ANTIMICROBIAL AGENTS

Molecular epidemiology of aminoglycoside resistance in *Acinetobacter* spp.

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Most aminoglycoside resistance in *Acinetobacter* spp. involves production of aminoglycoside-modifying enzymes. Previous studies have shown that the genes encoding these enzymes can be present on plasmids, transposons or within integron-type structures. To determine whether particular mechanisms of aminoglycoside resistance have developed in strains from specific geographical locations (with subsequent clonal spread), or whether common mechanisms have been acquired by genotypically distinct clinical isolates of *Acinetobacter* spp. throughout the world, a genotypically heterogeneous collection of 24 multiresistant clinical isolates of *Acinetobacter* spp. from 15 hospitals in 11 countries worldwide was studied. All were resistant to two or more aminoglycoside antibiotics. The full aminoglycoside resistance profile was determined for each isolate, allowing a putative enzyme content to be inferred, with subsequent confirmation of enzyme content and genetic location by polymerase chain reaction (PCR) and hybridisation techniques. All produced at least one aminoglycoside-modifying enzyme, most commonly AAC(3)-I and ANT(3'')-I in various combinations. Other enzymes found were AAC(3)-II, AAC(6')-I, ANT(2''), APH(3')-I and APH(3')-VI. None was confined to strains from a particular geographical area. Nine isolates transferred resistance mediated by AAC(3)-I, ANT(2'')-I, APH(3')-I or APH(3')-VI by conjugation to a sensitive strain of *A. baumannii*, but most resistance was non-transferable. PCR mapping revealed an integron location in six isolates for the *aac(3)-Ia* gene and in three isolates for the *ant(3'')-Ia* gene. Overall, the study demonstrated that similar aminoglycoside-modifying enzymes are found in unrelated isolates of *Acinetobacter* spp., and that particular genes are not restricted to specific areas of the world. The demonstration of certain genes on plasmids and integrons emphasises the probable importance of these structures in the dissemination of certain types of aminoglycoside resistance in *Acinetobacter* spp.

Introduction

Acinetobacter spp., particularly *A. baumannii*, cause severe nosocomial infections, especially in intensive care units [1]. Such infections have become difficult to treat as many strains are now resistant to multiple antibiotics, including the aminoglycosides, third-generation cephalosporins and fluoroquinolones [2]. Resistance rates to all the clinically useful aminoglycosides are higher in *Acinetobacter* spp. than any other pathogen group [3]. There are various mechanisms by which bacteria can become resistant to aminoglycosides, including alteration of the ribosomal target site and reduction of uptake [4, 5]; however, the production of aminoglycoside-modifying enzymes is

thought to account for most resistance in clinical isolates of *Acinetobacter* spp. [3]. Three classes of aminoglycoside-modifying enzymes exist, all of which have been found in *Acinetobacter* spp. [5]. These enzymes are the *O*-nucleotidyltransferases (ANT) and *O*-phosphotransferases (APH), that catalyse, respectively, the nucleotidylation (adenylation) and phosphorylation of the hydroxyl groups, and the *N*-acetyltransferases (AAC), that catalyse acetylation of amino groups, so rendering the antibiotic inactive. Hybridisation data have confirmed that some strains of *Acinetobacter* may contain several aminoglycoside resistance genes [3].

Many factors influence the dissemination of antibiotic resistance genes. Although certain species of *Acinetobacter* have been shown to be intrinsically resistant to some aminoglycosides, resistance genes have also been

found on plasmids, transposons and integrons [6–8]. The rapid emergence of resistance to aminoglycosides in clinical isolates of this genus has been attributed to their ability to acquire these resistance determinants. However, studies to date have concentrated on identifying particular enzymes and genetic determinants involved in resistance and, except on a local scale, have not investigated the epidemiological relationships of the resistant strains themselves. The purpose of the present study was to determine whether specific mechanisms of aminoglycoside resistance have developed in particular strains from defined geographical locations (with subsequent clonal spread), or whether common resistance mechanisms have been acquired by genotypically distinct clinical isolates of *Acinetobacter* spp. throughout the world. To address this question, the genotypic and epidemiological relationships of the isolates in this study were correlated with the aminoglycoside-modifying enzymes produced by the isolates and the genetic location of the responsible resistance determinants.

