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Epidemiology of Rifampin ADP-Ribosyltransferase (*arr-2*) and Metallo-β-Lactamase (bla_{IMP-4}) Gene Cassettes in Class 1 Integrons in *Acinetobacter* Strains Isolated from Blood Cultures in 1997 to 2000

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We characterized two new gene cassettes in an *Acinetobacter* isolate: one harbored the metallo-β-lactamase (IMP-4) gene bla_{IMP-4}, the other harbored the rifampin ADP-ribosyltransferase (ARR-2) gene *arr-2*, and both arrayed with the aminoglycoside acetyltransferase [AAC(6′)-Ib_7] gene cassette *aacA4* in two separate class 1 integrons. The epidemiology of these gene cassettes in isolates from blood cultures obtained from 1997 to 2000 was studied. Isolates bearing either the bla_{IMP-4} or the *arr-2* gene cassette or both represented 17.5% (10 of 57) of isolates in 1997, 16.1% (10 of 62) in 1998, 2.5% (1 of 40) in 1999, and 0% (0 of 58) in 2000. These two gene cassettes, probably borne on two separate integrons, were found in at least three genomic DNA groups, with evidence of clonal dissemination in the intensive care unit during 1997 to 1998. Seventeen of the 52 *Acinetobacter baumannii* (genomic DNA group 2) isolates from 1997 to 2000 harbored *intI1*, but only one was positive for these gene cassettes, whereas 20 of the 21 *intI1*-positive isolates of all other genomic DNA groups were positive for either or both of them. Reduced susceptibility to imipenem and rifampin was seen only in isolates harboring the bla_{IMP-4} and *arr-2* cassettes, respectively. The aminoglycoside phosphotransferase [APH(3′)-VIa] gene *aph(3′)-VIa* was detected in all 21 isolates for which the MIC of amikacin was ≥8 μg/ml, with or without *aacA4*, whereas *aacA4* alone was found in isolates for which the MIC of amikacin was 0.5 to 2 μg/ml. Significant differences between the 17 *intI1*-positive and 47 *intI1*-negative isolates belonging to genomic DNA group 3 from 1997 to 1998 in the MICs of amikacin, gentamicin, imipenem, sulfamethoxazole, and ceftazidime were observed (Mann-Whitney test, $P < 0.001$ to 0.01).

In the past decade, there has been an explosion of interest in the integron, a genetic element which facilitates gene dissemination among bacteria of different species and perhaps genera, thus establishing the possibility of a wide dispersal of antimicrobial resistance. These genetic elements are marked by the presence of two conserved regions between which gene cassettes are integrated at a recombination site known as *attI*. The 5′-conserved region of an integron contains an integrase gene, *intI*, and a promoter (P_c) which drives the expression of any integrated gene cassettes. Individual gene cassettes, existing in a free circularized form when not integrated, are comprised of an open reading frame (ORF) and a 59-base element (59-be) at the 3′ end. The 59-be is a family of recombination sites that act as substrates to integrase-mediated recombination (43). To date, over 70 resistance cassettes have been described (32, 45). According to the 3′-conserved sequence (3′-CS), integrons are divided into three classes. The majority of the resistance cassette-bearing integrons belong to class 1, and these carry, at the 3′-CS, the truncated quaternary ammonium compound resistance gene *qacEΔ1*, the *sul1* sulfonamide resistance gene, and *orf5*, of unknown function (20).

Acinetobacter spp. are increasingly important nosocomial pathogens worldwide and particularly in the Hong Kong region (5, 36, 61). The therapy of *Acinetobacter* infections is complicated by multidrug resistance against aminoglycosides, extended-spectrum cephalosporins, and fluoroquinolones (1, 5, 9, 14, 50). The integron was found to be highly prevalent (50 to 80%) among nosocomial strains of *Acinetobacter baumannii* (genomic DNA group 2) (18, 49), and its association with resistance dissemination has attracted much attention. An Italian study of 36 epidemiologically unrelated *A. baumannii* strains isolated in six hospitals over 11 years showed that 13 of the 16 class 1 integron-positive strains carried the same array of cassettes, prompting the hypothesis of horizontal transfer of the entire integron or ancient acquisition (17).

One of the major cassette-borne resistance genes reported in recent years is the bla_{IMP} gene encoding the metallo-β-lactamase IMP belonging to molecular class B (7). The clinical importance of IMP is highlighted by its ability to hydrolyze all penems, cephems, and carbapenems. To date, eleven bla_{IMP} alleles have been described, and most appeared to be linked to integrons. Six of the alleles have been found in acinetobacters from various localities worldwide (9, 10, 15, 22, 23, 27, 37, 44, 58, 63). The bla_{IMP-4} gene in acinetobacters isolated in our hospital and encoding an IMP-4 metallo-β-lactamase that has 95.6 and 89.3% amino acid sequence homology to IMP-1 and IMP-2, respectively (9), was previously described. Further characterization here showed that bla_{IMP-4} was borne on a cassette arraying with three other resistance gene cassettes, namely *qacG2*, *aacA4*, and *catB3*, in a class 1 integron. In

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TABLE 1. PCR primers used in this work

