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AmpC cephalosporinase hyperproduction in *Acinetobacter baumannii* clinical strains

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***Objective*: To compare the genetic environments of *ampC* genes in different *Acinetobacter baumannii* isolates showing different levels of β-lactam resistance.**

***Methods*: The patterns of β-lactam resistance and β-lactamase production were investigated for 42 *A. baumannii* clinical strains. The MICs of various β-lactams were determined in the presence or absence of the class C cephalosporinase inhibitor, cloxacillin (500 mg/L). The *ampC* gene and its 5′ adjacent sequence were analysed by PCR and DNA sequencing. An RT–PCR method was developed to evaluate *ampC* transcript levels.**

***Results*: Strains fell into three resistance groups: first, strains with a ceftazidime MIC ≤ 8 mg/L (20 strains, 47.6%); secondly, strains with a ceftazidime MIC 32 mg/L, which was reduced four-fold in the presence of cloxacillin (eight strains, 19%); and thirdly, strains with a ceftazidime MIC ≥ 256 mg/L, which did not decrease in the presence of cloxacillin (14 strains, 33.4%). In all of the resistant isolates (groups II and III), but not in any of the ceftazidime-susceptible isolates (group I), a 1180 bp insert showing all the characteristics of an insertion sequence was detected upstream from the *ampC* gene. Isolates having this insert overexpress *ampC*, according to RT–PCR experiments.**

***Conclusion*: Presence of an insertion sequence upstream of *ampC* in *A. baumannii* clinical isolates, possibly including a strong promoter, has the potential to cause over-expression of AmpC, resulting in high-level ceftazidime resistance.**

Keywords: AmpC hyperproducers, insertion sequences, *Acinetobacter baumannii*

Introduction

Members of the genus *Acinetobacter* are aerobic, non-fermenting Gram-negative bacilli, responsible for a wide spectrum of infections in immunocompromised hosts.

The *Acinetobacter baumannii* complex has emerged as some of the most important opportunistic pathogens within the hospital environment, being able to colonize and produce infections in most immunocompromised patients, especially from intensive care units[1] and/or in the context of serious underlying disease. Antimicrobial treatment of such severe infections is complicated by a widespread multidrug resistance pattern.

Different mechanisms of β-lactam resistance have been reported and identified in *A. baumannii*: β-lactamase production,[2] penicillin-binding protein alterations[3,4] and reduced penetration across the outer membrane.[5] Nevertheless, β-lactamase production is one of the main mechanisms of resistance to β-lactams in *Acinetobacter* spp. Such β-lactamases can be plasmid-mediated enzymes like TEM-1 or CARB-5 penicillinases,[6,7] integron-located class D oxacillinases,[8–10] class B metalloenzymes[11,12] or chromosomally encoded cephalosporinases (AmpC) belonging to class C β-lactamases.[13,14] The AmpC cephalosporinase was recently sequenced from a multi-resistant *A. baumannii* clinical strain (Ab RYC 52763/97) isolated during an outbreak in Spain.[14] This constitutively expressed enzyme does not share strong similarity with Enterobacteriaceae AmpC cephalosporinases.[15] As of yet there is no information about the regulation of *A. baumannii* AmpC expression.

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In this study, we examined the different β-lactam-resistance patterns encountered in 42 *A. baumannii* clinical strains and related this to AmpC expression. In order to investigate the changes in AmpC expression noted, we compared the genetic environment of the *ampC* gene in different strains showing different levels of β-lactam resistance.

Materials and methods

Bacterial strains

From January to December 2001, 42 strains of *A. baumannii* were isolated from 42 patients from different units at Nantes University Hospital. Identifications were performed with the Vitek2 system with an identification card for Gram-negative bacilli (ID-GNB) (bioMérieux, Marcy l'Étoile, France) and confirmed by overnight growth on Trypticase soy agar (bioMérieux) at 44°C.

Pulsed-field gel electrophoresis (PFGE)

DNA isolation from *A. baumannii* and PFGE were performed as previously described with some modifications[16] with a *Sma*I restriction. NCTC 8523 was used as DNA size marker. The interpretation of the banding pattern was performed according to Tenover *et al.*[16]

Susceptibility tests and MIC determination

Susceptibility to β-lactams was determined using the disc diffusion method (Bio-Rad, Marnes la Coquette, France) on Mueller–Hinton (MH) agar (Difco Laboratories, Detroit, MI, USA), according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie.[17]

MICs were determined in the presence or absence of 500 mg/L of cloxacillin by serial two-fold dilution tests in MH agar. Inocula of 10^4 cfu/spot from an 18 h culture in MH broth were applied with a Steers multiple-inoculum replicator.

