Role of ISAba1 and ISAba125 in governing the expression of \( \text{bla}_{\text{ADC}} \) in clinically relevant Acinetobacter baumannii strains resistant to cephalosporins

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Acinetobacter baumannii is a multi-resistant opportunistic nosocomial pathogen responsible for several outbreaks worldwide. It can cause several infections at various sites of the body. One of the main infections caused by this bacterium is ventilator-associated pneumonia in patients in intensive care units. Treating these infections is becoming difficult because of the high resistance to antimicrobial agents. This study compared the expression of the chromosomally encoded \( \text{bla}_{\text{ADC}} \) gene in isolates having ISAba1, ISAba125 and no insertion upstream of the \( \text{bla}_{\text{ADC}} \) gene in A. baumannii clinical isolates. It showed that the expression of \( \text{bla}_{\text{ADC}} \) was six times greater when ISAba125 was present upstream of the gene in comparison with the constitutively expressed \( \text{bla}_{\text{ADC}} \) gene with no insertion present upstream. The study indicated that ISAba125 has better promoters than ISAba1 and this is responsible for the overexpression of the \( \text{bla}_{\text{ADC}} \) gene as they share considerable homology to the well-established *Escherichia coli* promoters. The –10 box of ISAba125 formed a fusion promoter with the –35 box of the \( \text{bla}_{\text{ADC}} \) gene causing the \( \text{bla}_{\text{ADC}} \) gene to be significantly overexpressed. The ability to upregulate the expression of \( \text{bla}_{\text{ADC}} \) with the assistance of different insertion elements such as ISAba1 and ISAba125 has become an important factor in *A. baumannii* resistance to cephalosporins.

**INTRODUCTION**

The predominant mechanism of \( \beta \)-lactam resistance in Gram-negative bacteria is the synthesis of \( \beta \)-lactamases (Philippon *et al.*, 2002). Following the introduction of highly stable cephalosporins, the carbapenems and monobactams, resistance initially appeared in organisms such as *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens* and *Pseudomonas aeruginosa*, where mutations in the chromosome lead to the overproduction of the AmpC \( \beta \)-lactamase. This confers resistance to oxyimino- and 7-alpha-methoxy- cephalosporins and monobactams (Sanders, 1987). Class C \( \beta \)-lactamases produced by Gram-negative bacteria can hydrolyse many \( \beta \)-lactam antibiotics including the cephamsycins and oxyimino-cepahlosporins (Philippon *et al.*, 2002; Sanders, 1987; Caroff *et al.*, 2000). These enzymes can also hydrolyse monobactams, such as aztreonam, to a lesser extent (Bush *et al.*, 1995; Mammerson & Nordmann, 2007). *Acinetobacter baumannii* is a Gram-negative, non-fermenting coccobacillus, which is associated with serious nosocomial infections (Cisneros & Rodríguez-Bañó, 2002; Levin *et al.*, 2003). Antimicrobial treatment of such infections is difficult due to their multidrug resistance profile (Corvec *et al.*, 2003). The AmpC enzyme from multiresistant *A. baumannii* was first sequenced from a Spanish isolate (Bou & Martinez-Beltrán, 2000). This constitutively expressed AmpC enzyme shares low similarity with *Enterobacteriaceae* AmpC cephalosporinas (Corvec *et al.*, 2003). High-level expression of AmpC \( \beta \)-lactamase is a mechanism of chromosomally mediated resistance to cephalosporins in *A. baumannii* (Bou & Martinez-Beltrán, 2000). Transposable elements are important motors of genetic variability and they have diverse strategies to exercise their role in different species of bacteria (Mugnier *et al.*, 2009). ISAba1 has been associated with the expression of several antibiotic resistance genes such as \( \text{bla}_{\text{OXA-51-like}} \) and \( \text{bla}_{\text{ADC}} \) (Corvec *et al.*, 2003, 2007; Figueiredo *et al.*, 2009; Héritier *et al.*, 2006).

