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Transferable Amikacin Resistance in *Acinetobacter* spp. Due to a New Type of 3′-Aminoglycoside Phosphotransferase

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***Acinetobacter baumannii* BM2580 resistant to kanamycin and structurally related antibiotics, including amikacin, was isolated from a clinical specimen. A phosphocellulose paper-binding assay and DNA annealing studies indicated that resistance to aminoglycosides in BM2580 was due to synthesis of a new type of 3′-aminoglycoside phosphotransferase. The gene conferring resistance to kanamycin-amikacin in this strain was carried by a 63-kilobase plasmid, pIP1841, self-transferable to *A. baumannii*, *A. haemolyticus*, and *A. lwoffii* but not to *Escherichia coli*. The aminoglycoside resistance gene of pIP1841 was cloned in *E. coli*, where it was expressed.**

Acinetobacter baumannii, previously *A. calcoaceticus* subsp. *anitratus* (*Herellea vaginicola*) (5), is increasingly responsible for nosocomial infections, especially in intensive care units. Pulmonary and urinary tract infections and septicemia due to *A. baumannii* (17) are difficult to eradicate in part because of the multiple resistance of this organism to antibiotics.

A. baumannii is naturally resistant to cephalosporins (28) and to low levels of trimethoprim. Acquired resistance in this species is secondary to the presence of transposable elements (15) or of plasmids (21, 30, 41). Curiously, *Acinetobacter* spp. can easily acquire plasmids from *Escherichia coli* (8, 40), whereas the opposite transfer seems to be a rare event (18, 19).

Aminoglycoside resistance in *Acinetobacter* spp. is common and due to enzymes which modify the antibiotics. 3′-Phosphotransferase types I and II, 2′-acetyltransferase, 3-acetyltransferase type I, 6′-acetyltransferase, 9-adenylyltransferase, 3″,9-adenylyltransferase, and 2″-adenylyltransferase have been detected in this bacterial genus (2, 16, 19, 25, 29, 30, 34).

Since it is inactivated only by the 6′-acetylating enzyme, amikacin remained until recently the most active aminoglycoside for infections due to *Acinetobacter* spp. However, since October 1984, we have observed in France outbreaks of infections due to strains of *A. baumannii* resistant to kanamycin and structurally related aminoglycosides, including amikacin. We have studied the biochemical mechanism and the genetic basis of this resistance phenotype. Resistance to aminoglycosides in *A. baumannii* BM2580, a representative of this epidemic, was due to synthesis of a new type of 3′-aminoglycoside phosphotransferase [APH(3′)] which efficiently modifies amikacin.

MATERIALS AND METHODS

Bacterial strains and plasmids. The sources and properties of the strains are listed in Table 1. *A. baumannii* BM2580 was isolated in 1984 from a human urine sample. *A. baumannii* BM2582, *A. haemolyticus* BM2585, and *A. lwoffii* BM2587 are spontaneous mutants of wild-type strains susceptible to antibiotics. *Acinetobacter* strains were identified at the species level by the method of Bouvet and Grimont (5). *E. coli* HB101 harboring RP4 (Tra^+ Ap Km Tc) (14) or pUC18 (Tra^- Ap) (31) was from our laboratory collection.

Media. Brain heart infusion broth and agar (Difco Laboratories) and Mueller-Hinton agar (Diagnostics Pasteur) were used. All incubations were at 37°C.

Determination of MICs. The method of Steers et al. (38) with 10^4 CFU per spot was used to determine the MICs of the antibiotics.

Genetic techniques. Conjugation (7), transformation in *E. coli* (35) and in *Acinetobacter* spp. (37), and curing of antibiotic resistance traits with ethidium bromide (4) were performed as previously described. Antibiotic concentrations (in micrograms per milliliter) for selection were as follows: amikacin, 20; ampicillin, 100; chloramphenicol, 15; kanamycin, 20; nalidixic acid, 50; and rifampin, 50.

Assay for aminoglycoside-modifying enzymes. The bacterial extracts were prepared (12), and the enzymes were assayed by the phosphocellulose paper-binding technique (20) as previously described. The final concentration of aminoglycosides in the assay mixture was 66.7 μl/ml, and the reaction was allowed to proceed for 30 min at 30°C.