A. baumannii ATCC 19606 was used as a control strain.

β-Lactamase extraction and isoelectric focusing

After 18 h of culture in Trypticase soy broth (bioMérieux), bacterial pellets were obtained by 20 min centrifugation at 10 000**g**. After washing and resuspension in distilled water, bacterial pellets were sonicated in a Branson Sonifier 250 to disrupt bacterial cell walls (intermittent exposure of 5× 30 s). After centrifugation, the supernatant contained the β-lactamase crude extract. Isoelectric focusing was performed according to the method of Matthew *et al.*,[18] using an 8% acrylamide/bisacrylamide gel containing ampholines (pH 3.5–9.5). An iodine-starch agar gel, containing either benzylpenicillin or cloxacillin, was used to visualize the β-lactamase bands.

Detection of metallo-β-lactamase-producing strains

Metallo-β-lactamase production was detected by imipenem-EDTA and ceftazidime-EDTA double-disc synergy test. Clinical strains were inoculated on MH agar plates. Two 10 μg imipenem discs and two 30 μg ceftazidime discs (Bio-Rad) were placed on each plate, and 5 μL of a 0.5 M pH 8.0 EDTA solution were added onto one of each antibiotic disc. The inhibition zones were compared after 18 h of incubation at 37°C; metallo-β-lactamase production leads to a 6–12 mm diameter increase in the presence of EDTA.[19,20]

Detection of ampC by PCR

Genomic bacterial DNA was extracted from the 42 strains by heating a suspension of bacteria to 95°C for 5 min in a final volume of 50 μL of distilled sterile water. After centrifugation at 13 000**g**, the supernatants were used as DNA templates. PCR amplifications were performed in a DNA thermal cycler Perkin-Elmer 480 (Perkin-Elmer, Applied Biosystems, Cergy-Pontoise, France). A 663 bp fragment was amplified by PCR using primers ACI5 (5′-ACTTACTTCAACTCGCGACG-3′) and ACI6 (5′-TAAACACCACATATGTTCCG-3′) corresponding to nucleotides 486–505 and 1128–1148 of the structural *ampC* gene, respectively (GenBank accession number AJ009979). The PCR was performed in a final volume of 50 μL containing 10 mM Tris–HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each nucleotide, 0.5 μM of each primer, 2.5 U of *Taq* DNA polymerase (Promega, Charbonnières, France) and 10 μL of DNA extract. After a 90 s denaturation step at 94°C, the 30 cycle PCR procedure was 30 s denaturation at 94°C, 30 s annealing at 57°C and 60 s extension at 72°C, followed by a final extension step of 10 min at 72°C. The PCR products were detected on a 1.5% agarose gel.

Determination of the ampC genetic environment by inverse-PCR

Genomic DNA extraction was performed using QIAmp DNA MiniKit (Qiagen, Courtaboeuf, France) and DNA was digested with *Hin*dIII endonuclease for 3 h at 37°C. After heat inactivation of the enzyme, a ligation was performed using T_4DNA ligase in a final volume of 10 μL. After overnight incubation at 4°C, 10 μL of the ligation product was added to a PCR mixture containing two inverse primers (INV5 and INV6, opposite sense from ACI5 and ACI6). The PCR was performed as previously described, apart from an extension time increased to 2 min. A band of ~1300 bp was obtained and sequenced in one strand using INV5 as a primer.

Amplification and sequencing of ampC 5′ non-coding sequence

After inverse-PCR and sequencing the 5′ non-coding region of susceptible strains, a new primer, ACI10 (5′-GCTGAACGCGATAAACTTC-3′), located upstream from the *ampC* gene was synthesized, and used with a primer located inside the *ampC* gene ACI2 (5′-TAGTACTGCTATTTACGGCT-3′) corresponding to nucleotides 327–347 of the *ampC* gene. PCR was carried out as previously described.

Detection of integrons by integrase-PCR

Screening for the presence of class 1 integrons for each strain was realized as previously described with integrase-specific primers.[21]

RT–PCR analysis for ampC mRNA detection

Total mRNA was extracted from three *A. baumannii* strains representing each group (isolates 1, 2 and 3) using the Qiagen RNeasy Protect Bacteria Mini Kits (Qiagen). Five microlitres of DNase-treated RNA was reverse-transcripted in a final volume of 25 μL containing 50 mM of Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 400 μM of each nucleotide, 2 μM of the reverse primer (ACI6 and 16S2) and 200 U of M-MLV reverse transcriptase (Promega). The reaction mixture was incubated for 1 h at 42°C, followed by a 5 min incubation at 95°C. Ten microlitres of the cDNA was used for amplification of specific *ampC* mRNA, using the primers for *ampC* (ACI5/ACI6) and 10 μL for the 16S rRNA (16S1 5′-GGAGGAAGGTGGGGATGACG-3′ and 16S2 5′-ATGGTGTGACGGGCGGTGTG-3′)[22] under the same conditions: 5 min initial denaturation at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 55°C and 1 min extension at 72°C, and a final extension step of 7 min at 72°C. Both PCR products were detected on a 1.5% agarose gel. Each RT–PCR was performed in triplicate.

