene. The 3 strains had *bla*$_{\text{OXA-79}}$, *bla*$_{\text{OXA-80}}$ and *bla*$_{\text{OXA-82}}$ gene. The *bla*$_{\text{ADC}}$ gene was present in all the strains and, in all strains, IS*Aba1* was located upstream of the gene and giving each strain an MIC of 128mg/L of ceftazidime.

The IS*Aba825* sequence was seen 7bp (AAGTCTT) upstream of the *bla*$_{\text{OXA-51-like}}$ gene. It was oriented in 5'-3' direction with respect to the *bla*$_{\text{OXA-65}}$ gene and putative promoters -10 TATGAA and -35 TTGTCA were located 17bp apart from each other and were located 74bp and 97bp upstream of the *bla*$_{\text{OXA-65}}$ gene. There was no insertion sequence detected downstream for all strains making it a defunct transposon.

Conclusions:

These data indicates a first report on the presence of a novel insertion element IS*Aba825* present upstream of the *bla*$_{\text{OXA-51-like}}$ gene, besides the well known insertion sequence IS*Aba1*, that can cause over-expression of the *bla*$_{\text{OXA-51-like}}$ gene. The IS*Aba825* element may also have a better promoter than IS*Aba1* to cause *bla*$_{\text{OXA-51-like}}$ over-expression.

The role of Insertion Elements in control of antibiotic resistance in *Acinetobacter baumannii* isolated from sources throughout the world.

B. S. Lopes and S.G.B Amyes

University of Edinburgh, UK

Background: The aim of this study was to investigate the drug resistance profile and the frequency of insertion elements found in *A. baumannii* strains isolated from various sources around the world.

Methods: A collection of 85 epidemiologically unrelated *A. baumannii* isolates were studied. Their identity was confirmed by $bla_{\text{OXA-51-like}}$ PCR and by restriction analysis of the 16S-23S rRNA fragment. The isolates were screened for the presence of insertion sequence (IS) upstream of the $bla_{\text{OXA-51-like}}$ by PCR amplification and sequencing. Primers FU and RU were used to check for the insert upstream of the bla_{ADC} gene. A multiplex PCR for oxacillinases was also performed. MICs for imipenem and ceftazidime were determined by the agar dilution method and the results interpreted according to the BSAC guidelines.

Results: All the isolates were positive for $bla_{\text{OXA-51-like}}$ gene; 7% of the isolates harboured the $bla_{\text{OXA-23}}$ gene, 10% had the $bla_{\text{OXA-24}}$ gene and 36% had the $bla_{\text{OXA-58}}$ gene. The MIC90 of imipenem was 16mg/L and the MIC90 of ceftazidime was >256mg/L. It was observed that, 88% of the isolates were resistant to ceftazidime, of which 63% (8% having IS*Aba125* and 55% having IS*Aba1*) harboured an insertion sequence upstream of the bla_{ADC} gene. Furthermore, 26% isolates were resistant to imipenem, 33% exhibited intermediate level of resistance of imipenem and 22% of the isolates had an insert upstream of the chromosomal $bla_{\text{OXA-51-like}}$ gene (5% having IS*Aba825* and 17% having IS*Aba1*).

Conclusions: In this study we report the prevalence of IS*Aba1* along with the rarer IS*Aba125* and IS*Aba825*, which have been found in clinically relevant strains, upstream of the bla_{ADC} and $bla_{\text{OXA-51-like}}$ gene respectively. In summary, we conclude that IS*Aba125* and IS*Aba825* may govern the expression of bla_{ADC} gene and $bla_{\text{OXA-51-like}}$ gene in highly resistant clinical strains in the same manner as IS*Aba1*.

Ceftazidime resistance in *Acinetobacter baumannii* from the United Arab Emirates

A. F. Opazo[1], **B. S. Lopes[1]**, A. Sonnevend[2], T. Pal[2], A. Ghazawi[2], and S.G.B. Amyes[1].

University of Edinburgh, UK[1] and University of United Arab Emirates[2]

Background:
Acinetobacter baumannii is one of the most important microorganism causing infections in hospitalized patient. The remaining options for the treatment of serious infectious diseases causes by multi-resistant *A. baumannii* are carbapenems, cephalosporins and colistin. Resistance to cephalosporins has been associated with the insertion sequence IS*Aba1* providing a strong promoter to ADC-genes.