Materials and methods

Bacteria

Twenty-four *Acinetobacter* isolates from 15 different hospitals in 11 countries were studied (Table 1). All had been identified previously to the genomic species level by the tDNA profile method [10] and their genotypic relationships had been investigated by analysis of randomly amplified polymorphic DNA (RAPD) fingerprints obtained with M13 core region and DAF4 primers [9]. The isolates were stored at -70°C in Skim Milk Powder (Oxoid) 10% w/v in sterile distilled water and then subcultured on to Isosensitest Agar (Oxoid), containing appropriate selective aminoglycoside antibiotics, and incubated overnight at 30°C before analysis. The strains used in the plasmid transfer experiments were *Escherichia coli* K12 strain J53.2 (F^{-} , *pro*, *met*, *rif*^r) and a rifampicin-resistant mutant of *A. baumannii* type strain ATCC 19606.

Aminoglycoside resistance profiles (AGRPs)

Resistance profiles of the 24 isolates to 14 different aminoglycoside antibiotics were determined by a modified disk inhibition zone method as described previously [11]. Disks (Sanofi Diagnostics Pasteur) contained the following antibiotics ($\mu\text{g}/\text{disk}$): kanamycin (30), neomycin (30), netilmicin (30), tobramycin (10), amikacin (30), gentamicin (10), apramycin (100), fortimicin (100), 6'-netilmicin (100), 2'-netilmicin (100), isepamicin (30), 5'-episisomicin (10), spectinomycin (25) and streptomycin (30). Plates were incubated for 24 h at 30°C and the diameters of the inhibition zones were measured. Similarity coefficients were then calculated and subjected to cluster analysis by the DENDRON v2.3a program (Solltech Inc., Oakdale, IA, USA).

Table 1. Summary of the genotypic relationships of the *Acinetobacter* isolates included in the study

Isolate	Identification	Source
Group 1*		
CW1	<i>A. baumannii</i>	Nottingham, England
CW14	<i>A. baumannii</i>	Venlo, The Netherlands
CW27	<i>A. baumannii</i>	Cardiff, Wales
CW28	<i>A. baumannii</i>	East Glamorgan, Wales
Group 2		
CW13	<i>A. baumannii</i>	Rotterdam, The Netherlands
CW20	<i>A. baumannii</i>	Paris, France
CW22	<i>A. baumannii</i>	Paris, France
Group 3		
CW32	<i>A. baumannii</i>	Perth, Australia
G1	<i>A. baumannii</i>	Cape Town, South Africa
G5	<i>A. baumannii</i>	Cape Town, South Africa
Group 4		
S4	<i>A. baumannii</i>	Singapore
A1	<i>A. baumannii</i>	Buenos Aires, Argentina
Group 5		
B1	<i>A. baumannii</i>	Berlin, Germany
B5	<i>A. baumannii</i>	Berlin, Germany
Group 6		
D1	<i>A. baumannii</i>	Freiburg, Germany
D2	<i>A. baumannii</i>	Freiburg, Germany
Group 7		
ESP41	<i>A. baumannii</i>	Barcelona, Spain
ESP77	<i>A. baumannii</i>	Barcelona, Spain
Ungrouped		
CW5	<i>A. baumannii</i>	Nottingham, England
CW11	<i>Acinetobacter</i> sp. 3	Bristol, England
CW31	<i>Acinetobacter</i> sp. 3	Perth, Australia
S2	<i>A. baumannii</i>	Singapore
A16	<i>A. baumannii</i>	Buenos Aires, Argentina
I133	<i>A. baumannii</i>	Trieste, Italy

*Isolates were included in the same genotypic group if shown previously [9] to have a similarity coefficient (S_{AB}) value of $\geq 72\%$ following analysis of RAPD fingerprints.

Determination of aminoglycoside acetylating and adenylating activity

The method of Al-Asadi *et al.* [12] was used in conjunction with a cellulose phosphate paper-binding system [13] to determine the presence of aminoglycoside acetylating or adenylating activity in cell extracts. Suspensions of cells grown at 30°C in 50 ml of Nutrient Broth (Oxoid) were harvested by centrifugation at 4000 g for 15 min at 4°C and resuspended in 5 ml of Tris-HCl buffer, pH 7.6. The cells were disrupted in an MSE ultrasonic disintegrator ($6 \times 30\text{-s}$ bursts at an amplitude of $15\text{ }\mu\text{m}$, with 30 s between bursts) on ice. The resulting preparations were tested for their ability to bind radioactivity from $1\text{-}^{14}\text{C}$ -acetyl coenzyme A (Dupont) and $8\text{-}^{14}\text{C}$ -adenosine triphosphate (Amersham International) to gentamicin, tobramycin, amikacin and kanamycin, as described previously [12]. All tests were performed in duplicate on two occasions. Suitable facilities were not available for the determination of phosphorylating activity.

