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Cephalosporinase over-expression resulting from insertion of IS*Aba1* in *Acinetobacter baumannii*

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ABSTRACT

IS*Aba1*-like sequences were identified immediately upstream of the bla_{ampC} gene in ceftazidime-resistant *Acinetobacter baumannii* isolates, but were absent in ceftazidime-susceptible *A. baumannii* isolates. AmpC over-expression resulted from insertion of IS*Aba1*-like sequences upstream of bla_{ampC}. IS*Aba1* provided strong promoter sequences, and it was demonstrated that the change in the ribosome binding site sequence resulting from insertion of IS*Aba1* did not influence expression of the bla_{ampC} gene. Sequence analysis revealed that AmpC sequences of *A. baumannii* isolates were almost identical and that IS*Aba*1 elements had a high percentage of identity.

Keywords *Acinetobacter baumannii*, ceftazidime resistance, cephalosporinase, insertion sequence, promoter sequence, ribosome binding site.

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INTRODUCTION

The most common mechanism of resistance to β-lactams in *Acinetobacter baumannii* involves production of a naturally occurring AmpC-type cephalosporinase [1,2]. Insertion sequence (IS) elements have been located upstream of the bla_{ampC} gene of ceftazidime-resistant *A. baumannii* [3,4], and it has been shown that IS*1133*-like elements (designated IS*Aba1*) provide promoter sequences that enhance high-level expression of the bla_{ampC} β-lactamase gene [3,4]. Insertion of these elements upstream of the bla_{ampC} gene provides new promoter sequences (P_{out}) for the bla_{ampC} gene, which can replace the original promoter sequences (P_{ori}) that drive the expression of AmpC in the absence of any IS elements. The insertion event results in a nucleotide change inside the ribosome binding site (RBS) sequence of the bla_{ampC} gene. The aim of the present study was to study the detailed regulation of AmpC expression by these IS elements, and to evaluate the role of the nucleotide change in the RBS sequence of the bla_{ampC} gene resulting from the insertion event.

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MATERIALS AND METHODS

Bacterial strains and plasmids

A. baumannii CLA-1 was isolated at the Hospital Bicêtre (K.-Bicêtre, France) in 2001 (Table 1) [5]. Between 1998 and 2003, ten ceftazidime-susceptible and five ceftazidime-resistant isolates of *A. baumannii* were collected from different hospitals in Europe and Turkey (Table 1). These isolates were identified by the API 20NE system (bioMérieux, Marcy l'Etoile, France). Two additional reference strains were obtained from the Institut Pasteur, Paris, France (Table 1). *Escherichia coli* strains DH10B and JM109 were used for cloning experiments, together with plasmid pACYC184 [6]. pCR-Blunt II-TOPO (Invitrogen, Cergy Pontoise, France) was used for post-PCR cloning and site-directed mutagenesis, and pKK232-8 (Amersham Pharmacia Biotech, Saclay, France) was used as a promoterless reporter gene. Details of the plasmids used in this study are given in Table 2.

Antimicrobial agents and MIC determinations

The antimicrobial agents and their sources have been described previously [7]. Antibiotic-containing disks were used for detection of antibiotic susceptibility, with Mueller-Hinton agar plates (Sanofi Diagnostics Pasteur, Marnes-La-Coquette, France) and a disk-diffusion assay (http://www.sfm.fr). MICs were determined by agar dilution [7], with results interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) [8].

Table 1. Origin and properties of *Acinetobacter baumannii* isolates examined as part of this study

