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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].

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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

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