In this study, we examine the intergenic regions present upstream and downstream of the \( \text{bla}_{\text{ADC}} \) gene in cephalosporin-resistant *A. baumannii* isolates to identify any novel insertion sequences, besides the well-known ISAba1 that is responsible for the overexpression of *ampC* in these isolates. The rationale behind the search for an insertion element is

**Abbreviations:** ESBL, extended-spectrum \( \beta \)-lactamase; NMP, 1-(1-naphthylmethyl)-piperazine; OMP, outer-membrane protein.

The GenBank/EMBL/DDJB accession number for the ISAba125–\( \text{bla}_{\text{ADC}} \) gene sequence of A. baumannii strain 2 is J0701699.
largely because of the general assumption that only the association of ISAba1–blaADC leads to the overexpression of the blaADC gene (Ruiz et al., 2007).

METHODS

Bacterial strains. The A. baumanii strains used in this study were numbers 2 (UK), 8 (European clone 1, RUH 875, Dordrecht, The Netherlands), 16 (UK) and 21 (Germany). All the strains were kindly supplied by Dr Kevin Towner (Nottingham, UK).

Identification and typing. All the isolates were screened for the conserved 16S–23S rRNA intergenic spacer sequences by PCR with the restriction of the PCR product performed as described earlier (Dolzani et al., 1995). They were then screened for the presence of the blaOXA-51-like gene (Héritier et al., 2005).

Antimicrobial susceptibility testing. The four strains were tested for their susceptibility to imipenem, meropenem, ceftazidime, cefepime and gatifloxacin with and without the efflux pump inhibitor 1-(1-naphthylmethyl)-piperazine (NMP) (100 mg l\(^{-1}\)). MICs were determined by the agar double dilution method according to the British Society for Antimicrobial Chemotherapy (BSAC) methodology (BSAC, 2010). The results were interpreted according to the BSAC guidelines. The results for ceftazidime and gatifloxacin were interpreted with the established guidelines for P. aeruginosa as no criteria exist for A. baumanii. The result for cefepime was interpreted according to the guidelines for members of the Enterobacteriaceae. The reference strains used for MIC testing were A. baumanii ATCC 19606, Escherichia coli NCTC 10418, P. aeruginosa NCTC 10662 and Staphylococcus aureus NCTC 6571 (BSAC, 2010).

PCR amplification. The strains were screened for various different groups of oxacillinas as described previously by Woodford et al. (2006). Amplification of the entire blaOXA-51-like gene was performed using oxA-69A and oxA-69B primers as described previously by Héritier et al. (2005). The strains were screened for the blaADC gene using the ADC1 and ADC2 primers (Ruiz et al., 2007). ISADC1 and ISADC2 primers were used to screen for ISAba1 sequence present upstream of the blaADC gene (Ruiz et al., 2007). Primers FU (5′-GOGCCGTG-AATTCTTAAGTG-3′) and RU (5′-AGGCCATACCTGGCGACATCAT-3′) were used to amplify the intergenic region upstream of blaADC. The expected product size was approximately 360 bp if no insertion was present upstream of blaADC. Primers FD (5′-CAGCTTATGCTGTG-CTGGAT-3′) and RU (5′-GAGCTGCGCATATGGGAAGA-3′) were used to amplify the intergenic region downstream of blaADC. The expected product size without any insertion downstream was 267 bp. The PCR conditions were initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 s, 57 °C for 40 s and 72 °C for 50 s. The final extension was done at 72 °C for 6 min. A PCR for the amplification of blaTEM was performed (Wells et al., 2004). A PCR for the detection of ISAba1 was performed using the ISAba1A and ISAba1B primers described by Poirel & Nordmann (2006). The ISAba125 element was screened for in all the strains using the primers 125F (5′-GGG TAA TGC TCG TAT CGT-3′) and 125R (5′-TAG ACG TAG ACG TGG TCA-3′).