Table 1. MICs (mg/L) of different β-lactams for all strains of the study

Strains	TIC	TIC + CLA	TIC + CLO	CTX	CTX + CLO	CAZ	CAZ + CLO	FEP	FEP + CLO	IPM	IPM + CLO	Isoelectric focusing (pI)
ATCC 19606	8	8	<2	16	<1	8	8	32	8	1	0.5	>8
Group I[a]												
1	16	16	8	16	4	4	4	32	16	1	0.25	ND
6	16	16	4	8	2	<2	<2	4	0.5	0.5	0.125	ND
14	8	4	<2	4	<1	<2	<2	32	<0.25	1	<0.03	ND
16	>512	256	>256	32	64	8	8	256	128	4	1	5.4, >8
17	16	8	4	8	<1	<2	<2	4	0.5	0.5	0.125	>8
18	16	16	4	8	2	4	2	4	1	0.5	0.125	ND
20	<4	<2	<2	4	<1	<2	<1	<0.5	<0.25	0.25	<0.03	ND
24	16	8	4	8	<1	<2	<2	2	0.5	0.5	0.125	ND
26	>512	256	>256	16	8	8	8	128	64	1	0.25	5.4, >8
33	32	32	16	32	16	8	8	16	8	0.5	0.5	ND
37	32	32	16	32	16	8	8	16	8	0.5	0.5	ND
38	32	32	16	16	16	8	8	16	8	0.5	0.5	ND
48	>512	256	>256	16	8	8	8	32	32	2	0.25	5.4, >8
49	32	32	16	16	16	8	8	16	8	0.5	0.5	>8
50	16	16	8	8	4	<2	<2	4	2	0.5	0.125	>8
51	32	32	16	16	16	8	8	8	8	0.5	0.5	>8
54	32	32	16	16	16	8	8	16	8	0.5	0.5	>8
64	32	32	16	16	16	8	8	8	8	0.5	1	ND
65	<4	4	<2	<2	<1	<2	<2	16	<0.25	0.5	<0.03	ND
67	16	2	<2	4	<1	<2	<2	2	<0.25	0.5	<0.03	ND
Group II[a]												
2	32	32	8	256	4	32	8	32	16	2	0.125	>8
8	32	32	4	256	<1	32	8	32	8	4	0.5	>8
10	32	32	8	256	4	32	8	32	16	2	0.125	>8
19	32	32	4	256	<1	32	4	32	2	4	0.25	>8
21	32	32	4	256	<1	32	8	32	8	2	0.5	>8
43	32	32	<2	128	<1	32	2	32	<0.25	4	0.5	>8
66	32	32	4	256	<1	32	8	32	8	4	0.25	>8
72	32	<2	<2	256	<1	32	8	32	8	4	0.25	>8
Group III[a]												
3	>512	>256	>256	128	64	512	>256	64	32	32	32	6.3, >8
4	>512	>256	>256	256	64	256	>256	64	32	1	1	>8
7	>512	>256	>256	256	64	512	>256	64	32	1	1	>8
12	>512	>256	>256	128	64	512	>256	32	32	2	1	>8
22	>512	>256	>256	128	64	512	>256	32	32	32	32	6.3, >8
28	>512	>256	>256	>256	128	>512	>256	256	64	2	1	5.4, >8
35	>512	>256	>256	256	128	512	>256	64	32	32	32	6.3, >8
55	>512	>256	>256	128	128	512	>256	64	64	64	>32	6.3, >8
57	>512	>256	>256	128	128	512	>256	64	64	64	>32	6.3, >8
59	>512	>256	>256	128	128	512	>256	64	64	64	>32	6.3, >8
62	>512	>256	>256	128	128	512	>256	64	64	64	>32	6.3, >8
63	>512	>256	>256	128	64	512	>256	32	64	32	32	6.3, >8
68	>512	>256	>256	128	128	512	>256	64	64	64	>32	6.3, >8
70	>512	>256	>256	128	128	512	>256	64	64	64	>32	6.3, >8

TIC, ticarcillin; CLA, clavulanate; CLO, cloxacillin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; IPM, imipenem; ND, not detected.
[a]See text for details.

