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Dissemination of Amikacin Resistance Gene *aphA6* in *Acinetobacter* spp.

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The distribution of the *aphA6* gene, encoding a 3′-aminoglycoside phosphotransferase type VI, was studied by dot blot hybridization with 115 amikacin-resistant *Acinetobacter* strains from various geographical areas. Nucleotide sequences related to *aphA6* were found in 109 strains belonging to seven species. As inferred from results of Southern hybridization, dissemination of amikacin resistance in *Acinetobacter* spp. is due to a gene rather than a strain or plasmid epidemic.

Acinetobacter spp., and in particular *Acinetobacter baumannii*, are responsible for severe nosocomial infections in intensive care units. *Acinetobacter* infections are difficult to cure, since strains are often resistant to multiple antimicrobial agents. β-Lactams, quinolones, and aminoglycosides are the major groups of antibiotics used in therapy of infections due to this bacterial genus. *Acinetobacter* spp. are naturally resistant to cephalosporins (20) and to low levels of trimethoprim, and they can acquire plasmids (4, 8, 21) or transposons (6) or both which account for antibiotic resistance. Until recently, amikacin remained the most active aminoglycoside in the treatment of infections caused by *Acinetobacter* spp., although it was inactivated in certain strains by a chromosomal 6′-aminoglycoside-acetylating enzyme (15). However, since 1984, outbreaks of infections due to *Acinetobacter* spp. resistant to amikacin have been observed in France (12; L. Gutmann, personal communication). We recently described the inactivation of amikacin by a 3′-aminoglycoside phosphotransferase type VI [APH(3′)-VI] in *Acinetobacter* spp. (14) and determined the nucleotide sequence of the corresponding gene *aphA6* (18).

The purpose of this study was to evaluate the distribution of *aphA6* in *Acinetobacter* spp. and to determine if dissemination of amikacin resistance was due to a strain, plasmid, or gene epidemic.

MATERIALS AND METHODS

Bacterial strains. *A. baumannii* BM2580, BM2580-1, and BM2582, *Acinetobacter haemolyticus* BM2585, *Acinetobacter lwoffii* BM2587 (14), and *Acinetobacter calcoaceticus* C4318 (4) were described previously. *A. calcoaceticus* BD413 *trpE27* (ATCC 33308) was used in transformation experiments (13). *A. baumannii* BM2663 and BM2664 are wild strains that produce a 6′-aminoglycoside acetyltransferase. A total of 115 clinical isolates of *Acinetobacter* spp. resistant to amikacin (MIC ≥ 32 μg/ml) were collected from 17 hospitals (15 in France, 1 in Israel, and 1 in Tunisia). The strains were isolated in different geographical locations, in various wards, and at different times between 1984 and 1988.

Strain identification. The strains had properties that define the *Acinetobacter* genus (2), and identification at the genus level was confirmed by the transformation assay (13). The identification scheme used (2) was completed by biotyping based on the utilization of levulinate, citraconate, L-phenylalanine, phenylacetate, 4-hydroxybenzoate, and L-tartrate (3). Bacteriophage typing, with 35 bacteriophages which distinguish 121 phage types, was performed as described previously (23). The method of Steers et al. (25) with 10^4 CFU per spot was used to determine the MICs of antibiotics.

Genetic techniques. Conjugation (14) into *Escherichia coli* K802N, transformation (24) into *E. coli* HB101 or *A. calcoaceticus* BD413 (13), and curing of antibiotic resistance with ethidium bromide (1) were performed as described previously.

Preparation of DNA. Total DNA was prepared as described by Roussel and Chabbert (22). Large-scale isolation of plasmid DNA was by a modification of the procedure of Ingram et al. (10).

DNA-DNA hybridization. For dot blot and Southern hybridizations, DNA was immobilized on Nytran membranes (Schleicher & Schuell, Inc.). Prehybridization and hybridization were carried out for 5 and 15 h, respectively, at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.5% sodium dodecyl sulfate–0.05% nonfat dry milk (11). The 365-base-pair *Eco*RI-*Bgl*II fragment internal to *aphA6* (Fig. 1) cloned into pUC19 was separated by electrophoresis in low-melting-temperature agarose after endonuclease digestion, extracted (17), and radiolabeled (17).

Aminoglycoside-modifying enzyme assays. Bacterial extracts were prepared (5), and the enzymes were assayed by the phosphocellulose paper-binding technique (9), as described previously. The final concentration of aminoglycosides in the assay mixture was 66.7 μg/ml, and the reaction was allowed to proceed for 30 min at 30°C.