Detection of aminoglycoside resistance genes

Extraction of DNA. For extraction of whole-cell DNA, isolates were grown in nutrient broth overnight at 30°C , the cells were harvested by centrifugation at 8000 g for

10 min and the DNA was extracted with DNAzolTM (GibcoBRL) reagent, used according to the manufacturer's instructions. Portions (5 µl) of the resulting suspensions were applied to Nylon Plus Membranes (Qiagen, Crawley, W. Sussex) and the DNA was immobilised by exposure to UV light (120 mJ/cm² at 254 nm) in a UV Stratalinker (Stratagene) on filter paper saturated with 10 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Intact chromosomal DNA and plasmid DNA were prepared in agarose plugs and separated by pulsed-field gel electrophoresis by the method of Curran *et al.* [14]. Following electrophoresis, discrete bands of chromosomal and plasmid (if present) DNA were transferred to Nylon Plus Membranes and cross-linked as described previously [14].

DNA hybridisation. DNA immobilised on membranes was hybridised with biotin-labelled oligonucleotide probes for seven distinct genes encoding aminoglycoside resistance: *aac(3)-Ia*, *aac(3)-IIa*, *aac(6')-Ia*, *aac(6')-Ib*, *ant(2'')-Ia*, *ant(3'')-Ia* and *ant(3'')-Ib* (Table 2) [15–22]. In addition, a probe for *aph(3')-VIa* was prepared from a 347-bp *EcoRI-HindIII* internal *aph(3')-VIa* fragment that had been cloned into plasmid pUC18 (T. Lambert, unpublished results). A suitable DNA probe was not available to detect the APH(3')-I gene. Following plasmid DNA preparation [23] from a strain of *E. coli* carrying the recombinant plasmid, the probe was isolated by *EcoRI-HindIII* digestion, separated by electrophoresis, extracted with a Qiaex Gel Extraction Kit (Qiagen) and biotin-labelled with a Bio-Nick Labelling System (Gibco BRL). All positive hybridisation results were detected with a BluGene Nonradioactive Nucleic Acid Detection System (Gibco BRL), with pre-hybridisation, hybridisation and detection conditions as recommended by the manufacturer.

Plasmid transfer experiments

Conjugation experiments with the rifampicin-resistant and aminoglycoside-susceptible recipient strains of *E. coli* K12 and the type strain of *A. baumannii* were performed with a filter-mating method [24] and overnight incubation at 30°C. Possible transconjugants were selected on Isosensitest agar containing rifampicin 100 µg/ml and one of amikacin, gentamicin, kanamycin

or tobramycin 10 µg/ml. Transfer frequencies were expressed in terms of the number of transconjugants obtained/recipient cell.

Association of aminoglycoside resistance genes with integrons

The PCR was performed with primer sets comprising the 5'CS primer (Table 2) specific for the 5' conserved sequence of Tn21-type integrons [25] and a primer for each individual aminoglycoside resistance gene (Table 2). Amplification conditions comprised 35 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 55°C and 5 min of extension at 72°C. PCR products were analysed on agarose 1.5% w/v gels and the sizes of any PCR products were determined by comparison with the distances migrated by pGEM DNA Markers (Promega) run on the same gel.

Results

Comparison of aminoglycoside resistance profiles and genotypic relationships

Table 1 shows the sources and known genotypic relationships of the isolates included in the study, based on cluster analysis of RAPD fingerprints obtained with M13 and DAF4 primers, respectively [9]. Although some isolates (those in the same group) have a close genetic relationship, the overall collection of isolates was genotypically heterogeneous. The zones of inhibition of the various aminoglycosides for the clinical isolates and the sensitive type strain of *A. baumannii* are shown in Table 3. All 24 clinical isolates were resistant to four or more aminoglycosides, with some exhibiting resistance to up to 11 of the 14 aminoglycosides tested (Table 3). Most were resistant to gentamicin, tobramycin, kanamycin, spectinomycin and streptomycin; however, all were relatively sensitive to apramycin. Fig. 1 shows the cluster analysis results obtained following analysis of the zones of inhibition. When compared with Table 1, it was apparent that some closely related isolates from the same genotypic group were unrelated in terms of their resistance to aminoglycoside antibiotics, and *vice versa*. Thus, for example, isolates G1 and G5, which are related genotypically, are unrelated according to their AGRPs, but isolates CW22 and CW32, while not related