Isolate	Year of isolation	Place of isolation	β-Lactamase content	PCR detection		AmpC over-expression	Ceftazidime MIC values (mg/L)
				IS*Aba1*	IS*Aba1*/bla_{ampC}		
CLA-1[a]	2001	Paris, France	AmpC + OXA-40	+	+	+	>512
RYC 52763/97[b]	1997	Madrid, Spain	AmpC + OXA-24 + TEM-1	+	+	+	>256
AYE[c]	2001	Valenciennes, France	AmpC + VEB-1	+	+	+	>512
Ama-1	2001	Paris, France	AmpC + PER-1	+	+	+	512
S120	1998	Ankara, Turkey	AmpC	+	+	+	512
MK 8560/99	1999	Warsaw, Poland	AmpC	+	+	+	512
MK 8744/99	1999	Warsaw, Poland	AmpC	–	–	–	4
215[d]	1999	Montpellier, France	AmpC + OXA-23	+	–	–	4
KB-1	1998	Paris, France	AmpC	–	–	–	4
KB-2	2003	Paris, France	AmpC	–	–	–	4
KB-3	2003	Paris, France	AmpC	–	–	–	4
KB-4	2003	Paris, France	AmpC	–	–	–	4
KB-5	2003	Paris, France	AmpC	–	–	–	4
KB-6	2003	Paris, France	AmpC	–	–	–	4
KB-7	2003	Paris, France	AmpC	–	–	–	4
KB-8	1999	Paris, France	AmpC	–	–	–	4
CIP 7034T	1969	Institut Pasteur collection	AmpC	–	–	–	2
CIP 70.10	1950	Institut Pasteur collection	AmpC	–	–	–	2

[a]Data from [5].
[b]Data from [2].
[c]Data from [18].
[d]Personal unpublished data.

Table 2. Plasmids used in this study

Plasmid	Resistance	Characteristics	Origin
pACYC184	Cl, Tet	Cloning vector	New England Biolabs
pCR-Blunt II-TOPO	Kan	Post-PCR cloning vector	Invitrogen
pKK232-8	Amp	Reporter vector	Pharmacia
pAB +	Amp, Cl	pACYC184 containing the entire $bla_{ampc\ and}$ promoter P_{out}	This study
pAB	Am, Cl	pACYC184 containing the entire bla_{ampc}	This study
TOPO($P_{ori}RBS_{ori}$)	Kan	TOPO containing promoter P_{ori}	This study
TOPO($P_{ori}RBS_{out}$)	Kan	TOPO containing promoter P_{ori} mutated in its RBS	This study
TOPO($P_{out}RBS_{out}$)	Kan	TOPO containing promoter P_{out}	This study
TOPO($P_{out}RBS_{ori}$)	Kan	TOPO containing promoter P_{out} mutated in its RBS	This study
pKK232-8 ($P_{ori}RBS_{ori}$)	Amp, Cl	pKK232-8 containing promoter P_{ori}	This study
pKK232-8 ($P_{ori}RBS_{out}$)	Amp, Cl	pKK232-8 containing promoter P_{ori} mutated in its RBS	This study
pKK232-8 ($P_{out}RBS_{out}$)	Amp, Cl	pKK232-8 containing promoter P_{out}	This study
pKK232-8 ($P_{out}RBS_{ori}$)	Amp, Cl	pKK232-8 containing promoter P_{out} mutated in its RBS	This study

Cl, chloramphenicol; Tet, tetracycline; Kan, kanamycin; Amp, ampicillin.

PCR amplification

Primers preAB1 and preAB2, and preABprom + and preAB2 (Table 3) were used to amplify 1243-bp and 1507-bp fragments containing the entire AmpC coding sequence without and with the IS*Aba1* promoter sequences, respectively, using whole-cell DNA from *A. baumannii* CLA-1 as the template. Primers Pori1 and Pori2 (Table 3) were used to amplify a 100-bp fragment containing the -35 and -10 sequences of P_{ori}, as well as the original RBS (RBS_{ori}: TGAG) from the bla_{ampC} of *A. baumannii* (Fig. 1); primers Pout1 and Pout2 (Table 3) were used to amplify a 122-bp fragment containing the -35 and -10 sequences of P_{out}, as well as the RBS (RBS_{out}: GGAG) as modified by the insertion of IS*Aba1* (Fig. 1). PCRs were performed as described previously [5].

