Effect of frameshift mutagen acriflavine on control of resistance genes in *Acinetobacter baumannii*

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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 septicæmic shock. This study evaluated the role of acriflavine, a frameshift mutagen, on the movement of insertion sequence *ISAbal* in clinical isolates of *A. baumannii*, with the focus on changes in expression levels of the *bla*_{ADC} and *bla*_{OXA-51-like} genes. Resistance profiles were assessed with consideration of *ISAbal* acting as a promoter upstream of the *bla*_{ADC} or *bla*_{OXA-51-like} gene. *ISAbal* movement was observed in the acriflavine mutants Ab153M and Ab1225M. Ab153M exhibited an increase in the MIC values of carbapenems and ceftazidime, with *ISAbal* gained upstream of the *bla*_{ADC} and *bla*_{OXA-51-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 *ISAbal* upstream of the *bla*_{ADC} and *bla*_{OXA-51-like} genes in strain Ab1225M, and a significant decrease in *bla*_{OXA-51-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 *ISAbal* upstream of the *bla*_{ADC} and *bla*_{OXA-51-like} genes. A significant decrease in *bla*_{OXA-51-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 *ISAbal* is present upstream of an antibiotic resistance gene, it may not necessarily contribute towards the overexpression of antibiotic resistance genes (*bla*_{OXA-51-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.

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INTRODUCTION

Acinetobacter baumannii is one of the most difficult nosocomial pathogens to treat, similar to other bacterial pathogens such as methicillin-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). *ISAbal* has been associated with the expression of several antibiotic resistance genes including *bla*_{OXA-51-like} and *bla*_{ADC} (Corvec *et al.*, 2003, 2007). It is also known that frameshift products are involved in transposition of IS629, a member of the IS3 family (Chen & Hu, 2006; Mahillon & Chandler, 1998). *ISAbal* belongs to the IS4 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 *IS4Bsu1*, a member of the IS4 family in *Bacillus subtilis*, is responsible for the genetic instability of poly- γ -glutamic acid production and that *IS1999*, 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*_{OXA-51-like} and *bla*_{ADC} as a result of the transposition of *ISAbal*. This study also aimed to identify the changes in levels of expression of antibiotic resistance genes due to the movement of *ISAbal*.

METHODS

The bacterial strains used in this study were *A. baumannii* 153 (Ab153) (Nottingham, UK), *A. baumannii* 153 acriflavine mutant (Ab153M), *A. baumannii* 1225 (Ab1225) (Wrocław, Poland), *A. baumannii* 1225 acriflavine mutant (Ab1225M) and *A. baumannii* 1225 serially subcultured strain (Ab1225s) (Wrocław, 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'-TTGTACACACCGCCGTC-3') and primer 2 (5'-GGTACTTAGATGTTTCAGTTC-3'). The expected product size was 975 bp. Restriction of the product by the enzyme *AluI* produced fragments of 50, 125, 135, 165, 175 and 330 bp specific for *A. baumannii*. Restriction of the same product with *NdeI* 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*_{OXA-51-like} gene by PCR with the primers *oxa*-69A (5'-CTAATAATTGATCTACTCAAG-3') and *oxa*-69B (5'-CCAGTGATGGATAGATTATC-3') at an annealing temperature of 48 °C.

The expected product sizes were 975 bp for strains without *ISAbal* present upstream of the *bla*_{OXA-51-like} gene and 2155 bp for strains with *ISAbal* present upstream of the *bla*_{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'-ACGT-CGCGAGTTGAAGTAAGTT-3') with an annealing temperature of 51 °C. The expected product size was 751 bp if *ISAbal* 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 *ISAbal*.

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*_{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'-TCGGCTGATTTCTTGGTT-3') with an annealing temperature of 51 °C, producing a product of 451 bp (Ruiz *et al.*, 2007). The primers used for *bla*_{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'-TTAGTCTTGC-GACCGTACTC-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 μ g 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*_{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*_{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*_{OXA-51-like} gene revealed that *bla*_{OXA-66} was present in strain Ab153 and *bla*_{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*_{OXA-51-like} gene, giving a product size of 975 bp, whereas strain Ab153M gained the IS*Aba1* element upstream of the *bla*_{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*_{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*_{OXA-51-like} gene in Ab153M compared with Ab153. There was a fivefold decrease in expression of *bla*_{OXA-51-like} in Ab1225M compared with Ab1225, and a fourfold decrease in expression of *bla*_{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 <i>bla</i> _{OXA-51-like} gene*	Expression of <i>bla</i> _{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 *ISAbal* upstream of the *bla*_{OXA-51-like} or *bla*_{ADC} gene. The MIC values for the Ab153 and Ab1225 parental and mutant strains suggested that *ISAbal* may act as a promoter for the *bla*_{OXA-51-like} or *bla*_{ADC} gene. This is dependent upon *ISAbal* acting as a promoter rather than just being present upstream of the antibiotic resistance gene. This also addressed the fact that *ISAbal* 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 *ISAbal* 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*_{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 *ISAbal* may play a preferential role in increased expression of the *bla*_{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 *ISAbal* upstream of the *bla*_{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 *ISAbal* upstream of the *bla*_{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 *ISAbal* 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.

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