Forward and reverse primers	Sequence 5′–3′	Amplicon (size in bp)	Position	Remark	Reference
int1f imp12	CAG AAG ACG GCT GCA CTG AA AGT GTG TCC TGG GCC TGG	bla_{IMP-4} upstream (1,833)	−1359 to +474 (bla_{IMP-4} ATG)	Characterization of the upstream region of bla_{IMP-4} in strain 74510	This work
imp11 int3c	ATG AGC AAA GTT ATC TGT ATT CT AAA GCA GTC TTG ACC TGA	bla_{IMP-4} downstream (2,812)	+741 (bla_{IMP-4} ATG) to 2071 downstream of bla_{IMP-4}	Characterization of the downstream region of bla_{IMP-4} in strain 74510	This work
int2a int6b	AAC CGA GGA TGC GAA CCA CT CCG AGC CGC TCG TAT AG	*intI1-arr-2-aacA4*-3′CS (3,602)	+382 (*intI1* ATG) to +482 (*orf5* ATG)	Characterization of the regions flanking *arr-2* in strain 74510	This work
int2a imp12	AAC CGA GGA TGC GAA CCA CT AGT GTG TCC TGG GCC TGG	*intI1*-bla_{IMP-4} (1,008)	+382 (*intI1* ATG) to + 474 (bla_{IMP-4} ATG)	Combining with RFLP for detection of the *intI1*-bla_{IMP-4} sequence[a]	This work
int1b aac61bB	CAT CCA AGC AGC AAG CGC GTT A ACC CCG GIT TCT CGT AGC AT	5′ CS-*arr-2-aacA4* (1,182)	−131 (*arr-2* ATG) to +460 (*aacA4* ATG)	Combining with RFLP for the detection of the 5′ CS-*arr-2-aacA4* sequence[a]	This work
int1e int1c	TCG TAG AGA CGT CGG AAT GG CCG AGG CAT AGA CTG TAC AA	Class 1 integrase gene *intI1* (965)	−40 to +925 (*intI1* ATG)	For detection of the *intI1* gene	27
5cs 3cs	GGC ATC CAA GCA GCA AG AAA GCA GAC TTG ACC TGA	Entire integron (variable)	Variable	For confirmation of the presence of class 1 integron	27
ardra1 ardra2	TGG CTC AGA TTG AAC GCT TAC CTG TTA CGA CTT CA	16S rRNA gene (1,500)	Variable	ARDRA of acinetobacter for genomic DNA group determination	11
BL BR	TAT GAG TGG CTA AAT CGA T CCC GCT TTC TCG TAG CA	*aacA4* (395)	+115 to +509 (*aacA4* ATG)	For detection of the *aacA4* gene	39
aph1 aph2	ATA CAG AGA CCA CCA TAC AGT GGA CAA TCA ATA ATA GCA AT	*aph(3′)-VIa* (234)	+140 to +374 [*aph(3′)-VIa* ATG]	For detection of the *aph(3′)-VIa* gene	60

[a] See Table 2 for the expected sizes of the restriction fragments.

addition, we describe a second class 1 integron bearing the cassette array *arr-2–aacA4*. We examined the epidemiology of the bla_{IMP-4} and *arr-2* cassettes in acinetobacters from blood cultures isolated during 1997 to 2000. The fact that these cassettes were apparently capable of conferring increased resistance to imipenem and rifampin (important antibacterial and antimycobacterial agents) should cause concern. However, the integrons bearing bla_{IMP-4} and *arr-2* were readily recovered from blood culture isolates from 1997 to 1998 but apparently disappeared after 1999. This raises the question of the ability of these resistance genetic elements to persist, highlighting the need for further epidemiological studies to address such issues.

MATERIALS AND METHODS

PCR, RFLP, and DNA sequencing. All the oligonucleotide primers used in this work are summarized in Table 1. All the PCR mixtures (50 μl) contained 25 pmol of each primer, 2 μl of genomic DNA prepared by boiling a colony in 200 μl of sterile distilled water, 0.5 U of *Taq* (AP Biotech, Uppsala, Sweden), and the supplied buffer. Unless otherwise stated in the reference quoted, the thermocycle employed for the PCRs was 94°C for 2 min and then 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, followed by a final 3 min at 72°C. Amplicons were digested by the appropriate restriction endonucleases (Table 2) according to the supplier's instructions when restriction fragment length polymorphism (RFLP) analysis was performed. Restriction patterns were obtained by agarose (2%) gel electrophoresis and compared after ethidium bromide staining. When sequencing of PCR amplicons was required, amplicons were first cloned by using a TA cloning kit (Invitrogen Life Technologies, The Netherlands) and then sequenced by using a PCR sequencing kit with the ALFexpress DNA sequencer (AP Biotech) according to the manufacturers' instructions. All the DNA sequences determined were done at least twice from two PCR amplifications. Comparisons of DNA sequences and calculations of similarity were performed by the software ClustalW (56).

Isolates from blood cultures. Table 3 shows the number of acinetobacters obtained during the 4-year period 1997 to 2000 from blood cultures. Isolates from the same patient were included if the positive blood cultures were taken at least 7 days apart or yielded acinetobacters either of different genomic DNA groups or with different novel patterns produced by amplified ribosomal DNA

TABLE 2. Expected fragments in the PCR-RFLP screening for the *intI1-bla*$_{IMP-4}$ and 5′ CS-*arr-2-aacA4* sequences

Restriction enzyme	Expected fragments (bp)
For detection of the *intI1-bla*$_{IMP-4}$ (1,008-bp) sequence	
*Apa*LI	96, 912
*Bsm*AI	157, 851
*Hin*CII	57, 268, 288, 395
*Hind*III	292, 716
For detection of the 5′CS-*arr-2-aacA4* (1,182-bp) sequence	
*Bso*BI	73, 350, 759
*Bsp*HI	160, 599, 423

restriction analysis (ARDRA [11]) (Table 1). They were stored on nutrient agar slopes at room temperature until further examination. Isolates obtained in 1997 and the first 8 months of 1998 had been used in another study (21). No outbreak of *Acinetobacter* infection was noted during the study years. Methods of genus confirmation by the transformation assay of Juni and genomic DNA group determination by ARDRA have been described elsewhere (11, 25).