Construction of reporter plasmids and site-directed mutagenesis

The 1243-bp and 1507-bp PCR amplicons (see above) were cloned in the *Eco*RV-restricted plasmid pACYC-184 to yield plasmids pAB and pAB + (Table 2), respectively, which were obtained and expressed in *E. coli* DH10B. The PCR amplicons obtained with primers Pori1 and Pori2, and Pout1 and Pout2, were cloned in the PCR cloning vector pCR-Blunt II-TOPO, using the Zero Blunt TOPO PCR Cloning kit (Invitrogen, Cergy Pontoise, France) as recommended by the manufacturer, to yield plasmids TOPO($P_{ori}RBS_{ori}$) and TOPO($P_{out}RBS_{out}$) (Table 2). The Quick Change site-directed mutagenesis kit (Stragene, La Jolla, CA, USA) was used as recommended by the manufacturer to substitute a T for a G in TOPO($P_{ori}RBS_{ori}$), as in the RBS sequence modified by insertion of IS*Aba1*, and a G to a T in TOPO($P_{out}RBS_{out}$), as in the original RBS sequence of the bla_{ampC} gene. TOPO($P_{ori}RBS_{ori}$) plasmid DNA was used as template with primers Pori-mut1 and Pori-mut2 (Table 3) to generate plasmid TOPO($P_{ori}RBS_{out}$) (Table 2), and plasmid TOPO($P_{out}RBS_{out}$) was used as template with primers Pout-mut1 and Pout-mut2 (Table 3) to generate plasmid TOPO ($P_{out}RBS_{ori}$) (Table 2). Plasmid DNA from the four recombinant plasmids was digested with *Eco*RI, the corresponding inserts were purified by gel electrophoresis, blunt-ended, and ligated into *Sma*I-restricted pKK232-8 plasmid DNA (Amersham Pharmacia Biotech). This latter plasmid carried a gene encoding chloramphenicol acetyltransferase (CAT), the expression

Table 3. Sequences of primers designed for this study

Primer	Sequence (5′–>3′)
preABprom +	GACCTGCAAAGAAGCGCTGC
preAB1	ACAGAGGAGCTAATCATGCG
preAB2	GTTCTTTTAAACCATATACC
Pori1	CCATGTGCTATAACAACATA
Pori2	TGTCGTGTACAGAGGATTAG
Pout1	ATAAGATATAAGTCATTGAG
Pout2	TTTTTAAATCGCATGATTAG
Pori-mut1	GCATCTTTCTTTTTAAATAATTTAGGA GCTAATCCTCTGTACACG
Pori-mut2	CGTGTACAGAGGATTAGCTCCTAAATTAT TTAAAAAGAAAGATGC
Pout-mut1	CGTGTACAGATGAGCTAATCATGCG
Pout-mut2	CGCATGATTAGCTCATCTGTACACG
pKK1	TGCGAAGCAACGGCCCGG
pKK2	AAGCTTGGCTGCAGGTCGA
IS*Aba*1a	ATGCAGCGCTTCTTTGCAGG
IS*Aba*1b	AATGATTGGTGACAATGAAG

of which was measured to evaluate the strength of the cloned promoters and the effect of the RBS changes. The ligation products were used to transform competent cells of *E. coli* JM109. The sequence of the inserted fragment was checked using two primers from pKK232-8, pKK1 and pKK2 (Table 3) [9], and an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA).

Chloramphenicol acetyltransferase assays

Total protein concentration was measured by the Bio-Rad DC protein assay (Bio-Rad, Ivry-Sur-Seine, France). CAT activity was assayed using different recombinant plasmids and culture extracts by a sandwich ELISA test performed in a microtitre plate (CAT ELISA; Roche Applied Science, Meylan, France). Each measurement of CAT enzyme activity was performed in triplicate. *E. coli* JM109 and *E. coli* JM109 containing pKK232-8 without any insert were used as controls.

Southern blot experiments

Whole-cell DNA from *A. baumannii* isolates was digested with *Eco*RI and transferred to a Nylon membrane (Hybond N^+; Amersham Pharmacia Biotech) as described previously [5]. The membrane was hybridised with a probe consisting of an internal 389-bp fragment, amplified by primers IS*Aba*Ia and IS*Aba*Ib (Table 3), which was specific for IS*Aba1*. Southern hybridisation was performed using the ECL nonradioactive labelling and detection kit (Amersham Pharmacia Biotech) as recommended by the manufacturer.

RESULTS AND DISCUSSION

Cloning and nucleotide sequence of bla_{ampC} and IS*Aba*1

A. baumannii clinical isolate CLA-1 (resistant to all β-lactams) produced a carbapenem-hydrolysing oxacillinase (OXA-40) and an AmpC-type cephalosporinase [5]. While studying OXA-40, recombinant clones expressing an AmpC-type phenotype were obtained. A recombinant plasmid, pABC-1, conferring an AmpC-type β-lactam resistance phenotype in *E. coli*, possessed a 2201-bp insert. Following analysis of the nucleotide and deduced amino-acid sequences, an AmpC protein sequence was found that was identical to that of another *A. baumannii* strain (AbRYC52763/97), except for a single amino-acid change (lysine to arginine at position 38) [2]. This AmpC protein also shared 98% amino-acid identity with AmpC ABA-1 from *A. baumannii*. When this gene was integrated into *Oligella urethralis*, β-lactam resistance was conferred on the host [10].