Preparation of DNA. *Acinetobacter* strains were grown on solid medium for 18 h and suspended in 20 mM Tris–2 mM EDTA (pH 8.0). Rapid screening of plasmid DNA was by the method of Takahashi and Nagano (39). Preparation of high-molecular-weight total DNA was as previously described (13), and large-scale isolation of plasmid DNA was adapted from the procedure of Ingram et al. (22). *Acinetobacter* plasmids were separated by electrophoresis in horizontal slab gels (20 by 20 by 0.7 cm) containing 0.8% low-temperature-gelling agarose, and pIP1841 DNA was purified as previously described (26).

Hybridization. The plasmids used as probes for hybridization are listed in Table 2. Plasmid DNA that was ^{32}P-labeled in vitro (27) was hybridized to DNA immobilized on nitrocellulose filters (26).

Enzymes and reagents. Restriction endonuclease *Acc*I and T4 DNA ligase (Amersham Corp.) were used according to the recommendations of the manufacturer. Lysozyme was from Sigma Chemical Co., and ribonuclease A (bovine pancreas) was from Calbiochem-Behring. [1-^{14}C]acetyl coenzyme A, [α-^{32}P]ATP (triethylammonium salt), [γ-^{32}P]ATP (triethylammonium salt), and [U-^{14}C]ATP (ammonium salt) were obtained from the Radiochemical Centre, Amersham

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TABLE 1. Properties of the strains used

Strain	Relevant characteristic(s)	Source or reference
A. baumannii BM2580	Cm Km	Wild-type strain
A. baumannii BM2580-1	Cm	Curing of BM2580
A. baumannii BM2582	Nal Rif	Spontaneous mutant of wild-type strain
A. baumannii BM2582(pIP1841)	Nal Rif Km	Conjugation of BM2580 × BM2582
A. haemolyticus BM2585	Rif	Spontaneous mutant of wild-type strain
A. haemolyticus BM2585(pIP1841)	Rif Km	Conjugation of BM2580 × BM2585
A. lwoffii BM2587	Rif	Spontaneous mutant of wild-type strain
A. lwoffii BM2587(pIP1841)	Rif Km	Conjugation of BM2580 × BM2587
E. coli S17-1	*hsdR hsdM*$^+$ *recA pro* RP4-2 (Tc::Mu) (Km::Tn*5*) Sm Tp	36
A. baumannii BM2580-1::Tn*5*	Cm Km	Conjugation of S17-1 × BM2580-1
A. baumannii BM2580-1(RP4)	Ap Cm Km Tc	Conjugation of HB101(RP4) × BM2580-1
E. coli HB101	*hsdR hsdM recA13 supE44 lac24 leuB6 proA2 thiA rpsL20*	6
E. coli HB101(pAT235)	Km	

Corp. The antibiotics were provided by the following laboratories: Schering Corp. (gentamicins C1a, C1, C2, A, and B; sisomicin; 2″-deoxysisomicin; and netilmicin), Bristol Laboratories (ampicillin; kanamycins A, B, and C; amikacin; and 4′-deoxy-6′-*N*-methylamikacin [BBK311]), The Upjohn Co. (neamine, neomycins B and C, and spectinomycin), Eli Lilly & Co. (apramycin and tobramycin), Parke, Davis & Co. (butirosin and paromomycin), Roger Bellon (habekacin), A. Kowa (lividomycin), Meiji (ribostamycin), Pfizer Inc. (streptomycin), Winthrop Laboratories (nalidixic acid), and Le Petit (rifampin).

RESULTS

Plasmid-mediated characters expressed by *A. baumannii* BM2580. *A. baumannii* BM2580 encodes resistance to chloramphenicol, kanamycin, neomycin, butirosin, and amikacin. In curing experiments, resistance to aminoglycosides was lost en bloc (4 resistant out of 200 colonies tested), and one cured strain, BM2580-1, was studied further. Aminoglycoside resistance was transferred from *A. baumannii* BM2580 to *A. baumannii* BM2582, *A. lwoffii* BM2587, and *A. haemolyticus* BM2585 by conjugation at frequencies of 1.5×10^{-4}, 2.6×10^{-6}, and 1.2×10^{-8}, respectively. The transfer of chloramphenicol resistance was never observed, and we never succeeded in transferring any resistance to *E. coli* HB101 by mixed incubation. The MICs of aminoglycosides of the parental strain BM2580, its cured derivative BM2580-1, recipients, and transconjugants are shown in Table 3.