Enzymes and chemicals. Restriction endonucleases (Amersham Corp.) were used according to the recommendations of the manufacturer. Lysozyme was from Sigma Chemical Co., and RNase A (bovine pancreas) was from Calbiochem-Behring. Nick translation kit reagents were from Bethesda Research Laboratories, Inc. [α-^{32}P]dCTP, [1-^{14}C]acetyl coenzyme A, [α-^{32}P]ATP (triethylammonium salt), and [γ-

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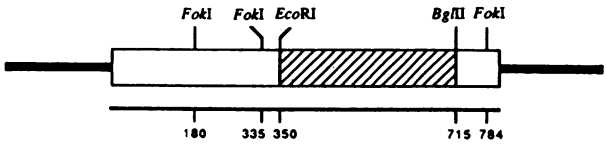

FIG. 1. Restriction map of the *aphA6* gene. Only relevant sites are indicated. Symbols: ▭, *aphA6*; ▨, intragenic probe used. Sizes are indicated in base pairs.

[32P]ATP (triethylammonium salt) were obtained from the Radiochemical Centre, Amersham Corp. Antibiotics were provided by the following laboratories: Beecham Laboratories, ticarcillin; Bristol Laboratories, kanamycins A, B, and C and amikacin; Eli Lilly & Co., tobramycin; Le Petit, rifampin; Parke, Davis & Co., butirosin; Pfizer Inc., streptomycin and tetracycline; Roger Bellon, habekacin and lividomycin; Schering Corp., gentamicins C1a, C1, C2, A, and B and netilmicin; The Upjohn Co., neamine and neomycins B and C; and Winthrop Laboratories, nalidixic acid.

RESULTS

Identification of strains. The properties of the strains studied are listed in Table 1. The 115 clinical isolates collected could be assigned to seven genospecies, and the 107 *A. baumannii* strains were subdivided into six biotypes. Three biotypes, 2, 6, and 9, were more common, with 25, 49, and 23 isolates, respectively. A total of 62 strains (54%) were typeable with phages and belonged to nine phage types, whereas 33 strains (29%) had bacteriophage susceptibility spectra that did not correspond to any phage type. The 20 remaining strains were resistant to all phages tested. Phage typing allowed us to put *A. baumannii* biotypes 2, 6, and 9 into six, five, and four lytic profiles, respectively. Among these, biotype 2 (atypical phage type) and biotype 6 (phage type 17) represented the largest groups, with 15 and 35 isolates, respectively. Phage type 117, which corresponds to the six strains from Israel, has not yet been observed in isolates of nosocomial origin. Heterogeneity of the strains was confirmed by analysis of total DNA digestion profiles of 14 isolates (Fig. 2A).

Susceptibility to antibiotics. The resistance phenotypes were obtained by disk susceptibility tests and, for certain antibiotics, by determination of MICs (Table 2). The majority of the strains were resistant to gentamicin, netilmicin, tobramycin, tetracycline, and ticarcillin but remained susceptible to habekacin. Only five strains were susceptible to gentamicin (MIC $\leq$ 0.5 μg/ml); they were also susceptible to netilmicin (MIC $\leq$ 1 μg/ml) and tobramycin (MIC $\leq$ 1 μg/ml) but were resistant to kanamycin, neomycin, butirosin, and amikacin which are substrates for the APH(3′)-VI. Six strains were resistant to habekacin (MIC $\geq$ 32 μg/ml), by synthesis of a 6′-aminoglycoside acetyltransferase.

Transfer of antibiotic resistance traits by conjugation. Amikacin resistance was transferred by conjugation, with frequencies ranging from 10^{-4} to 10^{-8}, from 44 isolates to *A. baumannii* BM2582. Selection for transfer of amikacin or kanamycin resistance revealed cotransfer of these two resistances plus neomycin and butirosin resistance. Acquisition of aminoglycoside resistance correlated with acquisition of the *aphA6* gene, as revealed by DNA-DNA hybridization (see below), and synthesis of an APH(3′)-VI enzyme, as detected by the phosphocellulose paper-binding assay, in seven transconjugants that were tested further. When transfer was not obtained (71 strains), *A. haemolyticus* BM2585 and *A. lwoffii* BM2587 were used as recipients, allowing us to obtain five additional transfers. For 46 strains, resistance to aminoglycosides that are substrates for the APH(3′)-VI enzyme was transferred. In one strain, resistance to gentamicin, netilmicin, streptomycin, sulfonamide, ticarcillin, and tobramycin was also transferred, whereas resistance to gentamicin, netilmicin, sulfonamide, and tobramycin was cotransferred to the two remaining strains. Transfer of aminoglycoside resistance to *E. coli* was not obtained.