An IS element, designated IS*Aba1*, was identified upstream of the bla_{ampC} gene. IS*Aba*1 was 1180 bp long, with terminal inverted repeats (IR) of 16 bp (Fig. 1), and belongs to the IS*4* family, according to the IS nomenclature proposed by Chandler and Mahillon [11]. The sequence of IS*Aba1* is available from the IS Biotoul website (http://www-is.biotoul.fr) with GenBank accession number AY758396. The inverted repeat left (IRL) is located just 9 bp upstream of the start codon of the *ampC* gene (Fig. 1). The transposition which occurred in *A. baumannii* CLA-1 generated a 9-bp duplication of the target sequence (Fig. 1). This is a likely reason for the ATG start codon being located immediately downstream of the direct repeat (DR) generated by the transposition event (Fig. 1). It is noteworthy that this duplication has modified the RBS by a single base, changing TGAG to GGAG (Fig. 1; position 1271–1274).

IS*Aba1* is very similar to the IS*1133*-like element located upstream of the aminoglycoside resistance gene *aacC2* identified in an *A. baumannii* isolate (five nucleotide changes) [12], or that found upstream of the bla_{ampC} gene identified in other *A. baumannii* isolates (five nucleotide changes) [3,4], and also to the partial sequence found upstream of the $phaB_{AC}$ sequence of *A. baumannii* RA3849 which encodes an aceto acetyl coenzyme A reductase (three nucleotide changes) [13]. The main difference was that IS*Aba1* had a single nucleotide deletion at position 584–585 (T-G at position 686–687 in Fig. 1, instead of TGG), compared with the IS*1133*-like element, that gave rise to a truncated transposase protein and overlapping of two open reading frames (ORFs) (Fig. 1).

IS*Aba1* shares 43% identity with IS*Dra1* from *Deinococcus radiodurans* R1, for which the ORF is a 327 amino-acid protein after taking the frameshift into account [14]. Sequences separated by 17 bp,

