

BACTERIAL GENETICS

Carriage of class 1 integrons and antibiotic resistance in clinical isolates of *Acinetobacter baumannii* from Northern Spain

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A collection of 70 clinical isolates of *Acinetobacter baumannii* from Bilbao in northern Spain was examined by PCR for the presence of class 1 integron structures. The organisms comprised 21 distinct RAPD genotypes, with 10 distinct antibiogram patterns. Four different integron structures were detected in a total of 59 (84%) of the 70 isolates, with two predominant integron structures found in 20 and 30 isolates each. No clear antibiogram differences could be correlated with the presence or absence of integron structures, but sequence analysis of two of the internal integron regions indicated homology with genes encoding ANT(2'') adenylyltransferase activity and AAC(6')-Ib acetyltransferase activity. Phenotypic analysis of aminoglycoside resistance profiles indicated that many isolates produced a combination of aminoglycoside-modifying enzymes, with most of the observed resistance to amikacin being associated with a gene encoding APH(3')-VI phosphotransferase, as detected by PCR. RAPD analysis indicated that all the Bilbao isolates producing APH(3')-VI were distinct from an epidemic integron-carrying and APH(3')-VI-producing *Acinetobacter* strain found in other regions of Spain. It is concluded that, although class 1 integrons are widely disseminated amongst clinical isolates of *A. baumannii* from the Bilbao region of Spain, at present they are not playing a major role in the dissemination of antibiotic resistance genes in this region.

Introduction

Members of the genus *Acinetobacter*, predominantly *A. baumannii*, are recognised as increasingly important nosocomial pathogens, especially in intensive care units, where they are associated particularly with nosocomial pneumonia [1]. Such infections are often difficult to treat because of the ability of acinetobacters to become rapidly resistant to multiple antibiotics, including aminoglycosides, expanded-spectrum cephalosporins, carbapenems and fluoroquinolones [1, 2]. Some strains are effectively untreatable, and the severity and difficulties associated with nosocomial infection by *A. baumannii* are related directly to the particularly high rates of resistance found [3].

As in other species, plasmids and transposons play an important role in the acquisition of antibiotic resistance by *Acinetobacter* spp. [4]. In addition, antibiotic resistance in *Acinetobacter* spp., particularly to aminoglycosides, has been associated increasingly with the presence of integrons [5–8]. These elements possess a site-specific recombination system enabling the insertion, deletion and re-arrangement of discrete genetic cassettes, often comprising antibiotic resistance genes, within the integron structure [9–13]. Several classes of integrons have been described [13], with class 1 integrons being the most common and widely distributed among gram-negative bacteria [14, 15]. Integrons have been found in isolates of *Acinetobacter* spp. from various locations worldwide [5, 7, 8] and it has been suggested that multiresistant isolates of *Acinetobacter* spp. may act as a reservoir of integron-associated antibiotic resistance genes, which could then spread to other pathogens in the hospital environment [8].

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The main aim of the present study was to determine

the prevalence of class 1 integrons among nosocomial isolates of *A. baumannii* from the city of Bilbao in northern Spain, and to examine whether there was any correlation between antibiotic resistance and carriage of class 1 integrons. The Hospital of Santa Marina, Bilbao, is a 200-bed institution specialising in respiratory illness. Infections caused by *A. baumannii* are a particular problem. An epidemic strain of *A. baumannii* carrying a class 1 integron has been reported previously in Barcelona and other regions of Spain [6, 8, 16] and the study also aimed to establish whether this strain had spread from Barcelona to Bilbao.

Materials and methods

Bacterial strains

In total, 70 isolates of *A. baumannii* were obtained from different patients attending the Hospital of Santa Marina, Bilbao, Spain, from June 1998 until April 1999. The specimens from which *A. baumannii* were isolated comprised 58 sputum samples, three blood cultures, one broncho-alveolar sample, one urine, three wound swabs, three pleural aspirates and one stool. All the isolates were identified initially with the API 32 system (bioMérieux, Marcy l'Etoile, France), with identification to the level of genomic species being confirmed by tDNA fingerprinting [17]. The isolates were stored at -20°C in nutrient broth containing glycerol 50% v/v before inclusion in this study. An integron-carrying strain of *A. baumannii* from Barcelona (generously donated by Professor J. Vila), designated previously as strain Ab41 [6] or strain ESP41 [8], was included in the study, as was the sensitive type strain of *A. baumannii*, ATCC 19606.