Table 2. Oligonucleotides for hybridisation and PCR analysis

Primer or probe	Nucleotide sequence (5' to 3')	Position in published sequence [reference]
5'CS	GGCATCCAAGCAGCAAG	1190–1206 [15]
<i>aac(6')-Ia</i>	TAATTGCTGCATTCCGC	797–781 [16]
<i>aac(6')-Ib</i>	TGTGACGGAATCGTTGC	432–416 [17]
<i>aac(3)-Ia</i>	AGCCCGCATGGATTGA	1375–1359 [18]
<i>aac(3)-IIa</i>	CCTCCGTTATTGCCTTC	118–102 [19]
<i>ant(3'')-Ia</i>	TCGATGACGCCAACTAC	464–448 [20]
<i>ant(3'')-Ib</i>	TCAATGACGCTTAGCAC	457–441 [21]
<i>ant(2'')-Ia</i>	CCGCAGCTAGAATTTTG	1341–1325 [22]

Table 3. Aminoglycoside resistance profiles and aminoglycoside acetylating and adenylating activity of the *Acinetobacter* isolates studied

Isolate	Zone sizes (mm) around disks containing (µg)												Adenylating activity					Acetylating activity				
	GEN (30)	TOB (10)	AMK (30)	KAN (30)	APR (100)	NET (30)	6'NET (100)	2'NET (100)	EPI (10)	ISP (30)	FOR (100)	NEO (30)	SPC (25)	STM (25)	GEN	TOB	KAN	AMK	GEN	TOB	KAN	AMK
Sensitive type	17	16	25	17	25	29	20	18	21	23	21	23	35	23	-	-	-	-	-	-	-	-
strain																						
CW1	5	15	17	17	17	15	23	23	15	17	5	15	0	5	-	-	-	-	+	-	-	-
CW5	5	15	19	5	17	13	15	15	15	15	5	11	0	5	-	-	-	-	+	-	-	-
CW11	5	11	11	11	19	5	7	5	11	9	5	17	7	32	+	+	+	-	+	+	+	-
CW13	5	15	11	5	27	26	22	20	22	9	25	11	0	21	+	+	+	-	-	-	-	-
CW14	5	19	22	5	24	22	19	18	19	23	5	9	0	17	-	-	-	-	+	-	-	-
CW20	5	9	19	11	19	15	18	14	15	17	17	17	0	5	+	+	+	-	-	-	-	-
CW22	5	5	19	5	21	21	25	25	21	21	25	17	4	5	+	+	+	-	-	-	-	-
CW27	9	9	13	7	21	5	20	5	11	9	5	13	0	5	-	-	-	-	+	+	+	-
CW28	9	9	11	7	21	5	20	5	9	7	5	13	0	5	-	-	-	-	+	+	+	-
CW31	7	7	11	9	21	9	20	5	9	9	5	15	5	34	-	-	-	-	+	+	+	-
CW32	11	11	19	13	21	21	23	24	21	25	23	17	7	22	+	+	+	-	-	-	-	-
G1	7	17	19	25	17	27	28	27	21	23	7	15	0	5	-	-	-	-	+	-	-	-
G5	19	21	21	25	15	16	5	5	13	15	13	13	0	18	-	-	-	-	-	-	-	-
S2	17	17	23	5	21	17	18	15	17	17	13	13	0	5	-	-	-	-	-	-	-	-
S4	9	9	23	5	22	17	12	17	13	13	15	17	0	5	+	+	+	-	-	-	-	-
A1	5	5	11	5	15	13	15	13	13	7	15	9	0	5	-	-	-	-	-	-	-	-
A16	5	7	13	5	21	5	5	5	5	17	19	9	0	21	+	+	+	-	+	+	-	-
B1	5	9	15	5	15	5	5	5	9	9	5	5	0	0	-	-	-	-	+	+	-	-
B5	5	9	15	5	11	5	5	5	9	9	5	9	0	0	-	-	-	-	-	-	-	-
D1	13	9	11	5	19	5	21	5	7	19	19	5	0	0	-	-	-	-	+	+	-	-
D2	15	9	17	5	19	5	21	5	7	15	23	5	0	0	-	-	-	-	-	-	-	-
ESP41	13	17	9	5	33	15	30	30	25	9	33	11	0	24	-	-	-	-	-	-	-	-
ESP77	13	5	5	5	19	20	18	18	17	17	5	9	0	17	-	-	-	-	+	-	-	-
I133	15	11	5	5	19	11	9	9	15	5	5	7	0	5	-	-	-	-	+	-	-	-