PFGE. Pulsed-field gel electrophoresis (PFGE) fingerprints were generated by using a contour-clamped homogeneous electric field electrophoresis apparatus (CHEF-MAPPER; Bio-Rad, Richmond, Calif.) as described in a previous study (21). The restriction endonuclease *Apa*I was used for the in situ digestion of intact *Acinetobacter* genomic DNA embedded in 1% agarose gel blocks prepared according to previously described methods (21). Samples were loaded into 1% certified PFGE grade (Bio-Rad) agarose gels and electrophoresed with 0.5× Tris-borate-EDTA buffer with an electric field of 6 V/cm, an included angle of 120°, and a pulse time of 5 to 35 s over 32 h at 14°C. Images of ethidium bromide-stained gels were digitized by use of a gel documentation system (Imagemaster; AP Biotech). Clusters of possibly related isolates were identified and interpreted by using the Dice coefficient of similarity and the unweighted pair group method (21, 55).

MICs of antimicrobials. The agar dilution method was used to determine the MICs of the following major classes of agents: amikacin, gentamicin, ceftazidime, nalidixic acid, tetracycline, sulfamethoxazole, and rifampin (all from Sigma, St. Louis, Mo.), ciprofloxacin (Bayer, Elberfeld, Germany), imipenem (Merck Sharp and Dohme, Hoddesdon, United Kingdom), netilmicin (Schering-Plough Corporation, Kenilworth, N.J.), and sulbactam (Pfizer, Sandwich, United Kingdom). Inocula of 10^4 CFU/spot were inoculated onto Mueller-Hinton agar plates with a multipoint inoculator (Dynatech Laboratories, Alexandria, Va.), and the plates were incubated at 35°C for 18 h. The MIC was defined as the lowest concentration which inhibited visible growth. Control strains *Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853 were included (35). Nonparametric tests (SPSS version 10) were used for statistical analysis.

Southern hybridization. In order to identify whether the integrons were plasmid borne, the extraction method described by Kado and Liu (26) was employed to obtain plasmids of over 50 Kb from acinetobacters. The plasmid extractions were then electrophoresed by using an agarose gel (0.7%), blotted onto nylon membranes following standard protocols (46), and hybridized with two probes. One probe was a *bla*$_{IMP-4}$ fragment (1 to 474 bp), and the other was an *arr-2* fragment (36 to 417 bp). A DIG system was used for signal detection, and the manufacturer's instructions were followed (Boehringer Mannheim, Mannheim, Germany).

Nucleotide sequence accession numbers. The sequences described in this article are available from the EMBL and GenBank databases under the accession numbers AF445082 and AY038837.

RESULTS

Class 1 integron bearing the *bla*$_{IMP-4}$*-qacG2-aacA4-catB3* cassette array. By use of a PCR method based on the published metallo-β-lactamase IMP-1 gene sequence (27), a variant of the gene (738 bp) from an acinetobacter (strain 74510) in a culture collection had previously been identified (9). Strain 74510 belonging to genomic DNA group 13TU was isolated from a wound in 1995. The metallo-β-lactamase was subsequently designated IMP-4, and the gene was designated *bla*$_{IMP-4}$ (9). From the presence of adjacent GTTRRRY integrase-dependent recombination motifs, *bla*$_{IMP-4}$ was speculated to be gene cassette borne (9).

In this work, a series of PCR primers were designed to amplify the flanking regions of *bla*$_{IMP-4}$, based also on the class 1 integron bearing the IMP-1 sequence (27). Two sets of primers produced positive results: (i) int1f and imp12 produced an amplicon of 1,833 bp, including 1,359 bp upstream of the IMP-4 gene; (ii) imp11 and int3c produced an amplicon of

TABLE 3. Acinetobacters in blood cultures from 1997 to 2000[a]

Genomic DNA group	No. of acinetobacters in blood cultures														
	1997					1998					1999			2000	
	Total	PCR positive for:				Total	PCR positive for:				Total	PCR positive for:		Total	PCR positive for:
		intI1	A	B	C		*intI1*	A	B	C		*intI1*	C		*intI1*
2	8	3				16	8	1			12	1		16	5
3	22	8	3	5		23	9	2	6	1	8			12	1
5											2			4	
8/9						1					2				
10	1					1									
12											2				
13TU	10					12					5			15	
15											1			3	
16											1				
17	4	1			1	1								1	
U	11	1			1	9					7	1	1	6	
Total[b]	56 (48)	13 (12)	3 (3)	5 (5)	2 (2)	63 (53)	17 (16)	3 (3)	6 (6)	1	40 (30)	2 (2)	1	58 (41)[c]	6 (5)

[a] Abbreviations: A, both the *intI1* -*bla*$_{IMP-4}$ and 5′ CS-*arr-2-aacA4* sequences; B, the *intI1-bla*$_{IMP-4}$ sequence; C, the 5′ CS-*arr-2-aacA4* sequence; U, unclassifiable by ARDRA.
[b] Numbers in parentheses indicate the numbers of patients.
[c] One isolate from 2000 was not done.