Antibiotic profiles

MICs for the isolates were determined by the broth micro-dilution method described by the National Committee for Clinical Laboratory Standards [18], with *Pseudomonas aeruginosa* strain ATCC 27853 as a control. The antibiotics tested were aztreonam, cefepime, cefotaxime, ceftazidime, imipenem, meropenem, ticarcillin, amikacin, gentamicin, ciprofloxacin, levofloxacin, norfloxacin and ofloxacin. Similarity coefficients (S_{AB} values) were calculated and subjected to cluster analysis as described previously [19], except that MIC values rather than inhibition zone sizes were used. Aminoglycoside resistance profiles were analysed further by a disk method, with 12 different drug analogues [20].

DNA fingerprinting

Total genomic DNA extracts of *A. baumannii* isolates were prepared [21] and then compared by analysis of randomly amplified polymorphic DNA (RAPD) fingerprints, obtained with primers AP3 [22] and ERIC2 [21]. Previous investigations have determined that good

discrimination between genetically unrelated groups of *Acinetobacter* spp. is achieved at an S_{AB} value of 0.7 [21]; therefore, isolates that clustered together with an S_{AB} value of >0.7 , with both primers, were considered to be closely related and to form a single RAPD genotype. Comparison of isolates from Bilbao and Barcelona was performed with M13 core primer, as described previously [21].

Detection and characterisation of class 1 integrons

Total genomic DNA extracts of *A. baumannii* isolates were used as templates in PCR experiments to detect the hot-spot insertion regions of class 1 integrons. Each PCR was performed with a Ready-To-Go PCR bead (Amersham Pharmacia Biotech, Little Chalfont, Bucks) in a final 25- μl volume containing 5 μl of DNA extract and 2.5 pmol each of primers 5'CS and 3'CS, as described previously [8]. Initial comparison of the inserted cassettes in the class 1 integron structures detected was performed by taking 5- μl portions of the primary PCR products and digesting them with *Hinf*I, as described previously [8].

Further PCR analysis of possible inserted cassettes was performed by taking total genomic DNA or initial integron PCR products and using the primers and methodology described by Vila *et al.* [16] to seek the *aph(3')-VIa* gene, which is reported to be responsible for much of the amikacin resistance found in *A. baumannii* isolates from Spain [16].

Preliminary sequence data

Cycle sequencing of PCR products, purified with a GFX PCR DNA Gel Band Purification Kit (Amersham), was performed with the 5'CS primer and a Cy5 Thermo Sequenase Dye Terminator Kit (Amersham), with both kits used as recommended by the manufacturer. DNA sequencing reactions were analysed on acrylamide (Premix Long Ranger Gel Solution; Flowgen, Ashby de la Zouch, Leics) 6% w/v gels in an automated ALFexpress DNA Sequencer (Amersham). The DNA sequences obtained were screened against known sequences contained in nucleic acid sequence databases by the BLAST (Basic Local Alignment Search Tool) on-line method (www.ncbi.nlm.nih.gov/BLAST/).

Results

DNA fingerprinting experiments and antibiograms

Twenty-one distinct RAPD genotypes of *A. baumannii* were identified among the 70 isolates included in the study; these are listed in Table 1, together with antibiogram data for one isolate of each type. Three genotypes (P, Q and R; Table 1) predominated, collectively accounting for 44 (63%) of the 70 isolates.