Results

β-*Lactam resistance and* β-*lactamase expression by clinical A. baumannii isolates*

The susceptibilities of 42 *A. baumannii* clinical strains, isolated in 2001, to β-lactams were determined (Table 1). Strains can be separated into three groups: 20 strains (47.6%) showing a ceftazidime MIC ≤ 8 mg/L make up group I; eight strains (19%) showing a ceftazidime MIC 32 mg/L, which was decreased in the presence of cloxacillin, make up group II; and 14 strains (33.4%) showing a ceftazidime MIC ≥ 256 mg/L, which was not decreased in the presence of cloxacillin, make up group III. In group III, 10 of the 14 isolates (71%)

Table 2. PCR of the *ampC* gene and the *ampC* genetic environment of the study strains

Strains	ACI5/ACI6[a]	ACI1/ACI2[b]	ACI10/ACI2[c]	Integrase
ATCC 19606	+	–	356 bp	ND
Group I[d]				
1, 6, 14, 17, 18, 20, 24, 33, 37, 38, 49, 50, 51, 54, 64, 65 and 67	+	–	356 bp	–
16, 26 and 48	+	–	356 bp	+
Group II[d]				
2 and 10	+	+	~1500 bp	+
8, 19, 21, 43, 66 and 72	+	+	~1500 bp	–
Group III[d]				
3, 4, 7, 12, 22, 28, 35, 55, 57, 59, 62, 63, 68 and 70	+	+	~1500 bp	+

ND, not done.
[a]ACI5, forward primer located inside the structural constitutive *ampC* gene; ACI6, reverse primer located inside the structural constitutive *ampC* gene.
[b]ACI1, forward primer located upstream from the *ampC* gene according to the sequence published by Bou & Martinez-Beltran;[14] ACI2, reverse primer located inside the structural constitutive *ampC* gene.
[c]ACI10, forward primer located upstream from the *ampC* gene according to the sequence we determined by inverse-PCR.
[d]See text for details.

were also resistant to the carbapenem, imipenem (MIC ≥ 32 mg/L), and the imipenem MICs were not modified in the presence of cloxacillin. All the group I and II strains were susceptible to imipenem, and its MIC decreased (twice for group I strains and three- to four-fold in group II strains) in the presence of cloxacillin. PFGE molecular typing of the 42 isolates showed that all group I and II isolates represent different strains. However, 75% of group III isolates were collected from a burns intensive care unit and presented a high degree of similarity (>90%) (data not shown).

Isoelectric focusing was performed on the crude β-lactamase extracts of all 42 isolates. For 12 strains (all in group I), no β-lactamase could be detected, probably because β-lactamase expression was below the sensitivity of the method. For 30 strains (71%), including all the group II and III strains, a basic β-lactamase band, most probably corresponding to the AmpC cephalosporinase (pI > 8), was detected. In all cases, the AmpC band intensity was higher for strains from groups II and III than from group I. Several strains showed an additional β-lactamase band with a pI of 5.4. These strains were also characterized by their resistance to ticarcillin, decreased by clavulanate, so the pI 5.4 band most probably represents a TEM-type β-lactamase. For 10 group III strains having an imipenem MIC ≥32 mg/L, a pI 6.3 band was also visible after revelation with an iodine–starch agar gel containing cloxacillin instead of benzylpenicillin. Tests for the presence of a metallo-β-lactamase in these strains was negative using imipenem-EDTA and ceftazidime-EDTA screening discs.

ampC gene upstream sequence and AmpC expression in clinical A. baumannii isolates

PCR tests for the *ampC* gene were positive for all 42 strains. However, using forward and reverse primers (ACI1 and ACI2) located in the 5′ non-coding sequence and *ampC* gene, respectively, previously published by Bou & Martinez-Beltran,[14] group I (ceftazidime susceptible) strains did not give any PCR amplification product, as shown in Table 2. By inverse PCR, we determined the immediate 5′ upstream region of the *ampC* genes of these group I strains. The sequence obtained is very different from the *ampC* upstream regions of group II and III (ceftazidime resistant) isolates, as shown in Figure1. A PCR was then performed with template DNA from all the strains using a forward primer anchored in the *ampC* upstream sequence from group I isolates (ACI10) and the *ampC*-targeted reverse primer used previously (ACI2). Following this reaction, group I strains yielded an amplicon of 356 bp, but group II and III isolates gave an ~1500 bp amplicon. These results suggested an insertion upstream from the *ampC* gene in groups II and III isolates. The 1500 bp product was sequenced on both strands. Compared with the group I isolates, an insertion sequence of ~1200 bp, with characteristic direct repeats of 9 bp (5′-GAGCTAATC-3′) and inverted repeats of 11 bp (5′-CTCTGTACACG-3′), was present in the 5′ non-coding region of all group II and III strains. An alignment of this sequence with the EMBL database (http://www.ncbi.nlm.nih.gov/BLAST/) revealed 100% identity with an upstream sequence of the $phaB_{AC}$ gene in *A. baumannii*, encoding an acetoacetyl-CoA reductase.[23,24] This sequence was also previously detected upstream of the ARI-1 gene, encoding OXA-23, an extended-spectrum class D β-lactamase, in *A. baumannii* strains.[25]