Detection of extended-spectrum β-lactamases (ESBLs). The isolates were screened for ESBL production using the cephalosporin and co-amoxiclav synergy disc method as described previously by Livermore & Brown (2001). The cephalosporins used for this assay were ceftazidime, cefotaxime, cefoxoamide and ceftazidime. A PCR was also performed for the detection of blpPER, blpVEB and blpGES (Moubarek et al., 2009).

Analysis of outer-membrane proteins (OMPs). The strains were grown overnight in nutrient broth and OMP extraction was performed as described previously by Bossi & Figueroa-Bossi (2007). The profiles were studied by SDS-PAGE using 15 % polyacrylamide gel. The gels were stained with Coomassie blue R-250 (Sigma). Approximately 100 μg protein was loaded into each well. Insertions causing disruption of carO gene (29 kDa OMP) were checked in all the isolates using the primers described by Mussi et al. (2005).

Efflux assay. The efflux assay was performed as described by Lee et al. (2003). The efflux inhibitor NMP was used at a concentration of 100 mg l\(^{-1}\). Fluorometric measurements were performed at 37 °C with a fluorescence spectrophotometer (POLARstar OPTIMA; BMG Labtech). Excitation and emission wavelengths of 468 and 499 nm were used.

Analysis of blaADC gene expression. Expression of the blaADC gene was studied by RT-PCR. The primers used for blaADC gene expression were ADC1 and ADC2 as described previously. Total RNA was extracted from isolates in the exponential growth phase using the RiboPure Bacteria kit (Ambion) and treated with the DNase I provided. cDNA was synthesized from 100 ng RNA using the Access Quick RT-PCR system kit (Promega). PCRs were run on an agarose gel and stained with gel red for visualization. PCR products were quantified using the Bio-Rad Quantity One Software 4.6.1. 16S rRNA was used as an internal control using primers described by Lin et al. (2009).

RESULTS

Identification and typing

The strains were identified as A. baumanii after amplification of the 16S–23S rRNA spacer region and restriction of the amplified product to obtain bands specific to A. baumanii using the enzymes AluI and NdeII as per the manufacturer’s guidelines.

Antimicrobial susceptibility testing

Table 1 shows the MICs of imipenem, meropenem, gatifloxacin, ceftazidime, cefepime and ceftazidime with and without the efflux pump inhibitor NMP for the strains used in this study. The MICs of meropenem were decreased fourfold after addition of the efflux pump inhibitor NMP in strains 2 and 16. 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. There was a twofold decrease in the MIC of gatifloxacin seen in strains 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 strains 2 and 16 were greater than that for strain 21, which was greater than that for strain 8.

PCR amplification and sequencing

All strains had the intrinsic blOXA-51-like gene. The primers oxA-69A and oxA-69B amplified the blOXA-51-like gene...
without any insertion upstream of the gene in all the strains. All four strains were positive for ISAba1 whereas only strains 2 and 16 were positive for ISAba125. Strains 2, 16 and 21 had the blaOXA-66 gene and strain 8 had the blaOXA-69 gene as the chromosomal blaOXA-51-like gene. All strains also had the blaADC gene. Strains 16 and 21 showed a positive result for the presence of ISAba1 upstream of blaADC with the primers described by Ruiz et al. (2007). Primers FU and RU used for the detection of sequence upstream of blaADC showed the presence of ISAba125 upstream of blaADC in strain 2 and ISAba1 in strains 16 and 21. Strain 8 did not have any insertion upstream of blaADC. Primers FD and RD amplified specific region downstream of blaADC but without any insertion element in any of the strains. Strains 2 and 16 were positive for blaTEM-1.

Detection of ESBLs

ESBLs could not be detected by the double disc synergy assay. All four isolates were negative for the blaPER, blaVEB and blaGES genes as determined by PCR.