Table 1. Antibiograms for *A. baumannii* isolates representing the 21 RAPD genotypes delineated during the study

RAPD genotype (number of isolates)	Representative isolate	Origin	MIC (mg/L)												
			TIC	CTX	CAZ	CFM	IMP	MEM	AZT	AMI	GEN	NOR	CIP	LEV	OFL
A (2)	SM10	Spt	128	128	128	128	>128	>128	128	32	>128	>128	64	16	64
B (1)	SM12	Spt	>128	16	>128	128	>128	>128	32	4	>128	>128	32	16	16
C (2)	SM13	B/c	32	1	>128	64	32	8	32	4	>128	>128	32	16	16
D (1)	SM15	Spt	1	1	1	32	8	2	4	4	>128	64	8	8	8
E (1)	SM16	Spt	128	64	>128	128	>128	>128	32	32	>128	>128	64	16	32
F (1)	SM17	Spt	4	1	1	64	2	2	8	2	>128	1	<1	2	2
G (1)	SM18	Spt	1	<1	<1	32	<1	<1	<1	4	>128	64	8	8	8
H (4)	SM20	Wnd	1	<1	<1	64	<1	<1	<1	1	>128	64	16	16	16
I (1)	SM21	Spt	32	4	<1	4	4	16	4	4	4	2	2	4	4
J (2)	SM25	Spt	16	64	<1	1	8	2	1	1	1	2	<1	2	2
K (1)	SM27	Spt	32	64	16	16	<1	2	32	8	>128	>128	128	32	32
L (1)	SM28	Spt	>128	>128	>128	>128	>128	>128	128	32	>128	>128	128	32	32
M (3)	SM29	Spt	>128	>128	>128	64	>128	>128	128	64	>128	>128	128	16	32
N (1)	SM30	Plp	>128	>128	>128	64	>128	>128	128	32	>128	>128	>128	16	32
O (1)	SM33	Spt	8	16	8	16	<1	<1	16	8	>128	>128	64	16	32
P (14)	SM36	Spt	>128	>128	>128	32	>128	>128	128	128	>128	>128	128	16	32
Q (23)	SM80	Spt	>128	>128	64	>128	>128	128	128	64	>128	>128	128	16	32
R (7)	SM49	Spt	128	64	128	128	32	16	32	32	>128	64	64	16	32
S (1)	SM52	Spt	16	4	4	8	4	16	16	32	2	4	4	16	16
T (1)	SM69	Spt	>128	>128	16	16	64	32	32	32	64	>128	64	16	32
U (1)	SM77	Spt	64	128	32	32	4	1	64	32	64	128	32	8	16

Spt, sputum; B/c, blood culture; Wnd, wound; Plp, pleural aspirate; TIC, ticarcillin; CTX, cefotaxime; CAZ, ceftazidime; CFM, cefepime; IMP, imipenem; MEM, meropenem; AZT, aztreonam; AMI, amikacin; GEN, gentamicin; NOR, norfloxacin; CIP, ciprofloxacin; LEV, levofloxacin; OFL, ofloxacin.

Minor variations in antibiograms were observed within each genotype, and the isolates listed are the most resistant representatives of each type. Antibiograms with an S_{AB} value of >0.9 were considered to be indistinguishable. On this basis, 10 distinct antibiograms were distinguishable among the 21 different genotypes, of which the most common was shared by 54 (77%) isolates belonging to 10 genotypes (A, B, E, L, M, N, P, Q, R and T). None of the other antibiograms was shared by more than two genotypes.

Detection of class 1 integrons by PCR

Amplification with primers specific for the 5' and 3' conserved regions of class 1 integrons yielded PCR products from 59 of the 70 isolates. Four predominant PCR products of 760, 550, 800 and 600 bp were visualised (Table 2). Additional faint PCR products of various sizes were visualised occasionally, but were not reproducible and were not investigated further. Four different *HinfI* fingerprint patterns, corresponding to the four predominant PCR products, were obtained and designated *a-d* (Table 2). The most common structures were *a* and *b*, found in 20 and 30 isolates, respectively. Structures *c* and *d* were detected in eight isolates and one isolate, respectively. Based on the sizes of the inserted cassette regions detected and the reported sizes of previously characterised gene cassettes [23], it seemed likely that each of the four integron structures contained one, or at most two, inserted gene cassettes. Integrons of the same internal structure were found in more than one *Acinetobacter* genotype, but no single genotype contained more than one type of integron structure (Table 2).