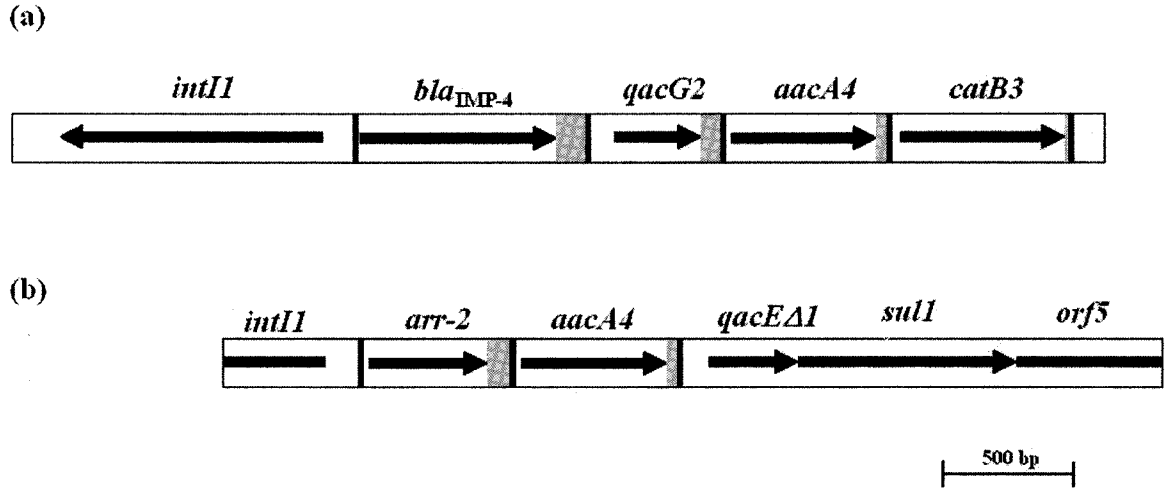

FIG. 1. Schematic representation of the sequences of the integrons bearing the bla_{IMP-4} cassette (a) and the *arr-2* cassette (b) determined in this work. The thick vertical lines show the borders of the gene cassettes. The arrows represent the ORFs, and the grey areas show the 59-be of individual cassettes.

2,812 bp, including 2,071 bp downstream of the IMP-4 gene (Table 1). DNA sequencing of the amplicons confirmed that bla_{IMP-4} was borne on a gene cassette in a class 1 integron. The 59-be of the bla_{IMP-4} cassette has the typical inverted repeat sequence related to a 59-bp consensus sequence (54).

Only one plasmid (estimated size, 275 Kb) was extracted from the isolate by using a method specialized for large plasmids (26). The presence of the IMP-4 sequence on this large plasmid was demonstrated by Southern hybridization of the extracted plasmid, with a bla_{IMP-4} fragment used as the probe (result not shown).

The sequence of the integron bearing the bla_{IMP-4} gene cassette identified in this work is schematically represented in Fig. 1. A typical class 1 integrase gene, *intI1*, was found upstream of the bla_{IMP-4} gene cassette, and downstream were three more gene cassettes: (i) a variant of *qacG* encoding a small protein that confers resistance to quaternary ammonium compounds (27), (ii) *aacA4* encoding an aminoglycoside acetyltransferase [aac(6′)-Ib_7] (28), and (iii) *catB3* encoding a chloramphenicol acetyltransferase (6). The *intI1* gene was identical to that of In1 except for the two strength-related hexameric motifs $TTGACA^{(-35)}$ and $TAAGCT^{(-10)}$ in the promoter P_c. While $TTGACA^{(-35)}$ was found to constitute a strong promoter, $TAAGCT^{(-10)}$ had previously been seen in a weak promoter (29). This hexamer combination has also been reported to exist in an integron in *Enterobacter aerogenes* before, but its exact strength is yet to be determined (38).

The bla_{IMP-4} gene had 95.6% nucleotide sequence identity to the bla_{IMP-1} gene initially found in Japan (37). Examination of all the 59-be linked to the published IMP alleles (bla_{IMP-1} in a class 1 integron [GenBank accession no. AJ223604] [27], bla_{IMP-1} in a class 3 integron [accession no. AF419627] [3], bla_{IMP-2} in a class 1 integron [accession no. AJ243491] [44], bla_{IMP-7} in a class 1 integron [accession no. AF318077] [15], bla_{IMP-8} in a class 1 integron [accession no. AF322577] [63]) revealed that the bla_{IMP-4} cassette 59-be is more closely related to that of bla_{IMP-7} (sharing 93% nucleotide sequence identity) than to that of bla_{IMP} (86% sequence identity), bla_{IMP-2} (44% sequence identity), and bla_{IMP-8} (27% sequence identity). However, the ORFs of bla_{IMP-4} and bla_{IMP-7} share a lower identity (89%) than their 59-be comparison.

The *qacG2* cassette downstream of bla_{IMP-4} including a long untranslated leading sequence is identical to the *qacG* cassette described for In31 (27) except for the 59-be (88% nucleotide sequence identity) (Fig. 2). Interestingly, the 59-be of *qacG2* is almost identical to the *qacE2* cassette found in a class 1 integron in *Aeromonas salmonicida* subsp. *Salmonicida* except for a 2-bp (CG) insertion between the 2L and 2R core sites (Fig. 2). The *qacE2* cassette has also a long untranslated leading sequence identical to *qacG2* in length and >99% identical in sequence, but the ORF of *qacE2* has two nucleotide differences translating into two amino acid changes (Phe13→Ser and Gly57→Ala). The long untranslated leading sequence is believed to be a secondary promoter containing two putative strength-related hexameric motifs reported also as existing in *qacG* in In31 (27).