RT–PCR analysis was conducted in order to estimate *ampC* transcript levels in the three different groups of strains. While a control reaction (measuring 16S rRNA levels) gave a similar strong signal for the three groups of strains, a large difference was obtained for the *ampC*-specific transcript (Figure 2). *ampC* mRNA is considerably more abundant in both group II and III strains, which have the *ampC* upstream insertion, than in group I strains, which do not.

The presence of integrase genes was confirmed by PCR in some of the strains, but there is no clear correlation with the presence of the *ampC* upstream insertion sequence (Table 2).

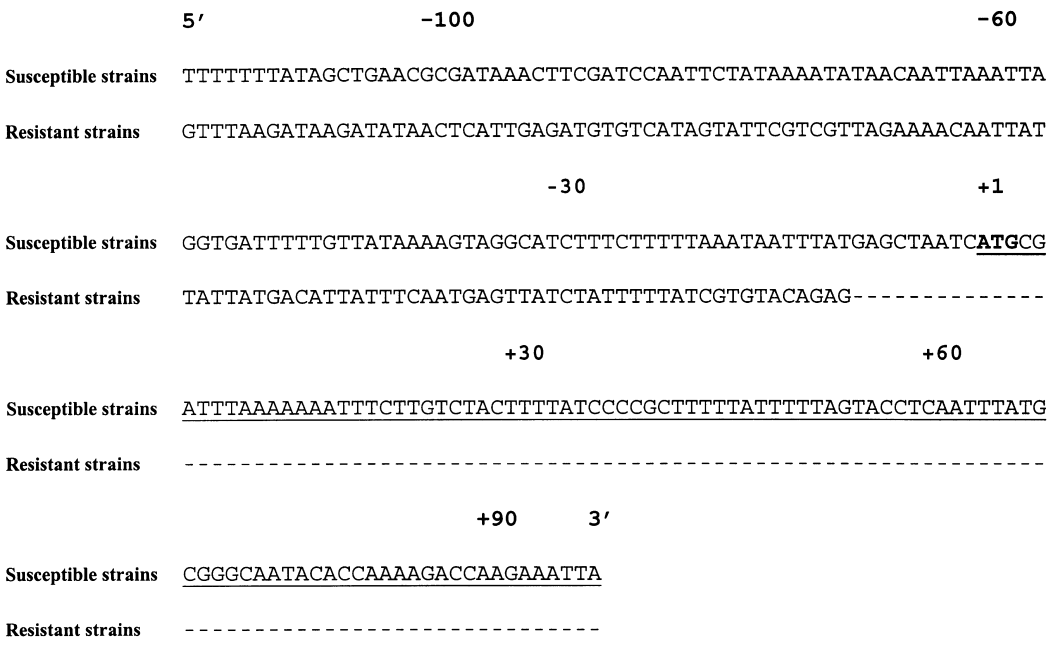

Figure 1. Comparison of the 5′ non-coding sequence of susceptible strains of *A. baumannii* determined by inverse-PCR and both group II and III resistant strains. The start codon is shown in bold type and the *ampC* gene sequence is underlined. Identical sequences are represented by dashed lines.

Discussion

In *Escherichia coli*, the main mechanism of overproduction of the constitutive AmpC enzyme is the acquisition of point mutations in the *ampC* promoter and attenuator regions.[26–29]

In *A. baumannii*, it appears that the presence of an ~1200 bp sequence upstream of *ampC* causes overexpression of AmpC. This 1200 bp sequence presents structural elements characteristic of an insertion sequence (IS): creation of direct repeats of 9 bp in the target sequence and presence of inverted repeats of 11 bp at each end of the inserted fragment.[30] RT–PCR analysis confirms the difference in the level of expression of the AmpC cephalosporinase. Furthermore, the RT–PCR results were in accordance with the ceftazidime MICs. Thus, the presence of the insertion sequence in both groups II and III probably increased the resistance. Accordingly, our experimental data suggest that overexpression of the *ampC* gene could be mediated by this IS, by providing a strong promoter upstream from the start codon ATG of the gene.