Transfer of aminoglycoside resistance by transformation. *A. calcoaceticus* BD413, a naturally competent strain, was transformed for amikacin resistance with total DNA of 7 of 11 strains tested. Aminoglycoside resistance in the transformants was due to acquisition of the *aphA6* gene and synthesis of the APH(3′)-VI enzyme. Other resistance characters were not cotransferred. In 9 of the 11 strains used as donors, amikacin resistance was not transferable by conjugation to *Acinetobacter* spp.

Curing of antibiotic resistance markers. Loss of amikacin resistance, which correlated with lack of hybridization with the *aphA6* probe and absence of synthesis of the 3′-phosphotransferase, was observed for seven of eight strains tested; five of the seven strains had transferable amikacin resistance. In one strain, loss of transferable amikacin resistance was associated with loss of resistance to gentamicin, netilmicin, streptomycin, sulfonamide, ticarcillin, and tobramycin.

Hybridization with the *aphA6* probe. We tested the presence of nucleotide sequences that were structurally related to *aphA6* in *Acinetobacter* strains by dot blot hybridization (data not shown). Homology with the probe was detected for 109 strains (95%) belonging to all the species studied (Table 1). Four *A. baumannii* isolates and the susceptible control strains did not hybridize with the probe. Total DNA of 14 isolates, with transferable amikacin resistance and various geographical origins, was digested with *Hin*dIII (Fig. 2A) and *Bam*HI (data not shown) and studied by Southern hybridization by using the same probe. There is no recognition site for these endonucleases in *aphA6* DNA (Fig. 1). The various sizes of the fragments hybridizing with the probe (Fig. 2B) suggest that the resistance gene is located in different genomic environments. The detection in certain strains, and in both restriction endonuclease systems, of two hybridizing DNA bands indicated that certain clinical isolates harbor two nontandemly arranged copies of *aphA6*. This finding was confirmed by further analysis of 10 of these strains by Southern hybridization following digestion with *Fok*I (Fig. 3). This enzyme generates a 450-base-pair DNA fragment which includes the entire probe (Fig. 1), and only this fragment was detected.

DISCUSSION

We have studied the contribution of the gene encoding a 3′-aminoglycoside phosphotransferase type VI to the incidence of amikacin resistance in *Acinetobacter* spp. by DNA-DNA hybridization using a specific probe. The 115 clinical isolates selected were epidemiologically unrelated and included 7 of the 12 *Acinetobacter* species (Table 1). *A. baumannii*, the species most involved in human pathology, predominated with 107 strains. Biotyping and phage typing (Table 1), resistance phenotypes (Table 2), and, for certain strains, analysis of total DNA restriction profiles (Fig. 2A), allowed distinction of the majority of the strains with the exception of two clusters. Dot blot hybridization experiments indicated that *aphA6*-related sequences were present in 109 strains (95%).

TABLE 1. Properties of the strains studied

Species (no. of strains)	No. of strains																				
	Phage type											Conjugation to *Acinetobacter* spp.[a]		Transformation to *Acinetobacter* spp.[b]			Plasmid curing			Hybridization with *aphA6*	
	17	40	75	76	86	91	113	117	121	Atyp[c]	Res[d]	+[e]	−[e]	+	−	NT[f]	+	−	NT	+	−
Acinetobacter baumannii biotype 1 (5)					1					4			5			5			5	5	
2 (25)	1		3	1		1				15	4	12	13			25	1	1	23	24	1
6 (49)	35						1	6		6	1	27	22	3	4	42	2		47	48	1
8 (1)										1			1			1			1		1
9 (23)	8								2	4	9	5	18	3		20	1		22	20	3
11 (4)		1								3		2	2			4	2		2	4	
Acinetobacter lwoffii (1)											1		1			1			1	1	
Acinetobacter junii (1)											1	1				1			1	1	
Acinetobacter haemolyticus (1)											1		1			1			1	1	
Acinetobacter sp. strain 3 (2)	2												2			2			2	2	
Acinetobacter sp. strain 11 (2)											2	1	1	1		1	1		1	2	
Acinetobacter johnsonii (1)											1	1				1			1	1	
Total (115)	46	1	3	1	1	1	1	6	2	33	20	49	66	7	4	104	7	1	107	109	6[g]

[a] *A. baumannii* BM2582, *A. haemolyticus* BM2585, *A. lwoffii* BM2587 (14), and *A. calcoaceticus* C4318 (4) were used as recipients.
[b] *A. calcoaceticus* BD413 *trpE27* was used as a recipient (13).
[c] Atyp, Atypical phage type: bacteriophage susceptibility spectrum that does not correspond to any of the defined phage types (23).
[d] Res, Resistant to all phages tested.
[e] +, Positive result; −, negative result.
[f] NT, Not tested.
[g] These strains encoded a 6′-aminoglycoside acetyltransferase.