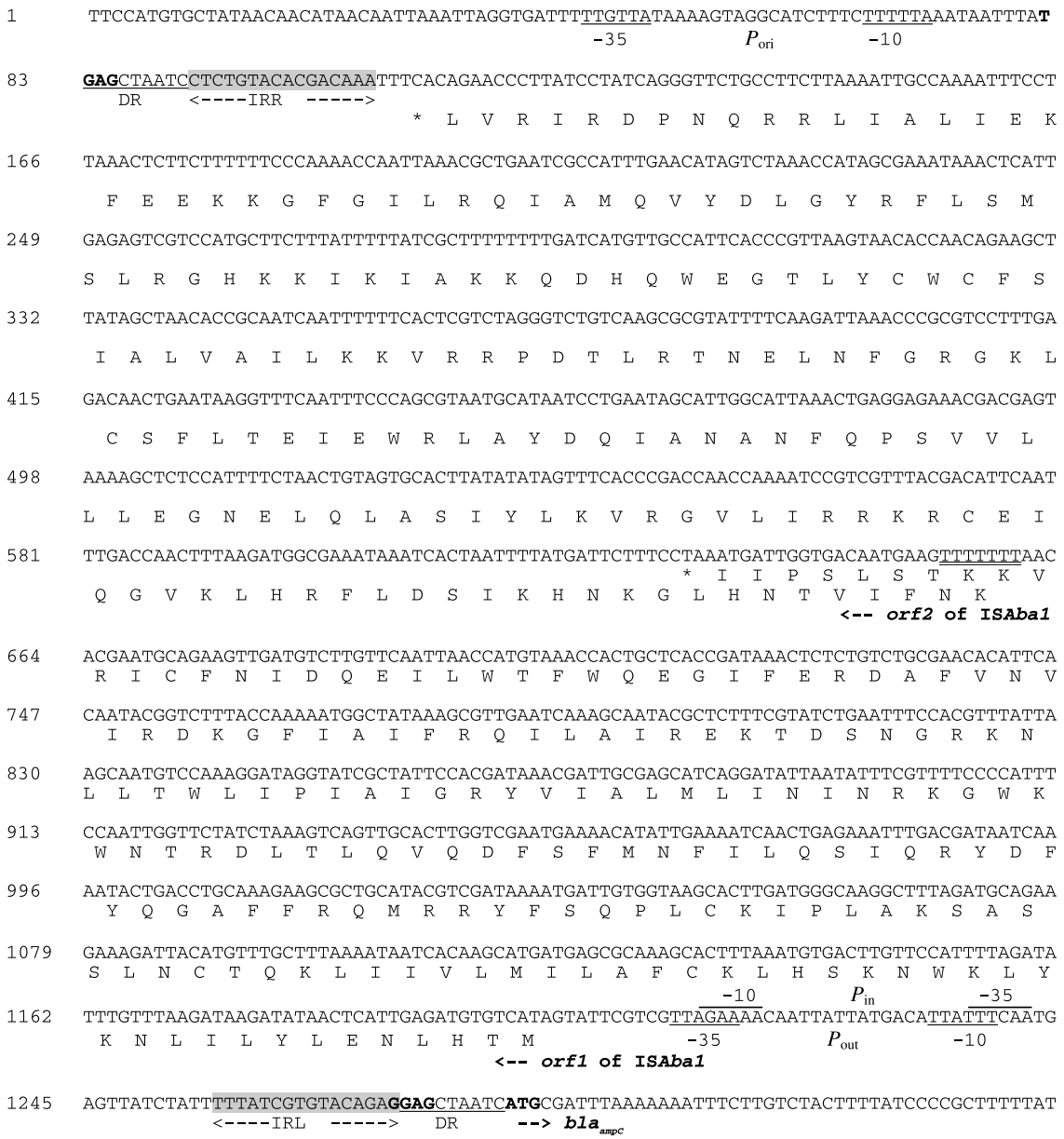

Fig. 1. Nucleotide sequence of the insertion sequence upstream of *bla*$_{ampC}$. The deduced amino-acid sequence is designated in a single letter code below the nucleotide sequence, direct repeat (DR) sequences are underlined, and inverted repeat sequences (IRR and IRL) are in grey. The frameshift site is double-underlined. The stars indicate stop codons. The -35 and -10 motifs of promoters are indicated. P_{in} is the promoter of the *orf1* transposase gene in IS*Aba1*, P_{out} the promoter of *bla*$_{ampC}$ provided by IS*Aba1*, and P_{ori} the putative host-cell promoter driving the expression of *bla*$_{ampC}$ in the absence of IS*Aba1*. The original ribosome binding site of *bla*$_{ampC}$, and that identified following insertion of IS*Aba1*, are indicated in bold characters.

constituting a P_{in} promoter for ORF1 (189 amino-acids) expression, were identified (-35 (TTGAAA) and -10 (TTTTCT); Fig. 1). ORF2 is a 178 amino-acid protein if the valine codon, located at position 644–646 in Fig. 1, is considered as the initiation codon. However, it is likely that a frameshift may occur in IS*Aba1* in the overlapping region, giving rise to a unique and probably functional transposase protein, as observed for other IS elements such as IS*911*, which has two consecutive ORFs [11,15,16]. A change in reading frame, when transcribing the *orf1* gene, could allow translation to continue in the *orf2* frame, giving rise to a protein comprising most of the

ORF1 and ORF2 polypeptides which may act as a transposase [15,17].

The sequence that allows backward shifting of the ribosome is an heptanucleotide that might correspond to the A AAA AAA motif identified in IS*Aba1* at positions 654–660 (T TTT TTT in Fig. 1) [14,16]. This frameshift may occur at a low frequency, leading to low-level expression of the functional transposase and thus to relative stability of this IS element.

Cloning of the bla_{ampC} gene with or without the P_{out} promoter

The -35 and -10 sequences (TTAGAA and TTATTT, respectively) separated by 16 bp, which are able to constitute a P_{out} promoter, were identified in the right-hand boundary of IS*Aba1* [3,4], being located 41 bp upstream of the start codon of the bla_{ampC} gene where they probably enhance its expression (Fig. 1) [3,4]. Antibiotic susceptibility testing revealed that *E. coli* DH10B (pAB+), with the insert containing the IS*Aba*1-mediated promoter sequences, had reduced susceptibility to most β-lactams compared with *E. coli* DH10B (pAB) (Table 4), including ticarcillin, piperacillin and aztreonam.