In *E. coli*, the crucial step of initiation of mRNA synthesis is strongly influenced by the sequence of two hexamers, the –35 and –10 boxes.[31] At these sites, consensus sequences have been defined for *E. coli*, respectively TTGACA and TATAAT, optimally separated by 17 bp. The closer sequences are to the consensus sequences, the stronger the promoter. The first two bases of the hexamers are known to be the best conserved ones among the various promoters sequenced.[31] No data are available about *A. baumannii* promoter consensus sequences. However, putative –35 and –10 boxes (TTGTTC and TATGAT) separated by 17 bp could be detected in the mobile element about 100 bp upstream of the start codon in the *A. baumannii ampC* gene. They differ from the *E. coli* consensus sequences by three bases and one base, respectively, the first bases being conserved.

Gene activation or inactivation by transposition of an IS element is a well-known phenomenon. Jaurin & Normark[32] described AmpC overproducing *E. coli* laboratory mutants with an IS*2* insertion sequence located in the promoter, leading to a 20-fold increase in promoter strength. On the other hand, in *E. coli*, enhanced efflux expression has been associated with insertion of IS*186* into the AcrAB repressor gene *acrR*, inducing reduced susceptibility to fluoroquinolones.[33] Our data confirm the role of mobile elements in modifying transcription rates of bacterial resistance genes.

Alignment with databases revealed 100% identity between the *ampC* upstream IS and the $phaB_{AC}$ upstream sequence, a mobile element previously found in the *A. baumannii* genome. Most interestingly, this same IS has been found upstream of the class D β-lactamase ARI-1.[25] To our knowledge, it is the first time that the same mobile element could be found upstream of both a class D oxacillinase gene usually located on integrons[34,35] and a class C gene like *ampC*. However, the same insertion sequence has been very recently detected upstream of the *aac(3)IIa* gene, encoding an aminoglycoside modifying enzyme.[36] This mobile element seems to be widespread, and

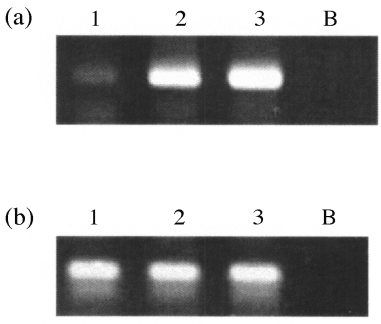

Figure 2. Comparison showing the intensity of the mRNA specific transcripts of the *ampC* gene (a) and 16S rRNA transcripts (b). Lane B, blank; lane 1, group I susceptible strain (isolate 1); lane 2, group II resistant strain (isolate 2); lane 3, group III resistant strain (isolate 3).

could represent a gene capture system, able to regulate different genes in *A. baumannii*.

It has also been shown recently that *Oligella urethralis* has integrated the *A. baumannii ampC* gene into its chromosome. The *ampC* gene in question carries the same upstream sequence as our group I strain.[37]

The distribution of the different resistance patterns in our study was very similar to that described by Danes *et al.*,[2] with nearly half of the strains being susceptible or moderately resistant to ceftazidime, and 33.4% showing a decreased susceptibility or resistance to imipenem. This resistance was not corrected by cloxacillin, so a mechanism other than AmpC β-lactamase overproduction must be involved. Carbapenem resistance in *A. baumannii* has been associated with modifications in outer membrane proteins[5] or penicillin-binding proteins,[3,4] and production of different β-lactamases.[2] Class B enzymes have been described in *A. baumannii*,[11,12] but the search for a metallo-enzyme was negative using an imipenem-EDTA or ceftazidime-EDTA test.[19,20] Furthermore, numerous studies have shown that imipenem resistance in *A. baumannii* was mostly linked to the production of a class D extended-spectrum oxacillinase.[8–10] These enzymes belong to two different clusters (OXA23/27 and OXA 24/25/26/40) and the genes encoding OXA-type β-lactamases are often located on class 1 integrons.[38] The integrase gene was detected by PCR in all the imipenem resistant strains of our study, but no oxacillinase gene could be detected with primers belonging to both clusters. Studies are in progress to understand the mechanism(s) underlying decreased susceptibility to imipenem of these strains.

References

1. Bergogne-Bérézin, E. & Towner, K. J. (1996). *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical and epidemiological features. *Clinical Microbiology Reviews* **9**, 148–65.