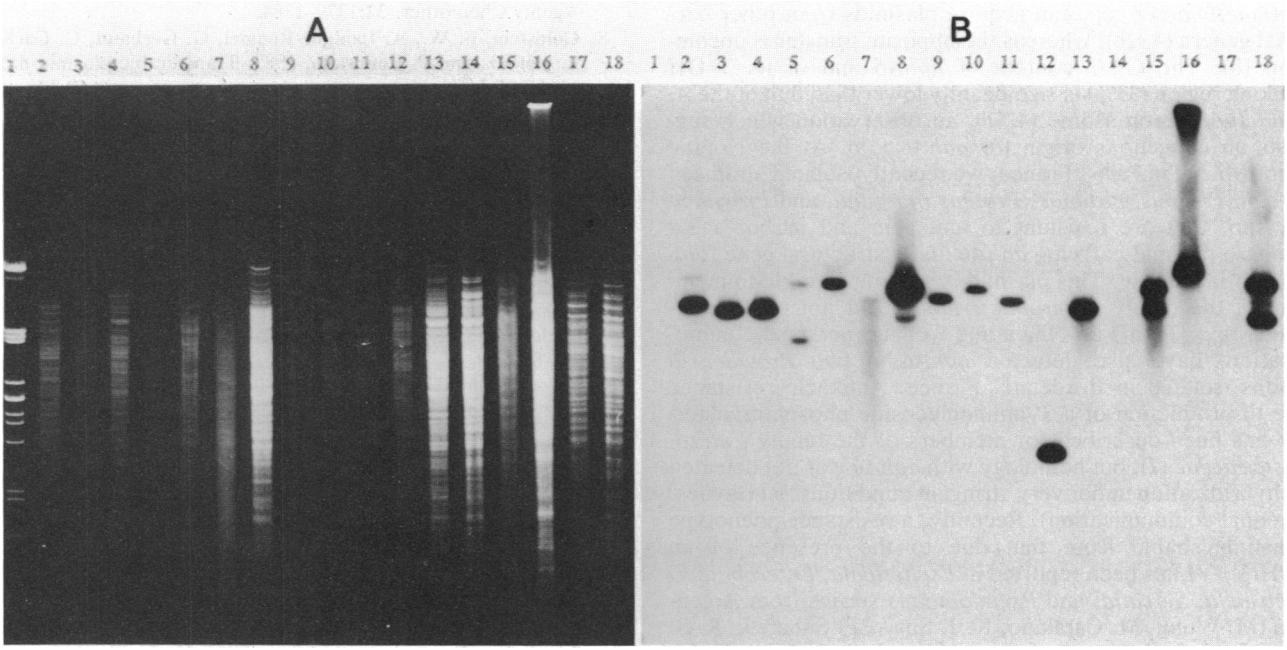

FIG. 2. Analysis of total DNA by agarose gel electrophoresis (A) and by hybridization (B). Total DNA was digested with *Hin*dIII, and the resulting fragments were separated by electrophoresis in a 0.8% agarose gel, transferred to a Nytran filter, and hybridized to the in vitro ^{32}P-labeled *aphA6* probe. Lanes: 1, bacteriophage λ DNA used as an internal standard; 2 to 13, DNA of wild *Acinetobacter* strains with transferable amikacin resistance; 14, strain BM2582 DNA used as a negative control; 15, strain C4318 DNA (pIP1841); 16, strain BM2582 DNA (pIP1841); 17, DNA of strain BM2580-1, cured derivative of strain BM2580, used as a negative control; 18, BM2580 DNA.

As already mentioned, differences in the epidemiological markers of the strains suggested that dissemination of amikacin resistance in *Acinetobacter* spp. was not due to an epidemic strain. The various profiles obtained by Southern hybridization in two different restriction endonuclease systems (Fig. 2B) do not favor the view of a plasmid epidemic. A gene epidemic seems more likely, and we have preliminary evidence of the transposability of *aphA6*.

Conjugation experiments indicated that in 49 strains (43%), amikacin resistance was borne by plasmids self-transferable to *Acinetobacter* spp. but not to *E. coli*. Although donors were resistant to multiple antibiotics (Table 2), only resistance to aminoglycosides that are substrates for the APH(3′)-VI enzyme was transferred in the vast majority of the cases. The six strains which did not hybridize to the probe were also resistant to habekacin and did not transfer aminoglycoside resistance to other *Acinetobacter* spp. These strains produced a 6′-aminoglycoside acetyltransferase which has been shown to be chromosomally encoded (21).