AMK, amikacin; APR, apramycin; ESP, episomicin; FOR, fortimicin; GEN, gentamicin; ISP, isepamicin; KAN, kanamycin; NEO, neomycin; NET, netilmicin; 2'NET, 2'-netilmicin; 6'NET, 6'-netilmicin; SPC, spectinomycin; STM, streptomycin; TOB, tobramycin.

Fig. 1. Dendrogram showing the results of cluster analysis of the clinical isolates based on zones of inhibition obtained with 14 different aminoglycoside antibiotics.

genotypically, are closely related according to their AGRPs.

Characterisation of aminoglycoside-modifying activity

Also listed in Table 3 are the aminoglycoside acetylating and adenylating activities found in each individual isolate included in the study. By examining and comparing the AGRPs and the acetylating or adenylating activities of the isolates with those expected of known aminoglycoside-modifying enzymes, it was possible to deduce the probable enzyme content of the isolates [26]. On this basis, seven different enzymes were produced by the collection of isolates (Table 4). Most resistance could be attributed to the production of one or more enzymes, although one isolate had an unusual resistance pattern that was attributed, in part at least, to a change in permeability. The most common enzyme appeared to be ANT(3'')-I (spectinomycin and streptomycin resistance), produced by 20 of the 24 isolates. AAC(3)-I (gentamicin and

fortimicin resistance) and ANT(2'') (gentamicin, tobramycin and kanamycin resistance) also appeared to be common, being produced by 12 and eight isolates, respectively. These two enzymes were considered to be responsible for most of the gentamicin resistance within the collection of isolates. Other common enzymes were APH(3')-I and APH(3')-VI, produced by nine isolates each. The enzymes occurred in various combinations of between one and four enzymes in each isolate, with the combinations of ANT(3'')-I with AAC(3)-I, and ANT(3'')-I with APH(3')-VI occurring the most frequently (in 10 and nine isolates each, respectively).

Correlation of inferred aminoglycoside-modifying enzymes with hybridisation data

Table 4 also shows the results obtained following hybridisation with the specific gene probes that were available. All the isolates failed to hybridise with the *aac(6')-Ia* and *ant(3'')-Ib* probes. Those isolates that exhibited a putative ANT(3'')-I profile all hybridised

Table 4. Aminoglycoside-modifying enzymes produced by the *Acinetobacter* isolates included in the study