Plasmid content of BM2580, its derivative BM2580-1, and a transconjugant. The plasmid DNAs from strains BM2580, BM2580-1, BM2582, and BM2582(pIP1841) (Table 1) were analyzed by agarose gel electrophoresis of crude bacterial lysates (Fig. 1). Plasmid pIP1841 encoded transferable aminoglycoside resistance and had a molecular size of approximately 63 kilobases (kb). The other plasmids were cryptic. Strain BM2580-1 was susceptible to aminoglycosides and had lost pIP1841. *A. baumannii* BM2582(pIP1841) was resistant to aminoglycosides after acquisition of pIP1841. The plasmid DNA from strain BM2580 was purified by ultracentrifugation, and plasmids were separated by electrophoresis in low-temperature-gelling agarose. Plasmid pIP1841 DNA was purified from the gel and used to transform BM2582. Transformants were resistant to aminoglycosides after acquisition of pIP1841 (data not shown).

Molecular cloning of the aminoglycoside resistance gene of pIP1841 in *E. coli*. Plasmid pIP1841 DNA digested with *Acc*I endonuclease and pUC18 DNA cleaved with *Acc*I and dephosphorylated were mixed, ligated, and introduced into *E. coli* HB101 by transformation (35), and clones were selected with ampicillin (100 μg/ml) and kanamycin (20 μg/ml). One of the transformants harboring the smallest plasmid was studied further, and its hybrid plasmid was called pAT235 (24). Plasmid pAT235 DNA was purified and analyzed by agarose gel electrophoresis after digestion with *Acc*I endonuclease (data not shown). Plasmid pAT235 consists of pUC18 plus a 1.8-kb pIP1841 DNA fragment.

Mechanism of resistance to aminoglycosides mediated by plasmid pIP1841. *A. baumannii* BM2580, BM2580-1, BM2582, and BM2582(pIP1841) and *E. coli* HB101 and HB101(pAT235) were examined for aminoglycoside-modifying activities. Strains harboring plasmid pIP1841 or pAT235 were found to contain APH but no adenylyltransferase nor acetyltransferase activities. The substrate profiles of the phophotransferases extracted from strains BM2580, BM2582(pIP1841), and HB101(pAT235) were very similar. That of BM2580 is shown in Fig. 2. That kanamycin B was modified but tobramycin (3′-deoxykanamycin B) was not indicates that the 3′-hydroxyl group was the site of phosphorylation [APH(3′)]. In pathogenic bacteria, APH(3′)s exist in three (I, II, and III) isozymic forms. These enzymes differ with respect to their substrate ranges, and the corresponding genes are not structurally related (43). Type I enzymes modify lividomycin A but not butirosin or amikacin; enzymes of type II modify butirosin but not lividomycin

TABLE 2. Plasmids used for hybridizations

Plasmid	Vector	Genotype	Insert (intragenic fragment)	Origin
pGH54	pBR322 derivative	*bla*(TEM-1) *aphA1*	513-bp[a] *Xho*I-*Hin*dIII from Tn*903 aphA1*	45
pAT234	pUC18	*bla*(TEM-1)	350-bp *Pst*I-*Sph*I from Tn*5 aphA2*	This study
pAT93	pBR322	*bla*(TEM-1)	530-bp *Hpa*II from pJH1 *aphA3*	42

[a] bp, Base pair.

TABLE 3. MICs of various aminoglycosides against *Acinetobacter* and *E. coli* strains

Strain	MIC (μg/ml)[a]				
	Kan	Neo	Tob	Liv	Ami
BM2580	1,024	64	0.5	4	256
BM2580-1	1	0.5	0.5	4	1
BM2580-1(RP4)	1,024	128	0.5	512	1
BM2580-1::Tn*5*	1,024	64	0.5	4	1
BM2582	2	1	0.5	8	2
BM2582(pIP1481)	2,048	128	0.5	8	512
BM2585	4	2	16[b]	8	4
BM2585(pIP1481)	2,048	256	16	8	1,024
BM2586	0.5	0.5	0.25	2	0.5
BM2586(pIP1481)	512	64	0.25	2	128
HB101	0.5	0.5	0.25	1	0.5
BH101(pAT235)	512	16	0.25	1	64

[a] Abbreviations: Kan, kanamycin; Neo, neomycin B; Tob, tobramycin; Liv, lividomycin; Ami, amikacin.
[b] *A. haemolyticus* is naturally resistant to low levels of tobramycin (T. Lambert, unpublished observation).