TABLE 2. MICs of antibiotics for the 115 amikacin-resistant *Acinetobacter* strains

Antibiotic	MIC (μg/ml)[a]		
	Range	50%	90%
Amikacin	32–>1,024	128	512
Gentamicin	0.5–512	64	256
Habekacin	1–256	2	8
Kanamycin	256–>1,024	1,024	>1,024
Netilmicin	1–≥256	8	64
Tetracycline	2–≥128	32	128
Ticarcillin	2–512	32	64
Tobramycin	1–≥256	8	64

[a] 50% and 90%, MIC for 50 and 90% of isolates, respectively.

Therefore, it appears that amikacin resistance in *Acinetobacter* spp. in France is mainly due to dissemination of the *aphA6* gene. This observation may also hold true for other ecosystems, since the clinical isolates from Israel and Tunisia studied also contained *aphA6*. In addition, a 70-kilobase plasmid encoding an APH(3′) in an amikacin-resistant *Acinetobacter* strain isolated in a Lebanese hospital has recently been reported (E. Gay, B. Hill, R. Gaynes, and R. Cooksey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, A-3, p. 1). The presence of two *aphA6* genes in certain strains is surprising. Among the three classes, acetyltransferases, nucleotidyltransferases, and phosphotransferases, of aminoglycoside-modifying enzymes, phosphotransferases confer the highest level of resistance, and a single copy of *aphA6* confers clinical resistance to the host (Table 2).

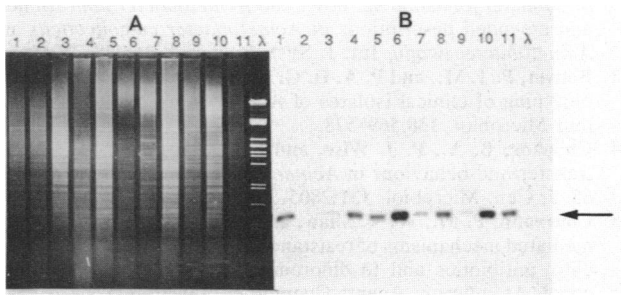

FIG. 3. Analysis of total DNA by agarose gel electrophoresis (A) and by hybridization (B). Total DNA of 10 geographically unrelated *Acinetobacter* strains (included in Fig. 2) was digested with *Fok*I, and the resulting fragments were separated by electrophoresis in a 0.8% agarose gel, transferred to a Nytran filter, and hybridized to the in vitro ^{32}P-labeled *aphA6* probe. Lanes 2, strain BM2580-1 DNA used as a negative control. Bacteriophage λ DNA digested with *Pst*I (lanes λ) was used as an internal standard. Arrow indicates the 450-base-pair fragment hybridizing to the probe.

Acinetobacter spp. can acquire plasmids from other bacterial genera (4, 26), whereas the opposite transfer is uncommon (8). The G+C content of *aphA6* and of its 3'-OH flanking region (33%) is significantly lower than that of the *A. baumannii* chromosome (42%), an observation which suggests an exogenous origin for *aphA6* (18). At the Hôpital Saint-Michel in Paris, France, we recently isolated strains of *E. coli*, *Proteus mirabilis*, *Proteus morganii*, and *Providencia* spp. that are resistant to amikacin and harbor a 29-kilobase plasmid carrying *aphA6* and a structural gene for a TEM β-lactamase. This plasmid is self-transferable to members of the family *Enterobacteriaceae* but not to *Acinetobacter* spp. Tran Van Nhieu and Collatz (personal communication) have also detected *aphA6* in two *Providencia* strains isolated in Bordeaux, France. Amikacin resistance due to production of a 3'-aminoglycoside phosphotransferase has been described for members of the family *Enterobacteriaceae* (7), but homology with *aphA6* was not detected by hybridization under very stringent conditions (R. Gaynes, personal communication). Recently, a resistance phenotype indistinguishable from that due to the presence of an APH(3')-VI has been reported in *Escherichia*, *Enterobacter*, *Klebsiella*, *Serratia*, and *Pseudomonas* strains from Argentina (M. Woloj, M. Catalano, K. J. Shaw, F. Sabatelli, R. S. Hare, and G. Miller, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 676, 1989). The extension of the substrate range of APH(3') enzymes to amikacin could have been anticipated because of wide use of the drug (16, 19).

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