Correlation of antibiotic resistance profiles with integron carriage and genotypes

Attempts were made to correlate the observed minor variations in antibiograms within each genotype with the presence or absence of integron structures, but no clear distinctions could be made. For example, only 10 of the 14 isolates that belonged to genotype P yielded a type *a* integron structure, and these 10 isolates varied slightly in antibiotic susceptibilities among themselves and from the four isolates that lacked an integron structure.

For screening purposes, preliminary DNA sequences were generated from the 5' end of purified PCR products corresponding to the inserted cassette regions in each of the four integron structures. Isolate SM36 (structure *a*) yielded a 550-base sequence that showed 95% base homology with the *aadB* gene cassette encoding ANT(2'') adenylyltransferase activity (accession no. U14415 [24]). Similarly, isolate SM49 (structure *c*) yielded a 650-base sequence that showed 93% base homology with an *aacA4* gene encoding AAC(6')-Ib acetyltransferase activity (accession no. AF043381). Taken together with the amplified external non-coding regions, the known sizes of these *aadB* and *aacA4* gene cassettes (591 bp and 637 bp, respectively [23]) were sufficient to account for the entire amplified regions (Table 2) of integron structures *a* and *c*. No significant database homology was detected with a 400-base sequence generated from isolate SM80 (structure *b*) or a 650-base sequence generated from isolate SM52 (structure *d*).

Phenotypic and genotypic analysis of aminoglycoside resistance genes

Members of the genus *Acinetobacter* frequently have complex combinations of aminoglycoside resistance mechanisms [20, 25]. Of the two gene cassettes identified here, *aadB*, encoding ANT(2''), confers resistance to gentamicin and tobramycin, whereas *aacA4*, encoding one of the AAC(6')-I family of enzymes, confers resistance to tobramycin, amikacin and netilmicin. Amikacin resistance in *Acinetobacter* spp. can be associated with production of either 6'-*N*-acetyltransferase type I (AAC(6')-I) enzymes [26] or 3'-*O*-phosphotransferase type VI (APH(3')-VI) [27]. To distinguish these possibilities, total DNA and integron PCR products from the representative isolates listed in Table 1, corresponding to the 21 different genotypes, were screened by PCR for the presence of the *aph(3')-VIa* gene. Of the 10 isolates that expressed resistance (MIC > 16 mg/L) to amikacin, eight (SM10, 16, 28, 29, 30, 36, 49 and 80) yielded a 234-bp PCR product characteristic of the *aph(3')-VIa* gene [16] when total, but not integron, DNA was used as a template. Thus, the *aph(3')-VIa* gene was widely distributed amongst the different genotypes, but did not form part of the integron structures identified. Isolate SM49 possessed both an *aph(3')-VIa* gene and an integron-associated

Table 2. Characteristics of the inserted cassette regions of the class 1 integron structures detected

Integron	Size (bp)	<i>HinfI</i> digestion products	Genotype (Number of isolates)
<i>a</i>	760	350, 220, 190	A (1), C (2), F (1), H (2), I (1), K(1), N(1), O (1), P (10)
<i>b</i>	550	550	B (1), L (1), M (3), Q (23), T (1), U (1)
<i>c</i>	800	550, 170	D (1), R (7)
<i>d</i>	600	300, 170, 110	S (1)

aacA4 gene. Two isolates (SM69 and 77) were resistant to amikacin, but lacked an *aph(3')-VIa* gene; these isolates were assumed to express an AAC(6')-I enzyme, but lacked the type *c* integron structure found in SM49. In contrast, isolate SM15 contained the type *c* integron structure, but was susceptible to amikacin (Tables 1 and 2).