The *aacA4* gene cassette is identical to the *aacA4* cassette previously found in a class 1 integron in *Campylobacter jejuni* (28). It contains the aminoglycoside acetyltransferase AAC(6′)-Ib_7 gene. The substrate-determining residue 102 is occupied by a serine; hence, it should confer resistance to tobramycin and slightly increased resistance to gentamicin but no resistance to amikacin (42). Compared to the first correctly described *aacA4*, the cassette has 2 extra C bases in a 2C-rich region in the 59-be (43). Also, the ORFs differ by one T→C accounting for the substrate-decisive Leu102→Ser and by one T→A changing the Val183 to Asp (43).

The ORF of the *catB3* cassette in this work differs from the *catB3* previously described for *Enterobacter* by one amino acid residue (Tyr81→Cys), and the two cassettes share 99% nucleotide sequence identity (6). Major differences were in the 59-be. The *catB3* in this work has a four-base deletion between the 2L and 2R core sites and a T→C base change between the 2R and 1R core sites.

The presence of 3′-CS typical of *sul1*-associated integrons

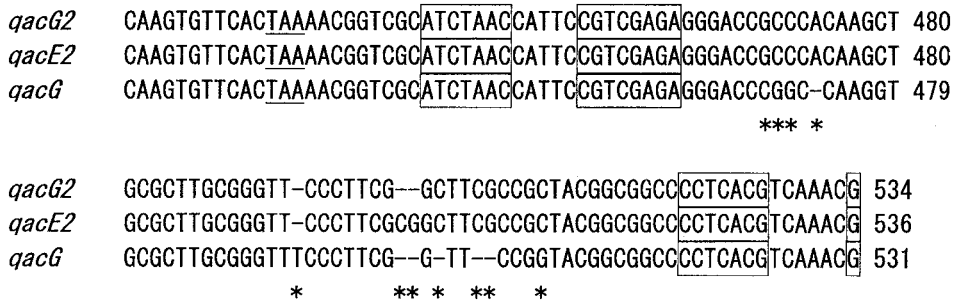

FIG. 2. Comparison of the 59-be of the gene cassettes *qacG2* (accession no. AF445082), *qacG* (AJ223604), and *qacE2* (AF327731). Underlining indicates stop codons of the ORFs, boxes show imperfect inverted repeat core sites, and asterisks indicate base differences. The base numbers shown correspond to the individual gene cassettes.

following the *catB3* gene cassette suggested that this integron contained only the four described cassettes (19).

Class 1 integron bearing the *arr-2–aacA4* cassette array. During the process of identifying sequences flanking the IMP-4 gene by using PCR mapping, a set of primers, int2a and int6b, resulted in an amplicon of 3,602 bp that did not contain bla_{IMP-4} (Table 1). Upon sequencing, the amplicon was found to be a fragment of a class 1 integron that contained two gene cassettes: (i) rifampin ADP-ribosyltransferase (*arr-2*) and (ii) the aminoglycoside acetyltransferase AAC(6′)-Ib_7 (*aacA4*). The sequence is schematically represented in Fig. 1. Three hundred eighty-two base pairs of a class 1 integrase gene *intI1* was found at the 5′ end of the PCR amplicon originally targeting the bla_{IMP-4}-*aacA4* integron. The P_c promoter has the hexameric motifs $TCGACA^{(-35)}$ and $TAAACT^{(-10)}$. $TAAACT^{(-10)}$ has previously been found to constitute a strong P_c promoter (29), but $TCGACA^{(-35)}$ has not been described before. However, this combination was previously reported as occurring in a sequence upstream to an aminoglycoside 3′-*N*-acetyltransferase gene, *aacC1*, described in *Serratia marcescens*; but the strength of it as a promoter is not known (GenBank accession no. S68049 [24]).

The *arr-2* cassette was found also in a class 1 integron in *Klebsiella pneumoniae* previously (GenBank accession no. AJ277027 [4]). It differs from another *arr-2* cassette reported to occur in *P. aeruginosa* and *E. coli* by one base (A→G), translating to one amino acid change (Lys98→Arg) (34, 57). The ribosyltransferase ARR-2 described in this work has 55% amino acid sequence identity to ARR-1 found in the naturally resistant *Mycobacterium smegmatis* (41). Related *arr-2* gene cassettes have also been reported from two other studies, both in the same geographical area as ours (16, 34).

The *aacA4* cassette encoding the aminoglycoside acetyltransferase AAC(6′)-Ib_7 is identical to that of the bla_{IMP-4}-bearing integron. Southern hybridization of the plasmid extract with an *arr-2* fragment used as a probe demonstrated the presence of this integron on the large plasmid (estimated size, 275 Kb), where the bla_{IMP-4}-bearing integron also resided (result not shown).

Distribution of bla_{IMP-4}, *arr-2*, and *intI1*. A PCR-based method targeting the *intI1*-bla_{IMP-4} fragment (1,008 bp) was designed to detect the *intI1*-bla_{IMP-4} sequence in our acinetobacters isolated from blood cultures in 1997 to 2000. The identity of the amplicons was confirmed by digestion by four restriction endonucleases (Table 2). Also, a similar approach with an amplicon of 1,182 bp digested by two restriction endonucleases was employed to detect the 5′-CS–*arr-2–aacA4* sequence (Table 2). Another PCR, previously described, was used to detect the *intI1* sequence (27).

Table 3 shows the numbers and genomic DNA groups of acinetobacters obtained from blood cultures from 1997 to 2000 and their distribution according to genomic DNA groups. Results of PCR to the class I integrase gene *intI1*, the *intI1*-bla_{IMP-4}, and/or 5′-CS–*arr-2–aacA4* sequences are also shown.