Analysis of OMPs

There were no significant changes seen in any of the OMP patterns produced by any of the strains used in this study by SDS-PAGE analysis as described earlier by Bossi & Figueroa-Bossi (2007). Insertional inactivation of carO was not seen in any of the strains, which also concur with the fact that all the strains had a very low imipenem MIC value, as it has been shown by Mussi et al. (2005) that one of the factors leading to carbapenem resistance is the loss or disruption of this 29 kDa OMP.

Efflux assay

The efflux activity of acriflavine, a substrate for AdeABC multidrug efflux pumps, was measured in the presence and absence of the efflux pump inhibitor NMP. It was observed that strains 2, 16 and 21 demonstrated active efflux to a certain extent. The fluorescence in strains 2, 16 and 21 increased gradually with respect to time except for that in strain 8.

Analysis of gene expression

Table 1 shows the level of expression of blaADC after the synthesis of cDNA. Normalization of DNA was done with 16S rRNA primers used as internal control. The expression studies on strains 2, 16, 21 and 8 indicate that strain 2 possessing ISAba125 upstream of blaADC produced a significant overexpression of blaADC, which was 2.1-fold greater than that for strain 16 having ISAba1 upstream of blaADC and 6.2-fold more than that for strain 8, which had no insertion element upstream of blaADC. Strain 16 had a ceftazidime MIC of >256 mg l⁻¹ with ISAba1 upstream of blaADC producing an expression 3-fold greater than that for strain 8, which had no insertion element upstream of

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Table 1. MICs of various antibiotics for the A. baumannii strains used in this study

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>blaOXA-51-like gene</th>
<th>blaADC gene (fold increase)</th>
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<tr>
<td></td>
<td>IPM</td>
<td>MEM</td>
</tr>
<tr>
<td>2</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td>16</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>21</td>
<td>0.5</td>
<td>0.5</td>
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Control of ADC β-lactamase in A. baumannii

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blaADC. Strain 21 had a ceftazidime MIC of 32 mg l\(^{-1}\) with ISAb1 upstream of blaADC, this correlated with an expression 2.4-fold greater than that for strain 8, which had no insertion element upstream of blaADC. These results are based on the mean increase or decrease in individual strains. Gene expression was measured three times under the same optimal conditions.

**DISCUSSION**

It has been previously reported that ISAb1 causes overexpression of blaADC in *A. baumannii* (Corvec et al., 2003; Héritier et al., 2006). All the strains reported here were resistant to ceftazidime, cefepime and gatifloxacin but sensitive to imipenem and meropenem. They did not have high levels of resistance to ceftazidime, cefepime and gatifloxacin but sensitive to imipenem and meropenem. They did not have any changes in their OMP profiles. Since the strains were also resistant to ceftazidime; however, strains 21 and 8 were far less resistant. The cephalosporin and co-amoxiclav synergy test was negative for all the strains. They were also negative for the presence of blaPER, blaVEB and blaGES genes (Moubareck et al., 2009).

Strain 22 had ISAb125 present upstream (Fig. 1) and the level of expression of the blaADC gene was 6.2 times greater than in strain 8, which had no insertion sequence present upstream of blaADC. In contrast, strains 16 (MIC of ceftazidime >256 mg l\(^{-1}\)) and 21 (MIC of ceftazidime=32 mg l\(^{-1}\)) had an ISAb1 sequence that was present upstream of blaADC, but the level of expression in these two strains was 3- and 2.4-fold greater than in strain 8, respectively, which did not harbour ISAb1 upstream of blaADC gene. It has been proved by Héritier et al. (2006) that −35 (TTAGAA) and −10 (TTTTTT) sequences separated by 16 bp constitute a promoter for ISAb1 and are located 41 bp upstream of blaADC.