Table 3 lists the inhibition zone sizes of aminoglycoside disks for four representative isolates carrying the four different integron structures. Isolate SM52 (integron structure *d*) did not differ significantly in behaviour from the sensitive type strain of *A. baumannii*. Therefore, it was concluded that the type *d* integron structure did not encode aminoglycoside resistance. After taking account of the resistance phenotype associated with *aph(3')-VIa* (already demonstrated by PCR in isolates SM36, SM49 and SM80), the profiles obtained also suggested production of ANT(2'') and an AAC(3) enzyme by isolate SM36, the production of AAC(3)-II by isolate SM80, and the production of AAC(6')-I and AAC(3)-II by isolate SM49. These results were consistent with the preliminary integron sequencing data in the two cases (isolates SM36 and SM49) where significant homology was identified.

Dissemination of the *aph(3')-VI* gene in Spain

Amikacin resistance in *Acinetobacter* spp. from different regions of Spain has previously been associated with the spread of an epidemic strain carrying the *aph(3')-VI* gene [16]. To determine whether the amikacin resistance found in Bilbao was associated, at least in part, with spread of the same epidemic strain, representatives (SM10, 16, 28, 29, 30, 36, 49 and 80) of the eight genotypes found to carry the *aph(3')-VIa* gene were directly compared in RAPD experiments with the epidemic strain ESP41 found in Barcelona and other regions of Spain. The RAPD profiles obtained with M13 core primer (Fig. 1) indicated that isolates SM28 and SM29 were indistinguishable from each other with this primer (although they were distinguished with primers AP3 and ERIC2),

but all eight Bilbao genotypes with the *aph(3')-VIa* gene were clearly distinguishable from strain ESP41.

Discussion

Class 1 integron structures were detected by PCR in 59 (84%) of 70 clinical isolates of *A. baumannii* from Bilbao. Although these clinical isolates were genetically diverse, with 21 distinct genotypes identified, only four integron structures – recognised on the basis of their *Hinf*I restriction endonuclease fingerprint profiles – were identified. These structures were disseminated amongst 18 genotypes, with types *a* and *b* being found in 20 and 30 isolates, respectively. These findings clearly indicate the spread of integron structures among different *A. baumannii* genotypes found in Bilbao. However, and in contrast to many integrons found in *A. baumannii* elsewhere in the world [8], the Bilbao structures seemed to have acquired only one (or at most two) inserted resistance gene cassettes.

Ten different antibiograms were seen amongst the 21 genotypes, but there were no clear phenotypic differences between isolates of the same genotype with and without an integron. Preliminary sequence analysis indicated the presence of cassettes encoding ANT(2'') adenylyltransferase and AAC(6')-Ib acetyltransferase activity for two of the integron structures (*a* and *c*, respectively). Aminoglycoside resistance determinants are the predominant gene cassettes found in integrons from gram-negative bacteria [10], including *Acinetobacter* isolates collected worldwide [8]. However, many multiply-resistant acinetobacters produce complex combinations of aminoglycoside-modifying enzymes, many of which are not encoded by integrons [20, 25], and analysis of disk inhibition tests indicated that this was the case for the present isolates.

As a strain of *A. baumannii* carrying a class 1 integron was reported to be widely disseminated in Barcelona and other regions of Spain [6, 8, 16], it was interesting to determine whether this strain had spread to the Bilbao region. The Barcelona strain encodes APH(3')-VI, a 3'-*O*-phosphotransferase that inactivates amikacin

Table 3. Aminoglycoside disk zone sizes and deduced aminoglycoside-modifying enzymes for representative isolates of *A. baumannii* carrying the four integron structures

Isolate (integron structure)	Aminoglycoside disk zone sizes (mm)												Inferred aminoglycoside -modifying enzymes
	GEN	TOB	AMK	KAN	APR	NET	6'NET	2'NET	EPI	ISP	FOR	NEO	
SM36 (a)	NZ	NZ	9	NZ	29	19	12	9	21	NZ	24	12	APH(3')-VI*, ANT(2''), AAC(3)-?
SM80 (b)	NZ	16	14	NZ	24	11	NZ	NZ	19	11	22	13	APH(3')-VI*, AAC(3)-II
SM49 (c)	NZ	NZ	16	NZ	25	NZ	16	NZ	NZ	13	24	11	APH(3')-VI*, AAC(6')-I, AAC(3)-II
SM52 (d)	13	13	24	14	26	25	22	22	23	24	29	23	None
ATCC 19606†	17	16	25	NZ	NZ	29	20	18	21	23	21	23	None

AMK, amikacin; APR, apramycin; EPI, episisomicin; FOR, fortimicin; GEN, gentamicin; ISP, isepamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; 6'-NET, 6'-netilmicin; 2'Net, 2'-netilmicin; TOB, tobramycin; NZ, no zone.