A or amikacin, whereas type III enzymes modify the three antibiotics in vitro. That butirosin and amikacin were substrates for phosphorylation and that lividomycin A was not indicates that the enzyme of BM2580 is of a new type. Plasmid RP4 (14) and transposon Tn*5* (1), which encode APH(3′) of types I and II, respectively, were introduced into BM2580-1 by conjugation (Table 1). As expected, these two genetic structures did not confer amikacin resistance to their new host (Table 3).

Analysis of DNA by hybridization. DNA of hybrid plasmids representative of the three types of APH(3′) (Table 2) was transferred to a nitrocellulose filter and hybridized to in vitro ^{32}P-labeled pAT235 insert DNA (Fig. 3). No homology was found between the 1.8-kb *Acc*I DNA fragment of pIP1841 and the genes encoding type I, II, and III enzymes. In the reverse experiment, nick-translated DNA fragments internal to the structural genes for the three types of enzymes did not hybridize to pAT235 (Fig. 3) nor to *A. baumannii* BM2580 and BM2582(pIP1841) plasmid DNA (data not shown).

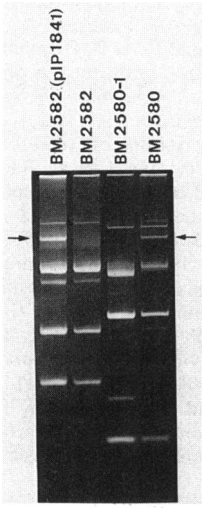

FIG. 1. Analysis of plasmid DNAs. DNAs extracted from the strains indicated at the top were fractionated by electrophoresis in a 0.9% agarose gel (18 by 13 by 0.4 cm) for 18 h at 3 V/cm. Plasmid pIP1841 is indicated by arrows. Plasmids pSF2124 (11 kb), Rs-a (39.5 kb), TP114 (62.5 kb), and pIP135-1 (70.4 kb) were used as molecular size standards.

DISCUSSION

A. baumannii BM2580 was a human clinical isolate resistant to kanamycin and structurally related antibiotics, in particular amikacin (Table 3). Aminoglycoside resistance was carried by pIP1841, a 63-kb plasmid, self-transferable to other *Acinetobacter* cells but not to *E. coli*. Resistance to aminoglycosides was correlated with the presence of pIP1841 DNA (Fig. 1).

Bacterial resistance to aminoglycosides is frequently mediated by aminoglycoside-modifying enzymes which are classified by the mechanism of their modification and the site on the antibiotic which they modify (15). In pathogenic bacteria, three types of 3′-phosphotransferases can be distinguished, particularly on the basis of their substrate range in vitro (43). The respective genes, although thought to diverge from a common ancestor (43), do not cross-hybridize (11). We established by in vitro substrate profile determina-

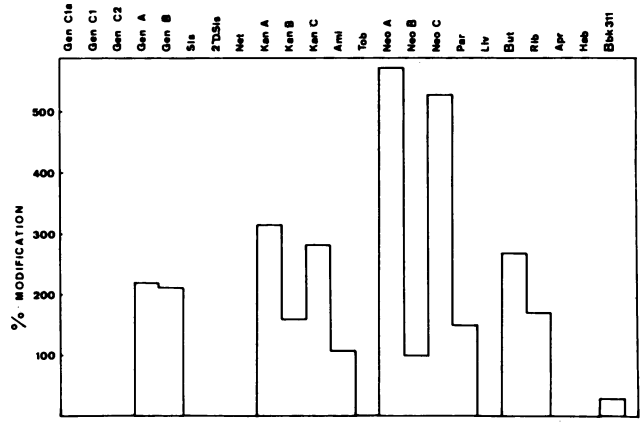

FIG. 2. Substrate profile of enzyme extracted from *A. baumannii* BM2580. Phosphorylation is expressed relative to neomycin B, defined as 100%. No acetylation, no phosphorylation, and no adenylation of the same aminoglycosides were detected in this strain. No aminoglycoside-modifying activity was detected in the susceptible strains BM2580-1, BM2582, and HB101. Abbreviations: Gen, gentamicin; Sis, sisomicin; 2″D.Sis, 2″-deoxysisomicin; Net, netilmicin; Kan, kanamycin; Ami, amikacin; Tob, tobramycin; Neo A, neamine; Neo B and Neo C, neomycins B and C; Par, paromomycin; Liv, lividomycin; But, butirosin; Rib, ribostamycin; Apr, apramycin; Hab, habekacin; Bbk311, 4′-deoxy-6′-*N*-methylamikacin.