Role of the nucleotide change in the RBS sequence of the bla_{ampC} gene

On the right-hand boundary of IS*Aba1*, other -35 (TTGTTA) and -10 (TTTTTA) sequences, separated by 18 bp, were identified in *A. baumannii* CLA-1. These probably correspond to the original P_{ori} promoter sequences of AmpC, and drive its expression in the absence of IS*Aba1* (Fig. 1). Since IS*Aba1* insertion gave rise to a T to G nucleotide change in the RBS sequence (Fig. 1; positions 1271–1274) of a sensitive strain of *A. baumannii*, the effect of this nucleotide change on expression at the level of translation was analysed.

A ten-fold increase in CAT activity was observed when P_{out} was present upstream of *ampC* when compared with the P_{ori} promoter sequences (Table 5). However, no difference in CAT activity was observed between RBS_{ori} and RBS_{out}.

The results of the CAT assays confirmed that IS*Aba1* supplied strong promoter sequences [3,4] and indicated that the change in the RBS sequence resulting from the insertion of IS*Aba1* did not influence the expression of bla_{ampC}.

Distribution of IS*Aba*1

As IS*Aba*1 located upstream of bla_{ampC} was involved in the increased level of β-lactam resistance, the presence of similar sequences was investigated by PCR in *A. baumannii* isolates with different β-lactam resistance patterns (Table 1). Sixteen clinical isolates and two reference strains of *A. baumannii* of different origins were analysed, including 12 ceftazidime-susceptible and six ceftazidime-resistant isolates (according to CLSI guidelines [8]) (Table 1). A previous study had demonstrated by pulsed-field gel electrophoresis [18] that none of the isolates were clonally related [19]. PCR amplicons were obtained with primers IS*Aba*1a and IS*Aba*1b (Table 3), which amplify an internal 389-bp fragment of IS*Aba*1, with seven isolates, including the six ceftazidime-resistant isolates and *A. baumannii* 215 (which produces OXA-23), while negative results were obtained with all the ceftazidime-susceptible isolates

Table 4. MICs of β-lactams for *Escherichia coli* DH10B and *E. coli* DH10B clones harbouring recombinant plasmids pAB + and pAB containing the entire AmpC coding sequence with or without promoter P_{out}

β-Lactam(s)	MICs (mg/L) *E. coli* DH10B (pAB+)	*E. coli* DH10B (pAB)	*E. coli* DH10B
Amoxycillin	>512	>512	4
Amoxycillin + CLA	>512	>512	4
Ticarcillin	64	8	4
Piperacillin	64	8	2
Piperacillin + TZB	32	4	2
Cephalothin	256	128	4
Cefuroxime	256	128	4
Cefoxitin	32	16	4
Ceftazidime	64	16	0.06
Cefotaxime	32	8	0.06
Cefepime	0.12	0.06	0.06
Cefpirome	0.25	0.12	0.06
Moxalactam	0.5	0.12	0.06
Aztreonam	16	0.25	0.12
Imipenem	0.25	0.12	0.06

CLA, clavulanic acid at a fixed concentration of 2 mg/L; TZB, tazobactam at a fixed concentration of 4 mg/L.

Table 5. Comparison of the strength of the *ampC* promoters P_{ori} and P_{out}, combined with the two different ribosome binding site sequences

Plasmid used to transform *E. coli* JM109	CAT activity (pg/μg)[a]
None	3 ± 1
pKK232-8	3 ± 1
pKK232-8($P_{ori}RBS_{ori}$)	47 ± 8
pKK232-8($P_{ori}RBS_{out}$)	48 ± 8
pKK232-8($P_{out}RBS_{ori}$)	398 ± 16
pKK232-8($P_{out}RBS_{out}$)	502 ± 16

[a]Mean ± standard deviation of three determinations of CAT activity.