2. Danes, C., Navia, M. M., Ruiz, J. *et al.* (2002). Distribution of β-lactamases in *Acinetobacter baumannii* clinical isolates and the effect of Syn 2190 (AmpC inhibitor) on the MICs of different β-lactam antibiotics. *Journal of Antimicrobial Chemotherapy* **50**, 261–4.

3. Fernandez-Cuenca, F., Martinez-Martinez, L., Conejo, M. C. *et al.* (2003). Relationship between β-lactamase production, outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates of *Acinetobacter baumannii*. *Journal of Antimicrobial Chemotherapy* **51**, 565–74.

4. Gehrlen, M., Leving, H., Cullman, W. *et al.* (1991). Imipenem resistance in *Acinetobacter baumannii* is due to altered penicillin-binding proteins. *Chemotherapy* **37**, 405–12.

5. Clark, R. B. (1996). Imipenem resistance among *Acinetobacter baumannii*: association with reduced expression of a 33–36 kDa outer membrane protein. *Journal of Antimicrobial Chemotherapy* **38**, 245–51.

6. Paul, G., Joly-Guillou, M. L., Bergogne-Bérezin, E. *et al.* (1989). Novel carbenicillin-hydrolysing β-lactamase (CARB-5) from *Acinetobacter calcoaceticus* var. *anitratus*. *FEMS Microbiology Letters* **59**, 45–50.

7. Vila, J. (1998). Mechanisms of antimicrobial resistance in *Acinetobacter baumannii*. *Reviews in Medical Microbiology* **9**, 87–97.

8. Afzal-Shah, M., Woodford, N. & Livermore, D. M. (2001). Characterization of OXA-25, OXA-26, and OXA-27, molecular class D β-lactamases associated with carbapenem resistance in clinical isolates of *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy* **45**, 583–8.

9. Bou, G., Oliver, A. & Martinez-Beltran, J. (2000). OXA-24, a novel class D β-lactamase with carbapenemase activity in an *Acinetobacter baumannii* clinical strain. *Antimicrobial Agents and Chemotherapy* **44**, 1556–61.

10. Lopez-Otsoa, F., Gallego, L., Towner, K. J. *et al.* (2002). Endemic carbapenem resistance associated with OXA-40 carbapenemase among *Acinetobacter baumannii* isolates from a hospital in northern Spain. *Journal of Clinical Microbiology* **40**, 4741–3.

11. Chu, Y. W., Afzal-Shah, M., Houang, E. T. *et al.* (2001). IMP-4, a novel metallo-β-lactamase from nosocomial *Acinetobacter* spp. collected in Hong-Kong between 1994 and 1998. *Antimicrobial Agents and Chemotherapy* **45**, 710–4.

12. Tysall, L., Stockdale, M. W., Chadwick, P. R. *et al.* (2002). IMP-1 carbapenemase detected in an *Acinetobacter* clinical isolate from the UK. *Journal of Antimicrobial Chemotherapy* **49**, 217–8.

13. Perilli, M., Felici, A., Oratore, A. *et al.* (1996). Characterization of the chromosomal cephalosporinases produced by *Acinetobacter lwoffii* and *Acinetobacter baumannii* clinical isolates. *Antimicrobial Agents and Chemotherapy* **40**, 715–9.

14. Bou, G. & Martinez-Beltran, J. (2000). Cloning, nucleotide sequencing, and analysis of the gene encoding an AmpC β-lactamase in *Acinetobacter baumannii*. *Antimicrobial Agents and Chemotherapy* **44**, 428–32.

15. Barlow, M. & Hall, B. G. (2002). Origin and evolution of the AmpC β-lactamases of *Citrobacter freundii*. *Antimicrobial Agents and Chemotherapy* **46**, 1190–8.

16. Tenover, F. C., Arbeit, R. D., Goering, R. V. *et al.* (1995). Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *Journal of Clinical Microbiology* **33**, 2233–9.

17. Comité de l'Antibiogramme de la Société Française de Microbiologie. (2003). Communiqué 2003. [Online]. http://www.sfm.asso.fr/nouv/general.php?pa=2 (6 August 2003, date last accessed).

18. Matthew, M., Harris, A. M., Marshall, M. J. *et al.* (1975). The use of analytical isoelectric focusing for detection and identification of β-lactamases. *Journal of General Microbiology* **88**, 169–78.

19. Lee, K., Chong, Y., Shin, H. B. *et al.* (2001). Modified Hodge and EDTA-disk synergy tests to screen metallo-β-lactamase-producing strains of *Pseudomonas* and *Acinetobacter* species. *Clinical Microbiology and Infection* **7**, 88–91.