The bla_{IMP-4} cassette linked to *intI1* was found only in genomic DNA groups 3 and 2 (*A. baumannii*), and the 5′-CS–*arr-2–aacA4* was found in genomic DNA groups 3 and 17 as well as in an unclassifiable isolate. Isolates bearing either the *intI1*-bla_{IMP-4} sequence or the *arr-aacA4* sequence or both were found mainly in 1997 and 1998, representing 17.5% of the isolates in 1997, 16.1% in 1998, and 2.5% in 1999. Of the 52 *A. baumannii* isolates from 1997 to 2000, 17 (33%) harbored *intI1* but only 1 was PCR positive for *intI1*-bla_{IMP-4} and 5′-CS–*arr-2–aacA4*; whereas 20 of the 21 *intI1*-positive isolates of all other genomic DNA groups were associated with the presence of *intI1*-bla_{IMP-4} and/or 5′-CS–*arr-2–aacA4* sequences.

Figure 3 shows the relatedness of all the genomic DNA group 3 isolates from 1997 to 1998. The five isolates harboring both bla_{IMP-4} and *arr-2* cassettes were grouped into two clusters of four isolates and one isolate, linked together at a Dice coefficient of similarity of ≤65% (Fig. 3). Four of these five isolates were obtained from the ICU. The 11 isolates harboring the bla_{IMP-4}cassette only were divided into four clusters linked together at a Dice coefficient of similarity of ≤65%, indicating that they were possibly not related (Fig. 3). Six of these 11 isolates came from the ICU. PFGE of all genomic DNA group 2 isolates from 1997 to 1998 showed that the single isolate containing both cassettes was linked to others with a Dice coefficient of similarity of <65% (data not shown).

MIC studies. With the exception of the unclassifiable isolate from 1999 (Table 4), reduced susceptibility to imipenem and rifampin was seen only in isolates harboring the *intI1*-bla_{IMP-4} and 5′-CS–*arr-2–aacA4* sequences, respectively. The MIC of imipenem for the 17 isolates bearing *intI1*-bla_{IMP-4} was 1 to 16 μg/ml. For three of the four isolates bearing 5′-CS–*arr-2–aacA4* only, the imipenem MIC was 0.03 to 0.25 μg/ml. The MIC of imipenem for the remaining isolate (1999, unclassifiable) was >32 μg/ml. The MIC of rifampin for the 10 isolates

TABLE 4. Comparison of MICs for genomic DNA group 3 isolates from blood cultures 1997-2000[a]

Antibiotic	MIC (μg/ml) for isolates:									
	Bearing *intI1-bla*$_{IMP-4}$ and/or 5′CS-*arr-2-aacA4* (n = 17)[b]					Not bearing either *intI1-bla*$_{IMP-4}$ or 5′CS-*arr-2-aacA4* (n = 41)[c]				
	Geomean	MIC$_{50}$	MIC$_{90}$	Min MIC	Max MIC	Geomean	MIC$_{50}$	MIC$_{90}$	Min MIC	Max MIC
AMK	18.08	32	64	0.50	64	1.33	2	4	0.06	64
GEN	1.44	1	8	0.06	64	0.61	1	2	0.06	128
NET	1.45	1	8	0.25	32	0.71	1	2	0.06	8
IPM	3.69	4	8	0.25	16	0.15	0.13	0.25	0.03	1
CIP	0.42	0.50	2	0.03	32	0.33	0.25	1	0.02	128
SUL	1.08	1	2	0.50	4	0.84	1	2	0.06	4
TET	1.57	2	4	0.13	128	1.66	2	4	0.13	256
SMX	128	128	128	128	128	16.83	8	1,024	0.25	1,024
RIF	6.80	2	128	1	128	1.93	2	4	0.25	32
CAZ	96.22	128	128	16	256	8.56	8	32	0.50	128
CHL	128	256	256	16	256	47.55	64	256	0.50	512

[a] Geomean, geometric mean of MIC; MIC$_{50}$ and MIC$_{90}$, MICs at which 50 and 90% of isolates are inhibited, respectively; Min MIC, minimum MIC; Max MIC, maximum MIC; AMK, amikacin; GEN, gentamicin; NET, netilmicin; IPM, imipenem; CIP, ciprofloxacin; SUL, sulbactam; TET, tetracycline; SMX, sulphamethoxazole; RIF, rifampin; CAZ, ceftazidime; CHL, chloramphenicol. By the Mann-Whitney test, for isolates bearing the *intI1-bla*$_{IMP-4}$ and/or 5′CS-*arr-2-aacA4* sequences versus isolates not bearing those sequences, $P < 0.001$ to 0.01 for AMK, GEN, IMP, SMX, CAZ; for other agents, $P > 0.05$.

[b] Fifteen isolates were tested for NET, and 5 were tested for CHL.

[c] Forty-one isolates were tested for all agents except CHL, for which 21 were tested.

bearing 5′-CS–*arr-2–aacA4* was ≥32 μg/ml, and that for the 11 nonbearers was 1 to 2 μg/ml. The MICs of 11 antimicrobial agents for genomic DNA group 3 isolates positive for both or either of the *intI1-bla*$_{IMP-4}$ and 5′-CS–*arr-2–aacA4* sequences were compared with those for isolates from 1997 to 2000 negative for those sequences (Table 4). There is a significant difference between the 17 *intI1*-positive and the 48 *intI1*-negative isolates (1997 to 2000) in the MICs of amikacin, gentamicin, imipenem, sulfamethoxazole, and ceftazidime (Mann-Whitney U test, $P < 0.001$ to 0.01) (Table 4). Similar statistical results were obtained when the 17 *intI1*-positive isolates were compared with 27 *intI1*-negative isolates (1 isolate was not done) from 1997 to 1998. The numbers of isolates in other genomic DNA groups were too small for statistical analysis (data not shown).