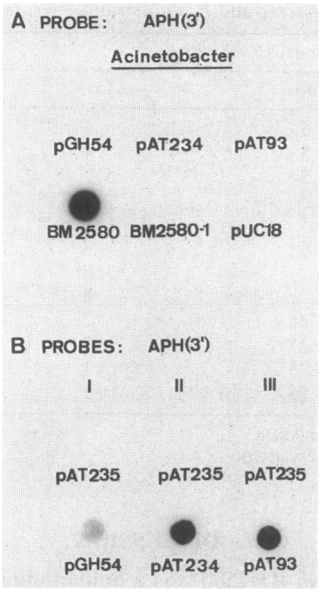

FIG. 3. Analysis of DNA by dot blot hybridization. (A) DNAs of plasmids representative of APH type I (pGH54), type II (pAT234), and type III (pAT93) were transferred to nitrocellulose sheets and hybridized to pAT235 insert DNA labeled with ^{32}P in vitro. Total DNAs from wild-type strain BM2580 and from its cured derivative BM2580-1 served as positive and negative controls, respectively. The absence of hybridization with pUC18 DNA indicates that the probe was not contaminated with vector DNA. (B) In the reverse experiment, the inserts of plasmids pGH54, pAT234, and pAT93 were used as probes and hybridized to immobilized pAT235 DNA. Homologous reactions were used as positive controls.

tion (Fig. 2) and by DNA-annealing studies (Fig. 3) that resistance to kanamycin-amikacin in BM2580 was due to synthesis of a new type of APH(3′).

Among the APH(3′)s detected in clinical isolates, only type III, which is apparently confined to gram-positive cocci (43), and *Campylobacter* species (23) modify amikacin in vitro (9). However, because of poor affinity for this substrate, this isozyme does not confer amikacin resistance to its original hosts (9) nor to *E. coli* after cloning in vitro (10). By contrast, the new type of APH(3′) present in BM2580 efficiently modifies amikacin in vitro (Fig. 2) and confers high-level resistance to this antibiotic when present in various *Acinetobacter* species or in *E. coli* (Table 3). Like the enzyme in BM2580 (Fig. 2), the type II phosphotransferase which is found exclusively in gram-negative bacteria (43) inactivates kanamycin and butirosin but not lividomycin. However, as opposed to the BM2580 activity, amikacin is not a substrate for this isozyme, and cells harboring the type II phosphotransferase remain susceptible to the drug in vivo (Table 3).

Amikacin-resistant variants of bacteria encoding the type II activity have been obtained in vitro after a gene dosage effect (3), increased production of enzyme (32), or decreased aminoglycoside uptake (33). Transposon Tn*5*, which encodes a type II enzyme (Table 2), does not confer amikacin resistance when introduced into *A. baumannii* BM2580-1 (Table 3), and we were unable to obtain spontaneous mutants of this strain resistant to the drug.

Amikacin resistance is not new in *Acinetobacter* spp., and a 6′-aminoglycoside acetyltransferase, apparently chromosomally encoded, has been described for this bacterial genus (29). Approximately 15% of *Acinetobacter* spp. isolated in Parisian hospitals resist amikacin (Meyran et al. personal communication). The vast majority of the strains studied harbor a transferable APH(3′) similar to that of BM2580 (T. Lambert and G. Gerbaud, unpublished observation). This new type of enzyme appears, therefore, to be (at least in our country) the major cause of resistance to amikacin in *Acinetobacter* spp. Analysis of the genes encoding aminoglycoside-modifying enzymes facilitates the study of resistance gene transfer between bacteria under natural conditions (44). In an attempt to understand the emergence and dissemination of kanamycin-amikacin resistance in *Acinetobacter* spp. by phosphorylation, we are determining the sequence of the APH(3′) determinant.

Although *Acinetobacter* spp. can acquire plasmids belonging to various incompatibility groups from the family *Enterobacteriaceae* (8), the transfer of plasmids from the *Acinetobacter* genus to *E. coli* has been reported only twice (18, 19). We obtained full expression of the APH(3′) gene from BM2580 in *E. coli* (Table 3), irrespective of the relative orientation of the insert and the cloning vector. Therefore, the transfer barrier may not result from lack of expression of *Acinetobacter* genes in heterologous systems but from a defect(s) in conjugation, plasmid replication, or both in the new host.

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