20. Yong, D., Lee, K., Yum, J. H. *et al.* (2002). Imipenem-EDTA disk method for differentiation of metallo-β-lactamase-producing clinical isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *Journal of Clinical Microbiology* **40**, 3798–801.

21. Koeleman, J. G. M., Stoof, J., Van Der Bijl, M. W. *et al.* (2001). Identification of epidemic strains of *Acinetobacter baumannii* by integrase gene PCR. *Journal of Clinical Microbiology* **39**, 8–13.

22. Martineau, F., Picard, F. J., Roy, P. H. *et al.* (1996). Species-specific and ubiquitous DNA-based assays for rapid identification of *Staphylococcus epidermidis*. *Journal of Clinical Microbiology* **34**, 2888–93.

23. Schembri, M. A., Bayly, R. C. & Davies, J. K. (1994). Cloning and analysis of the polyhydroxyalkanoic acid synthase gene from an *Acinetobacter* sp.: evidence that the gene is both plasmid and chromosomally located. *FEMS Microbiology Letters* **118**, 145–52.

24. Schembri, M. A., Bayly, R. C. & Davies, J. K. (1995). Phosphate concentration regulates transcription of the *Acinetobacter* polhydroxyalkanoic acid biosynthetic genes. *Journal of Bacteriology* **177**, 4501–7.

25. Donald, H. M., Scaife, W., Amyes, S. G. B. *et al.* (2000). Sequence analysis of ARI-1, a novel OXA β-lactamase, responsible for imipenem resistance in *Acinetobacter baumannii* 6B92. *Antimicrobial Agents and Chemotherapy* **44**, 196–9.

26. Caroff, N., Espaze, E., Gautreau, D. *et al.* (2000). Analysis of the effects of –42 and –32 *ampC* promoter mutations in clinical isolates of *Escherichia coli* hyperproducing AmpC. *Journal of Antimicrobial Chemotherapy* **45**, 783–8.

27. Corvec, S., Caroff, N., Espaze, E. *et al.* (2002). –11 Mutation in the *ampC* promoter increasing resistance to β-lactams in a clinical *Escherichia coli* strain. *Antimicrobial Agents and Chemotherapy* **46**, 3265–7.

28. Nelson, E. C. & Elisha, B. G. (1999). Molecular basis of AmpC hyperproduction in clinical isolates of *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **43**, 957–9.

29. Jaurin, B., Grundström, T. & Normark, S. (1982). Sequence elements determining *ampC* promoter strength in *E. coli*. *EMBO Journal* **1**, 875–81.

30. Mahillon, J. & Chandler, M. (1998). Insertion sequences. *Microbiology and Molecular Biology Reviews* **62**, 725–74.

31. Harley C. B. & Reynolds, R. P. (1987). Analysis of *Escherichia coli* promoter sequences. *Nucleic Acids Research* **15**, 2343–61.

32. Jaurin, B. & Normark, S. (1983). Insertion of IS2 creates a novel *ampC* promoter in *Escherichia coli*. *Cell* **32**, 809–16.

33. Jellen-Ritter, A. S. & Kern, W. V. (2001). Enhanced expression of the multidrug efflux pumps AcrAB and AcrEF associated with insertion element transposition in *Escherichia coli* mutants selected with a fluoroquinolone. *Antimicrobial Agents and Chemotherapy* **45**, 1467–72.

34. Gallego, L. & Towner, K. J. (2001). Carriage of class 1 integrons and antibiotic resistance in clinical isolates of *Acinetobacter baumannii* from Northern Spain. *Journal of Medical Microbiology* **50**, 71–7.

35. Gombac, F., Riccio, M. L., Rossolini, G. M. *et al*. (2002). Molecular characterization of integrons in epidemiologically unrelated clinical isolates of *Acinetobacter baumannii* from Italian hospitals reveals a limited diversity of gene cassette arrays. *Antimicrobial Agents and Chemotherapy* **46**, 3665–8.

36. Segal, H., Thomas, R. & Gay Elisha, B. (2003). Characterization of class 1 integron resistance gene cassettes and the identification of a novel IS-like element in *Acinetobacter baumannii*. *Plasmid* **49**, 169–78.

37. Mammeri, H., Poirel, L., Mangeney, N. *et al*. (2003). Chromosomal integration of a cephalosporinase gene from *Acinetobacter baumannii* into *Oligella urethralis* as a source of acquired resistance to β-lactams. *Antimicrobial Agents and Chemotherapy* **47**, 1536–42.

38. Naas, T. & Nordmann, P. (1999). OXA-type β-lactamases. *Current Pharmaceutical Design* **5**, 865–79.