Methods:
3 strains of *A. baumannii* isolated in Al Ain, UAE, named Nm55, Nm57 and Nm128, were studied. Nm57 was isolated from an intravenous catheter tip of an adult patient, Nm55 and Nm128 were both isolated from sputum samples taken 15 weeks apart from a 6 year old patient. They were confirmed as A. baumannii by PCR detecting the $bla_{\text{OXA-51-like}}$ gene and sequencing the *rpoB* gene. Genotypic characterization was performed by pulsed-field gel electrophoresis (PFGE). Antibiotic susceptibility and determination of minimal inhibitory concentration (MIC) of ceftazidime were performed according to BSAC guideline. Detection of bla_{OXA} genes was performed by multiplex-PCR and detection of IS*Aba1* and ADC was done by standard-PCR.

Results:
All the strains were designated as identical according to PFGE pattern.All strains were resistant to carbapenems, cefotetan, aztreonam, cefoperazone, cefepime, cefotaxime and cefpodoxime. Strain Nm55 was resistant to ceftazidime, while Nm57 was intermediate and Nm128 was susceptible. All the strains harbour both OXA-64 chromosomal and the OXA-23 plasmid ß-lactamases. IS*Aba1* was upstream of the $bla_{\text{OXA-23}}$ genes in all three strains and was responsible for carbapenem resistance. All the strains harboured the same bla_{ADC} gene but in no case was there a defined IS element upstream.

Conclusions:
This study shows a rare example of an *A. baumannii* strain that was resistant to carbapenems but was sensitive to ceftazidime. When the strain progressed to ceftazidime resistance this was not by the reported method of an insertion of an IS*Aba1*-like element upstream bla_{ADC} gene providing the necessary promoter.

The role of IS*30* in the expression of the *bla*$_{ADC}$ gene in *Acinetobacter baumannii*

B. Lopes, A. Hamouda and S. Amyes.
University of Edinburgh, UK

Objective:
Multidrug-resistant *Acinetobacter baumannii* is a major nosocomial pathogen that is rapidly evolving and developing resistance to all major classes of antibiotics. It is identified by the presence of intrinsic *bla*$_{OXA-51-like}$ gene and restriction of 16S-23S rRNA intergenic spacer sequences. The aim of this study was to investigate the role of insertion sequences related to the expression of cephalosporinanses present in *A. baumannii.*

Methods:
A panel of 17 geographically diverse *A. baumannii* isolates was screened for the presence of insertion sequence upstream and downstream of *bla*$_{ADC}$ gene. The isolates were confirmed as *A. baumannii* by *bla*oxa-51-like PCR and by restriction analysis using the enzymes *Alu*I and *Nde*II. The MICs of ceftazidime were determined by the BSAC guidelines. The strains were checked for ESBL production by synergy disc assay between cephalosporins and augmentin (co-amoxiclav).

Results:
The MICs for ceftazidime showed that 4 strains had an MIC of > 256mg/L, 7 strains had an MIC of 128mg/L, 1 strain had an MIC of 64mg/L and 5 strains had an MIC of 32mg/L. None of the strains produced ESBLs as tested by synergy between augmentin and cephalosporins such as ceftazidime, cefotaxime, cefipime and cefpodoxime. PCR results showed that IS*Aba1* was present upstream of *bla*$_{ADC}$ gene in 12 strains (MICs ranging from 32-128mg/L). Out of the five strains having MIC of 32mg/L one strain did not have IS*Aba1* present upstream of *bla*$_{ADC}$ gene. The 4 strains with MICs >256mg/L harboured IS*30* upstream of *bla*$_{ADC}$ gene. There was no insertion sequence detected downstream for all 17 strains making it a defunct transposon. The IS*30* insert was observed 126bp upstream of *bla*$_{ADC}$ gene. Although the region just upstream of *bla*$_{ADC}$ gene had a functional promoter, the IS*30* element also had a promoter for the transposase it carries and this may also increase the expression of the *bla*$_{ADC}$ gene.