*The presence of the gene encoding APH(3')-VI was confirmed by PCR. †Sensitive type strain of *A. baumannii*.

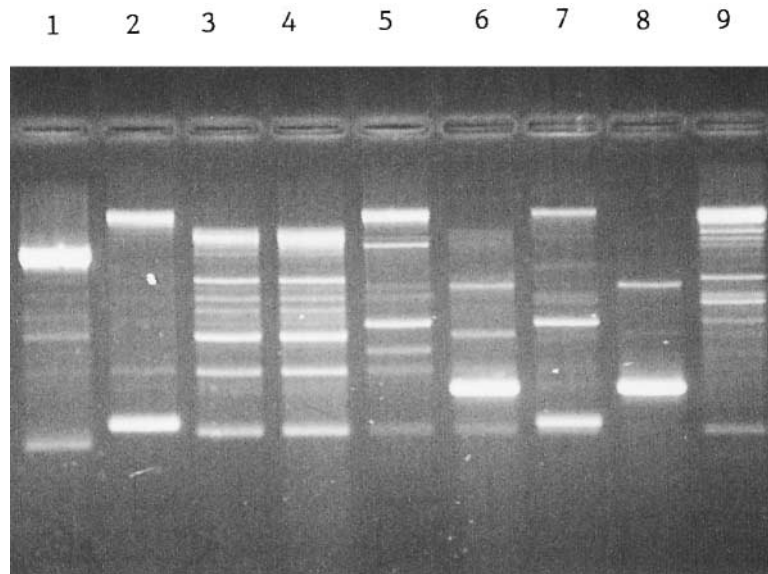


Fig. 1. Comparative RAPD profiles obtained with M13 primer for *A. baumannii* isolates carrying the *aph(3')-VI* gene. Lanes: 1, isolate SM10; 2, SM16; 3, SM28; 4, SM29; 5, SM30; 6, SM36; 7, SM49; 8, SM80; 9, ESP41 (epidemic isolate from Barcelona).

[27]. This gene was detected by PCR in eight of the 10 genotypes found in Bilbao that expressed resistance to amikacin. However, all these genotypes were clearly distinguished from the Barcelona strain. As in France [28], it seems that *aph(3')-VIa* has spread to several different genotypes, including two predominant types in Bilbao, and that spread of a single resistant strain is not implicated, as in other areas in Spain [16]. Work in France suggests that *aph(3')-VIa* may be transposable [28] or associated with plasmids that are self-transmissible among some *Acinetobacter* strains, or both [27, 28]. Preliminary experiments indicate that many of the Bilbao isolates carry diverse collections of plasmids of various molecular sizes (unpublished results).

It was concluded that although class 1 integrons appear to be widely disseminated amongst clinical isolates of *A. baumannii* from the Bilbao region of Spain, these structures are not playing a major role in the dissemination of antibiotic resistance genes. Clinical isolates of *A. baumannii* from diverse worldwide locations seem to share resistance mechanisms with other genera, although many resistance plasmids from other genera are unstable in *A. baumannii*. Thus, despite their limited role at present, the presence of integrons in 84% of the clinical isolates examined in Bilbao is a cause for concern, as these structures may enhance the ability of these organisms to respond rapidly to the challenge of new antibiotics, enabling otherwise unstable resistance genes to be incorporated easily into the bacterial chromosome or into indigenous *Acinetobacter* plasmids. Isolates of *A. baumannii* from some centres are already extremely difficult to combat therapeutically [1], and the widespread occurrence of integrons in this species can only add to the problem.

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