Isolate	Enzyme content deduced from AGRPs	Genes detected by DNA–DNA hybridisation
CW1	AAC(3)-I + ANT(3'')-I	<i>aac(3)-Ia</i> * + <i>ant(3'')-Ia</i> *
CW5	AAC(3)-I + APH(3')-I† + ANT(3'')-I	<i>aac(3)-Ia</i> * + <i>ant(3'')-Ia</i> *
CW11	AAC(3)-I + AAC(6')-I + ANT(2'')	<i>aac(3)-Ia</i> + <i>aac(6')-Ib</i> + <i>ant(2'')-Ia</i>
CW13	ANT(2'') + ANT(3'')-I + APH(3')-VI	<i>ant(2'')-Ia</i> + <i>ant(3'')-Ia</i> + <i>aph(3')-VIa</i>
CW14	AAC(3)-I + ANT(3'')-I + APH(3')-I†	<i>aac(3)-Ia</i> * + <i>ant(3'')-Ia</i> *
CW20	ANT(2'') + ANT(3'')-I	<i>ant(2'')-Ia</i> + <i>ant(3'')-Ia</i>
CW22	ANT(2'') + APH(3')-I†	<i>ant(2'')-Ia</i>
CW27	AAC(3)-I + AAC(6')-I + ANT(3'')-I + APH(3')-VI	<i>aac(3)-Ia</i> * + <i>aac(6')-Ib</i> + <i>ant(3'')-Ia</i> + <i>aph(3')-VIa</i>
CW28	AAC(3)-I + AAC(6')-I + ANT(3'')-I + APH(3')-VI	<i>aac(3)-Ia</i> * + <i>aac(6')-Ib</i> + <i>ant(3'')-Ia</i> + <i>aph(3')-VIa</i>
CW31	AAC(3)-I + AAC(6')-I + APH(3')-I†	<i>aac(3)-Ia</i> * + <i>aac(6')-Ib</i>
CW32	ANT(2'') + APH(3')-I†	<i>ant(2'')-Ia</i>
G1	AAC(3)-I + ANT(3'')-I	<i>aac(3)-Ia</i> + <i>ant(3'')-Ia</i>
G5	ANT(3'')-I + Impermeability	<i>ant(3'')-Ia</i>
S2	ANT(3'')-I + APH(3')-I†	<i>ant(3'')-Ia</i>
S4	ANT(2'') + ANT(3'')-I	<i>ant(2'')-Ia</i> + <i>ant(3'')-Ia</i>
A1	ANT(2'') + ANT(3'')-I + APH(3')-VI	<i>ant(2'')-Ia</i> + <i>ant(3'')-Ia</i> + <i>aph(3')-VIa</i>
A16	ANT(2'') + ANT(3'')-I + AAC(3)-II + APH(3')-VI	<i>aac(3)-IIa</i> + <i>ant(2'')-Ia</i> + <i>ant(3'')-Ia</i> + <i>aph(3')-VIa</i>
B1	AAC(3)-I + ANT(3'')-I + APH(3')-VI	<i>aac(3)-Ia</i> + <i>ant(3'')-Ia</i> + <i>aph(3')-VIa</i>
B5	AAC(3)-I + AAC(6')-I + ANT(3'')-I + APH(3')-VI	<i>aac(3)-Ia</i> + <i>aac(6')-Ib</i> + <i>ant(3'')-Ia</i> + <i>aph(3')-VIa</i>
D1	ANT(3'')-I + APH(3')-I†	<i>ant(3'')-Ia</i>
D2	ANT(3'')-I + APH(3')-I†	<i>ant(3'')-Ia</i>
ESP41	ANT(3'') + APH(3')-VI	<i>ant(3'')-Ia</i> + <i>aph(3')-VIa</i>
ESP77	AAC(3)-I + ANT(3'')-I + APH(3')-I†	<i>aac(3)-Ia</i> + <i>ant(3'')-Ia</i>
I133	AAC(3)-I + ANT(3'')-I + APH(3')-VI	<i>aac(3)-Ia</i> + <i>ant(3'')-Ia</i> + <i>aph(3')-VIa</i>

*Integron-associated genes.
†No DNA probe was available to detect the gene encoding APH(3')-I.

with the *ant(3'')-Ia* gene probe. This was also found to be the case for AAC(3)-I and *aac(3)-Ia*, AAC(6')-I and *aac(6')-Ib*, ANT(2'')-I and *ant(2'')-Ia*, and APH(3')-VI and *aph(3')-VIa*. Thus the hybridisation results were in complete agreement with the enzyme content inferred from the AGRPs. A DNA probe was not available to detect the APH(3')-I gene, but there were no positive hybridisation results that could not be accounted for by reference to the enzyme profiles.

Location of aminoglycoside resistance genes

Transfer of aminoglycoside resistance from the clinical isolates to *E. coli* K12 was not detected (transfer frequency of $< 2 \times 10^{-10}$). However, of the 24 strains, nine transferred one or more resistances to a sensitive strain of *A. baumannii* at frequencies of between 5×10^{-6} and 1.6×10^{-4} (Table 5). Hybridisation of DNA separated on pulsed-field gels with the available probes revealed that two *ant(2'')-Ia* genes were present on plasmids detected in two of the 24 isolates (isolates CW32 and A16), but DNA probes for 41 of the total of 55 genes detected were found to hybridise to the intact chromosome. The remaining 12 genes may have been located on plasmids that were not separated by pulsed-field gel electrophoresis; indeed, nine were shown to be transferable in conjugation experiments (Table 5). In addition, the location of the genes encoding resistance mediated by the APH(3')-I enzyme could not be deduced because of the lack of an available probe. PCR analysis with the 5'CS primer and the primer for the *aac(3)-Ia* gene yielded a product of 200 bp from six isolates (Table 4). Three of these isolates also yielded a product of 2.7 kb with the 5'CS primer and that specific for the *ant(3'')-Ia* gene (Table 4). However,

Table 5. Aminoglycoside resistance genes transferred from clinical isolates to the sensitive type strain of *A. baumannii*

