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

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 septicemia shock. This study evaluated the role of acriflavine, a frameshift mutagen, on the movement of insertion sequence ISAb1 in clinical isolates of A. baumannii, with the focus on changes in expression levels of the blaADC and blaOXA-51-like genes. Resistance profiles were assessed with consideration of ISAb1 acting as a promoter upstream of the blaADC or blaOXA-51-like gene. ISAb1 movement was observed in the acriflavine mutants Ab153M and Ab1225M. Ab153M exhibited an increase in the MIC values of carbapenems and ceftazidime, with ISAb1 gained upstream of the blaADC and blaOXA-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 ISAb1 upstream of the blaADC and blaOXA-51-like genes in strain Ab1225M, and a significant decrease in blaOXA-51-like gene expression and, to a lesser extent, in blaADC 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 ISAb1 upstream of the blaADC and blaOXA-51-like genes. A significant decrease in blaOXA-51-like gene expression was observed in Ab1225s, whereas the expression of blaADC 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 ISAb1 is present upstream of an antibiotic resistance gene, it may not necessarily contribute towards the overexpression of antibiotic resistance genes (blaOXA-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.

INTRODUCTION

Acinetobacter baumannii is one of the most difficult nosocomial 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 supplicative 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-lactamas), class C (Acinetobacter derived cephalosporinases AmpC/blaADC) and class D (the oxacilinases: 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...
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). ISAba1 has been associated with the expression of several antibiotic resistance genes including blaOXA-51-like and blaADC (Corvec et al., 2003, 2007). It is also known that framshie products are involved in transposition of IS629, a member of the IS3 family (Chen & Hu, 2006; Mahillon & Chandler, 1998). ISAba1 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 ISAba1, 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 framshie mutagen was evaluated with respect to its effect on the control of blaOXA-51-like and blaADC as a result of the transposition of ISAba1. This study also aimed to identify the changes in levels of expression of antibiotic resistance genes due to the movement of ISAba1.

**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'-TTTCAGTTC-3') and primer 2 (5'-GGTACACAGTGATGATTAC-3'). The expected product size was 975 bp. Restriction of the product by the enzyme Aldl 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 blaOXA-51-like gene by PCR with the primers oxa-69A (5'-CTTAATTTGATCTACTCAAG-3') and oxa-69B (5'-CCAGTGATGATAGATTATC-3') at an annealing temperature of 48 °C. The expected product sizes were 975 bp for strains without ISAba1 present upstream of the blaOXA-51-like gene and 2155 bp for strains with ISAba1 present upstream of the blaOXA-51-like gene (Héritier et al., 2005).

The strains were screened for the blaADC gene with primers ISADC1 (5'-GTTGCACCTTGGTGATAGTAAA-3') and ISADC2 (5'-ACGTCGCGATTGGTAAGTT-3') with an annealing temperature of 51 °C. The expected product size was 751 bp if ISAba1 was located upstream of the blaADC 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 ISAba1.

**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 blaOXA-51-like and blaADC genes was analysed by RT-PCR. The primers used for blaADC were ADC1 (5'-CCCGCAACGACAGTGAT-3') and ADC2 (5'-TCCGGTGATTTCCGTT-3') with an annealing temperature of 51 °C, producing a product of 451 bp (Ruiz et al., 2007). The primers used for blaOXA-51-like were 51F (5'-TTTCAGTTC-3') and 51R (5'-TTCCCTTCTCCGTGAACAC-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'-GACGTACTCTCGCAAGATAACG-3') and 16S-R (5'-TTAGCTTCTCGGACCGACT-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 (Boshi & Bosi-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 ISAb1 element was present upstream of the bla$_{OXA-51-like}$ gene. If the ISAb1 element was not present upstream, the fragment size was 975 bp, which was the size obtained for strain Ab153. ISAb1 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 ISAb1 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**

ISAb1 movement was recorded in Ab1225M and Ab153M after acriflavine treatment had been performed. It was observed that strain Ab1225M lost its ISAb1 element, previously present upstream of the bla$_{OXA-51-like}$ gene, giving a product size of 975 bp, whereas strain Ab153M gained the ISAb1 element upstream of the bla$_{OXA-51-like}$ gene with a product size of 2155 bp.

ISAb1 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. ISAb1 movement was not seen in the subcultured strain Ab1225s and its features remained identical with respect to the parental strain Ab1225. The ISAb1 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 ISAb1 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>
<thead>
<tr>
<th>Strain</th>
<th>MIC (mg l$^{-1}$)</th>
<th>Expression of bla$_{OXA-51-like}$ gene*</th>
<th>Expression of bla$_{ADC}$ gene*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Imipenem</td>
<td>Meropenem</td>
<td>Piperacillin/tazobactam</td>
</tr>
<tr>
<td>Ab153</td>
<td>0.25</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>Ab153M</td>
<td>2</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>Ab1225</td>
<td>2</td>
<td>4</td>
<td>64</td>
</tr>
<tr>
<td>Ab1225M</td>
<td>0.25</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>Ab1225s</td>
<td>0.5</td>
<td>2</td>
<td>16</td>
</tr>
</tbody>
</table>

*Fold increase/decrease.
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 ISAba1 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 ISAba1 may act as a promoter for the *bla*_{OXA-51-like} or *bla*_{ADC} gene. This is dependent upon ISAba1 acting as a promoter rather than just being present upstream of the antibiotic resistance gene. This also addressed the fact that ISAba1 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 ISAba1 present upstream of the *bla*_{ADC} gene. This is supported by the fact that resistance to oximinocephalosporins is mainly due to hyperproduction of AmpC-type β-lactamases (Rodriguez-Martinez *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 ISAba1 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 ISAba1 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 blaOXA-51-like and blaADC 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 blaADC 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.

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