Detection of the *aph(3′)-VIa* and *aacA4* genes in isolates from 1997 to 1998. There were 21 isolates for which the MIC of amikacin was ≥8 μg/ml; 15 of them were positive by PCR for both *aph(3′)-VIa* (60) and *aacA4*, and 6 were positive only for *aph(3′)-VIa*. Seventy-six isolates for which the MIC of amikacin was ≤0.12 to 4 μg/ml were examined in a similar manner; all were negative for *aph(3′)-VIa*, but five isolates (MIC of amikacin, 0.5 to 2 μg/ml) were positive for *aacA4* PCR and all of them were carriers of either or both cassettes on the two integrons described. None of the PCR products was sequenced to determine the exact *aacA4* type.

DISCUSSION

To date, over 70 cassettes capable of conferring antimicrobial resistance have been characterized (45), including the largest class 1 integron found so far, In53, in an *E. coli* isolate, containing nine different antibiotic resistance genes of different classes (34). In comparison, there are only a few epidemiological studies of specific resistance integrons. The carriage of integron may vary among different types of clinical specimens and different genera (13, 30, 47, 48, 59, 62). In our study, 33% (17 of 52) of *A. baumannii* (genomic DNA group 2) isolates from 1997 to 2000 carried class 1 integron but only 1 isolate carried both the *bla*$_{IMP-4}$ and *arr-2* cassettes (Table 3); whereas, for other genomic DNA groups, *intI1* carriage appeared to be closely related to the presence of *bla*$_{IMP-4}$ and *arr-2–aacA4* (Table 3). No isolate belonging to genomic DNA group 13TU, another member of the *Acinetobacter calcoaceticus-A. baumannii* complex, was PCR positive for *intI1*, or *intI1-bla*$_{IMP-4}$, or 5′-CS–*arr-2–aacA4* sequences (5). In studies of resistant nosocomial isolates of *A. baumannii*, 70 to 80% were found to be PCR positive for the *intI1* gene (14, 40). As reported by others, we found that integron carriage was significantly associated with reduced susceptibility to the aminoglycosides, β-lactams, ciprofloxacin, and cotrimoxazole tested (30).

In a previous study, it was reported that *bla*$_{IMP-4}$ was present in the Prince of Wales Hospital culture collections of acinetobacters as early as 1994 (9). Cassettes of other *bla*$_{IMP}$ alleles (IMP-2, IMP-7, and IMP-8) have since been described for other members of *Enterobacteriaeae* and as integron borne (15, 44, 63). Further characterization of *bla*$_{IMP-4}$ here showed that it was also integron cassette borne. Acinetobacters that were PCR positive for either or both of the *intI1-bla*$_{IMP-4}$ and 5′-CS–*arr-2–aacA4* sequences were nearly all from patients who had stayed in an ICU in which there had not been an overt change in the practice of antibiotic prescription over the study period. The two integrons bearing these gene cassettes were found in at least three genomic DNA groups (Table 3), indicating the presence of horizontal dissemination. There was also evidence of longitudinal dissemination of possibly clonally related isolates during 1997 to 1998 (Fig. 3). The four isolates that were PCR positive for both *intI1-bla*$_{IMP-4}$ and 5′-CS–*arr-2–aacA4* formed a cluster with a Dice coefficient of similarity of >80% (Fig. 3) (21). Another cluster with a similar Dice coefficient of similarity was formed by the three isolates that were PCR positive for *intI1-bla*$_{IMP-4}$ only (Fig. 3).

It is not clear why the carriage of both integrons bearing the *intI1-bla*$_{IMP-4}$ and/or 5′-CS–*arr-2–aacA4* sequences had declined from blood culture isolates by 1999. There was also a