According to Héritier et al. (2006), the intergenic region also harbours −35 (TTGTTA) and −10 (TTTATT) sequences separated by 18 bp, which constitute a promoter for blaADC in the absence of an insertion sequence. ISAb125 was found 66 bp upstream of the blaADC gene and was oriented in the opposite direction to the blaADC 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 the −10 box with the sequence TATAAA, which was fused with the −35 promoter of blaADC, was seen 17 bp downstream. The blaADC gene was present 39 bp downstream of the new −10 box. The ISAb125 sequence had a 4/6 and 2/6 match, respectively, with the *E. coli* consensus −35 (TGTGAC) and −10 (TATAAA) whereas ISAb125 had a 4/6 and 5/6 match, respectively, with the *E. coli* consensus. This suggests that ISAb125 is able to provide a better promoter than ISAb1. As the −10 box of ISAb125 is fused with the −35 box of blaADC, it can form a fusion promoter to drive very strong expression of blaADC and the expression results support this claim. ISAb1, which drives the expression of blaADC, is also shown to be present in the opposite orientation (3′→3′) with blaADC, as shown by

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**Fig. 1.** Alignment of ISAb125 with various regions upstream and downstream of the *A. baumannii* chromosomal blaADC gene (not to scale).
Hérétier et al. (2006), hence ISAb125 present in the opposite orientation (3′–5′) may also act in a similar way to drive the expression of bla_{ADC}. Mussi et al. (2005) have proved that ISAb125 causes insertional inactivation of the CarO OMP in A. baumannii strains, leading to a carbapenem-resistant profile. The insertion element ISAb125, first identified in A. baumannii, is widely distributed and has also been associated with gene variants of the bla_{ADC} carbapenemase in the family Enterobacteriaceae (Hornsey et al., 2011; Nordmann et al., 2012; Pfeifer et al., 2011). The results obtained in this study show that IS elements such as ISAb125 could modulate antibiotic resistance depending on the antibiotic treatment of the patient as the isolates used in this study were of clinical origin. The movement of ISAb125 upstream of an antibiotic resistance gene can result in significant overexpression of the target resistance gene. Furthermore, ISAb125 can also cause the duplication of bla_{ADC} facilitating its spread between phylogenetically unrelated bacterial species that belong to the same commensal flora. It is not surprising that IS elements can cause the spread of resistance genes between different species of bacteria, as it has already been shown by Mammeri et al. (2003) that ISOur1 of Oligella urethralis is believed to have been co-transferred with the cephalosporinase gene bla_{Ab1} from A. baumannii. The presence of ISAb125 seen in strain 2 may be related to its evolution from the European clone II, which has the element upstream of the bla_{ADC}. This is not surprising as strain 2 and the European clone II possess the same bla_{OXA-51-like} gene and bla_{ADC} gene and share 93.5% relatedness as analysed by PFGE (data not shown).

Moreover, the ISAb125 sequence was also associated with other strains isolated from Europe which have the bla_{OXA-66} gene of the European clone II and which are clonally related but have either one or more amino acid changes in the bla_{ADC} gene (unpublished data). This study provides an insight into the insertion sequence ISAb125 belonging to the IS30 family (Siguier et al., 2006), which is involved in significant overexpression of the bla_{ADC} gene, greater than that of the previously known sequence ISAb1 found upstream of the bla_{ADC} gene, and this may be due to efficient control mechanisms employed by the repressor activator systems that are responsible for regulation of ISAb125. Our results also suggest that the repressor control system in ISAb125 is different from that of ISAb1 and hence the contribution towards a greater cephalosporin resistance with strong hydrolytic activity of ceftazidime. The widely used ISADC1 and ISADC2 primers (Ruiz et al., 2007) unfortunately have a drawback in that they could only amplify a specific product related to the insertion element ISAb1 upstream of the bla_{ADC} gene. With the newly designed primers, we tested 37 European strains in our collection that had varied levels of resistance to cephalosporins: 48% were positive for the ISAb1–bla_{ADC} combination and 10% were positive for the previously unnoticed ISAb125–bla_{ADC} combination (B. S. Lopes and S. G. B. Amyes, unpublished data). Hence, this study also provides a new set of primers which can span the intergenic regions upstream and downstream of the bla_{ADC} gene and thus aid in studying any novel insertions that may occur with the progression of time.

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REFERENCES