Isolate	Gene or enzyme activity transferred	Transfer frequency*
CW1	<i>aac(3)-Ia</i>	2×10^{-5}
CW5	<i>aac(3)-Ia</i>	5×10^{-6}
CW13	<i>ant(2'')-Ia</i> + <i>aph(3')-VIa</i>	$6.4 \times 10^{-5} + 9.6 \times 10^{-5}$
S2	APH(3')-I	5.4×10^{-5}
A1	<i>ant(2'')-Ia</i> + <i>aph(3')-VIa</i>	$5.6 \times 10^{-5} + 1.6 \times 10^{-4}$
A16	<i>ant(2'')-Ia</i>	1.3×10^{-4}
ESP41	<i>aph(3')-VIa</i>	3.9×10^{-5}
ESP77	APH(3')-I + <i>aac(3)-Ia</i>	1.6×10^{-5}
I133	<i>aac(3)-Ia</i> + <i>aph(3')-VIa</i>	$4.7 \times 10^{-5} + 4.9 \times 10^{-5}$

*Number of transconjugants/recipient cell.

the other isolates carrying the *aac(3)-Ia* or *ant(3'')-Ia* genes (six and 14 isolates respectively), did not yield integron-associated products. No other integron-associated products were detected with any of the other primer combinations investigated.

Discussion

The primary aim of this study was to investigate whether particular aminoglycoside-modifying enzymes produced by clinical isolates of *Acinetobacter* spp. had evolved in and were confined to specific genotypes of this genus (with subsequent clonal spread), or whether the same resistance mechanisms were being acquired independently by unrelated genotypes, and if so, the nature of the genetic processes involved. The isolates included in the study were obtained from worldwide locations and were known from a previous study [9] to be genotypically heterogeneous. However, based on the

current consensus similarity value of 72% used to group genotypically related isolates of *Acinetobacter* [11, 27], seven related groups of isolates could be distinguished (Table 1). When the cluster analysis results based on aminoglycoside inhibition zones (Fig. 1) were compared with the genotypic groupings, it was apparent that isolates sharing the same AGRPs were found in different genotypic groups, indicating that the same resistance mechanisms may be shared by diverse groups of isolates.

To examine this point further, the aminoglycoside-modifying enzyme content of the various isolates was first deduced by analysis of the AGRPs and aminoglycoside-modifying activity found in cell extracts, and was then confirmed by the use of specific aminoglycoside gene probes. The hybridisation results were in complete agreement with the enzyme content inferred for each isolate from its AGRP. Seven different enzymes were produced by the collection of isolates (Table 4), often in combination. AAC(3)-I and ANT(2''), found in several genotypic groups, were responsible for most of the gentamicin resistance observed. Similarly, the other five enzymes were also found in genotypically unrelated isolates. Thus, based on the limited number of isolates examined, it appeared from these results that the same aminoglycoside resistance genes were distributed amongst several genotypically distinct groups of isolates. Attempts to transfer resistance from the clinical isolates to *E. coli* were unsuccessful, but transfer of certain resistance genes was detected from nine isolates to a sensitive type strain of *A. baumannii* (Table 5). Potential mobilisation of transposons from the chromosome of the resistant clinical isolates to other bacteria remains to be investigated, but the *aac(3)-Ia* and *ant(3'')-Ia* genes were shown by PCR analysis to be associated with Tn21-type integron structures in six isolates. Integrons are novel genetic elements that include the determinants of a site-specific recombination system capable of capturing and mobilising genes that are contained in discrete mobile gene cassettes [28]. They are thought to be involved in the spread and formation of new combinations of antibiotic resistance determinants in a wide range of gram-negative bacteria [29] and can be located either on plasmids or can become stably inserted into the chromosome as part of transposition-type processes [30, 31].

In conclusion, this study demonstrated that similar aminoglycoside-modifying enzymes are found in unrelated isolates of *Acinetobacter* spp. and that particular genes are not restricted to specific areas of the world. It seems that certain aminoglycoside resistance genes have become disseminated in *Acinetobacter* spp. by a range of genetic mechanisms, including transfer on plasmids and integron-type structures. Such genetic mechanisms have already been shown to be involved in the global dissemination of mercury-resistance amongst environmental isolates of *Acinetobacter* spp. [32]. The

association of these various genetic structures with resistance to antibiotics other than aminoglycosides, and their importance in the dissemination of such resistance, remains to be determined.

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