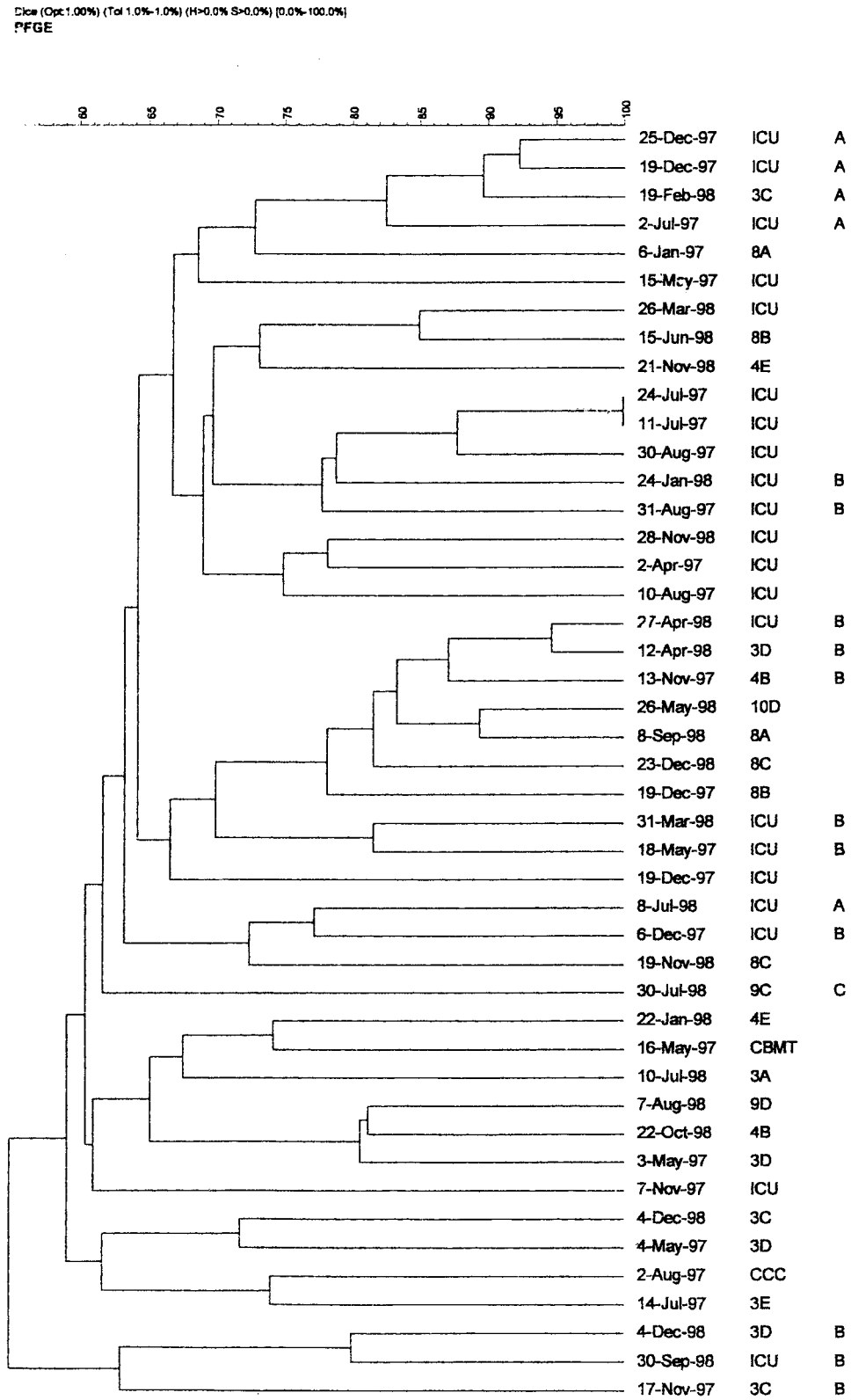

FIG. 3. Dendrogram based on Dice coefficients of similarity for 45 genomic DNA group 3 isolates from blood cultures obtained in 1997 and 1998. The dendrogram was constructed by the unweighted pair group method with arithmetic averages UPGMA (BioNumerics; Applied Maths, Sint-Martens-Latem, Belgium). A, isolates bearing both the *intI1-bla*$_{IMP-4}$ and 5′-CS-*arr-2-aacA4* sequences; B, isolates bearing the *intI1-bla*$_{IMP4}$ sequence (one isolate was not studied); C, isolates bearing the 5′-CS–*arr-2*–*aacA4* sequence; 3C, 8A, 8B, 4E, CBMT, CCC, etc., designations of wards.

corresponding fall in the carriage of class 1 integrons (Table 3) by isolates from 1999 to 2000. No specific containment measures for infection control were implemented in the ICU during this period, as these isolates were not clustered temporally. It is worth noting that the imipenem MIC for *intI1-*bla_{IMP-4}-positive isolates was only modestly raised (1 to 16 μg/ml). Integron-borne resistance to older antibiotics such as trimethoprim and the early aminoglycosides, e.g., *dfrIa-aadA1a* and *dfr12-orfF-aadA2*, is frequently conserved, suggesting that these gene cassettes have become preserved and stably integrated over a long period of time (8, 31). Should there be a genetic linkage of the two resistance cassettes discussed here to other resistance determinants, removal of selective pressure may not bring about a reduction of resistance within a useful time (12). To devise effective preventive programs, there is therefore a need to understand fully the appearance, maintenance, and decline of antibiotic resistance.

Gene cassettes bearing resistance to rifampin have been reported from two other studies, both in the same geographical area as ours (16, 34). In our hospital, the *arr-2* gene cassette appeared to be a major contributor to rifampin resistance in acinetobacters at the time of study, since for all *arr-2* positive isolates the MIC of rifampin was ≥32 μg/ml whereas for all negative isolates it was ≤8 μg/ml.

In clinical isolates of *Acinetobacter* spp. the production of aminoglycoside-modifying enzymes is thought to account for most of the resistance (2, 33, 50, 51). PCR and hybridization data showed that some strains contained more than one aminoglycoside resistance gene (50, 52, 53). Of the aminoglycoside-modifying enzymes detected in *Acinetobacter* so far, only the APH(3′)-VI and some versions of AAC(6′)-I enzymes confer resistance to amikacin (39, 60). The *aph(3′)-VIa* gene encoding the APH(3′)-VIa phosphotransferase was detected in all of our 21 isolates for which the MIC of amikacin was ≥8 μg/ml with or without *aacA4* gene cassettes. The exact identification of the *aacA4* cassettes encountered in the blood culture isolates is uncertain because the amplicons were not sequenced.

The results of our study on blood culture collections of acinetobacters over a 4-year period show that the bla_{IMP-4}-bearing and *arr-2*-bearing integrons, apparently residing on a large plasmid(s), were disseminated horizontally in three different genomic DNA groups and longitudinally mainly in the ICU during 1997 to 1998. They were associated with increased resistance to imipenem and rifampin. Their carriage became undetectable in 1999 to 2000, suggesting that they were yet to be established firmly as resistance determinants in *Acinetobacter* and judicial use of antimicrobials may delay such an outcome. Nevertheless, further studies similar to ours should be carried out for a better understanding of the impact of integrons on the persistence of antimicrobial resistance in clinical practice.

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