Conclusions:
These data indicate the presence of a novel insertion element IS*30* besides IS*Aba1* that can cause overexpression of *bla*$_{ADC}$ gene. The isolates with IS*30* may encode novel ADC variants having significant hydrolysing activity against ceftazidime. The IS*30* element may also have a better promoter than IS*Aba1* to cause *bla*$_{ADC}$ gene overexpression.

The utilisation of gluconic acid in certain strains of *Acinetobacter baumannii*

B. Lopes, A. Hamouda and S. Amyes.
University of Edinburgh, UK

Background:
A characteristic feature of glucose oxidizing Acinetobacters is seen with blood agar having aldose sugar. The aim of this study was to evaluate the nature of brown pigment produced by *Acinetobacter baumannii.*

Methods:
Two (A/B) *A. baumannii* strains were isolated from diabetic patient and identified by $bla_{\text{OXA-51-like}}$ PCR and restriction analysis of 16S and 23S r-RNA spacer sequences using *Alu*I and *Nde*II. MICs for to imipenem , meropenem and ceftazidime were estimated by BSAC guidelines. Isosensitest (IST)/M9 glucose broth was used for the growth of strains. Growth of the strains was monitored over 48 hour period using IST broth having gluconic acid concentration range from 0.1 to 4%. The inhibitory activity of the pigment produced by strain A was checked by ditch plate method.

Results:
The MICs for IPM, MEM, CTZ and FEP were 0.5, 0.5, 8 and 4mg/L respectively for strain A and B. Both the strains were positive for $bla_{\text{oxa-51-like}}$ and bla_{ADC} but negative $bla_{\text{oxa-23/40/58}}$ and for metallo-beta-lactamases. It was seen that the strain A produced a brown pigment in presence of gluconic acid. It also grew better than strain B over a period of 48 hours in the presence of IST broth containing gluconic acid concentration ranging from 0.1 to 4%. Both strains did not produce any pigment in Nutrient glucose broth. The brown pigment produced by strain A did not have any inhibitory effect against *S.aureus* NCTC6571, *Ps. aeruginosa* NCTC10662, *A. baumannii* ATCC19606 and *E.coli* NCTC10418. The glucose dehydrogenase enables both the strains to form 6-phosphogluonate which is free to enter the Entner-Doudouroff pathway hence there is no pigment production seen in both the strains when M9 glucose broth is used. On the other side human blood being enriched with nutrients including gluconic acid helps strain A to survive better by converting gluconic acid to 2,5 diketogluconate which leads to the formation of brown pigmentation. It is also possible that strain B lacks gluconic acid dehydrogenase which could lead to the block in successive steps making glycolysis the preferential path for metabolism. In strain B it is also possible that the enzyme 2 keto gluconate dehydrogenase is absent which converts 2 keto gluconate to 2, 5-diketo gluconate which is responsible for brown pigmentation. Strain A can metabolise glucose by glycolysis as well as Entner Doudouroff pathway. It is also possible that strain A follows the pathway for brown pigment production converting excessive gluconic acid to 2, 5 diketogluconate depending on the gluconic acid concentration as the intensity of absorbance increased exponentially from lower concentration to higher. 2, 5 diketogluconate being a precursor for ascorbic acid production may offer protection against antioxidant stress. For strain B glycolysis may be the only option for metabolism unless gluconic acid dehydrogenase is present by which it can enter the ED pathway bypassing the step for pigment production which is due to functional 2-ketogluconate dehydrogenase.

Conclusions:
The survival of *A. baumannii* A in gluconic acid enriched medium helps it to survive better than *A.baumannii* B. Excess gluconic acid in strain A leads to brown pigmentation which may offer protection against antioxidant stress**.** The results show that strain A has multiple routes of metabolism which offers it better chance for survival than strain B.