(Table 1). Using the preABprom+ and preAB2 primers, which anneal upstream of bla_{ampC} in IS*Aba1* (Table 3), positive results were obtained for the six ceftazidime-resistant isolates, but not for the bla_{OXA-23}-positive *A. baumannii* isolate. In this latter strain, the IS element identified had 99% nucleotide homology with IS*Aba*1 over the 972-bp sequence upstream of bla_{OXA-23}, with the same overlapping of two ORFs. Thus, as suggested by Segal *et al.* [20], IS*Aba1* might also play a role in expression and acquisition of various antibiotic resistance genes, such as carbapenem-hydrolysing oxacillinase genes.

Using a 389-bp internal probe for IS*Aba*1, Southern blot experiments were negative for the ceftazidime-susceptible *A. baumannii* isolates, but yielded between one and nine positive signals with the ceftazidime-resistant isolates and the bla_{OXA-23}-positive *A. baumannii* isolate (Fig. 2). Thus, the ceftazidime-resistant isolates possessed several copies of IS*Aba*1, one copy being located upstream of the bla_{ampC} gene. While this work was in progress, Segal *et al.* [20] also identified several copies of IS*Aba*1 in *Acinetobacter* isolates containing this element.

Interestingly, IS*Aba*1 was identified upstream of the bla_{ampC} gene of *A. baumannii* isolates expressing PER-1 and VEB-1 extended-spectrum β-lactamases, indicating a dual mechanism for ceftazidime resistance, involving both AmpC hyper-production caused by IS*Aba1* insertion and acquisition of a broad-spectrum β-lactamase gene. Whereas inducible AmpC expression has been reported for most AmpC-positive Gram-negative bacterial species [6], this is not the case for *A. baumannii.* Induction experiments, performed with imipenem or cefoxitin as inducers and cephalothin as substrate [21], confirmed the absence of AmpC induction in *A. baumannii*. These results are consistent with the absence of a LysR-type regulatory gene located upstream of the bla_{ampC} gene.

As reported for AmpC genes from *Pseudomonas aeruginosa* [22,23], the AmpC sequences from different isolates of *A. baumannii* were almost identical (no more than two amino-acid substitutions), regardless of the level of β-lactamase expression or the presence of IS*Aba*1. Sequence analysis of the seven IS*Aba*1 elements also revealed a high degree of identity (99%), with none of the nucleotide changes occurring in the area of the putative frameshift site.

The association between IS*Aba1* and bla_{ampC} or bla_{OXA-23} adds to the diversity of IS elements as sources of β-lactamase expression (10, 18, 24–34). Further work should determine whether isolates of *A. baumannii* can be delineated into two subspecies, with the first being ceftazidime-susceptible and lacking an IS*Aba1*-like sequence, and the second being ceftazidime-resistant as a result of the insertion of IS*Aba1*, which then became disseminated throughout the chromosome.

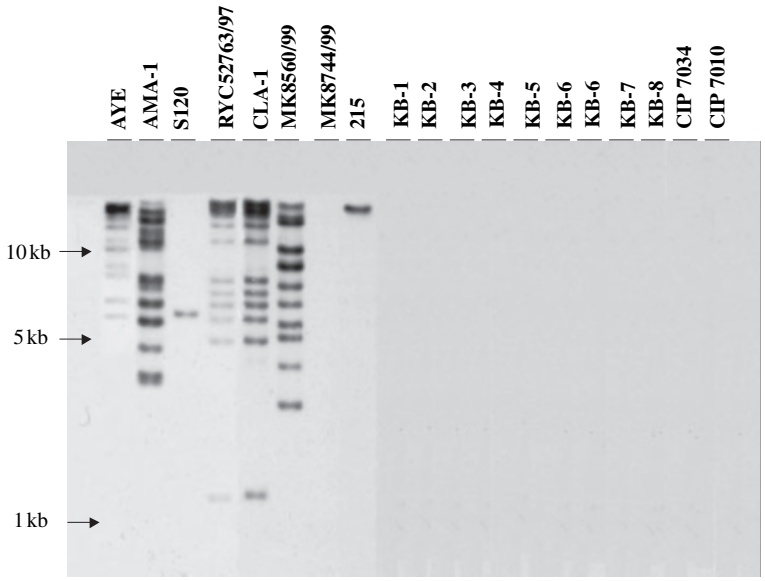

Fig. 2. *Eco*RI-restricted genomic DNA from the 18 *Acinetobacter baumannii* isolates following hybridisation with a probe consisting of an internal 389-bp fragment of IS*Aba1*.

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