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Appearance of Amikacin and Tobramycin Resistance due to 4′-Aminoglycoside Nucleotidyltransferase [ANT(4′)-II] in Gram-Negative Pathogens

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Following the use of amikacin as the principal aminoglycoside at a Denver hospital, amikacin resistance appeared first in *Pseudomonas aeruginosa* and then in *Escherichia coli*, *Klebsiella pneumoniae*, and other enteric organisms from debilitated and compromised patients who had spent time in intensive care units and who had been treated with multiple antibiotics, usually including amikacin. In a *P. aeruginosa* isolate, resistance to amikacin and tobramycin was transferable by the IncP-2 plasmid pMG77, while in *E. coli* and *K. pneumoniae* resistance was carried by the transmissible plasmids pMG220, pMG221, and pMG222 belonging to the IncM group. Isolates and transconjugants produced an enzyme with adenylyltransferase activity with substrates having a 4′-hydroxyl group, such as amikacin, kanamycin, neomycin, Sch 21768, isepamicin (Sch 21420), or tobramycin, but not with aminoglycosides lacking this target, such as dibekacin, netilmicin, sisomicin, or gentamicin C components. Genes encoding the 4′-aminoglycoside nucleotidyltransferase [ANT(4′)] activity were cloned from pMG77, pMG221, and pMG222. A DNA probe prepared from the ANT(4′) found in *P. aeruginosa* hybridized with the ANT(4′) determinant found in *E. coli*. A probe for the ANT(4′) from *Staphylococcal* spp., which differs in its modification of substrates, like dibekacin, that have a 4″- but not a 4′-hydroxyl group, failed to hybridize with the gram-negative ANT(4′) determinant, which consequently has been termed ANT(4′)-II.

Amikacin was designed to be resistant to many of the enzymes that modify other aminoglycoside antibiotics. Emergence of resistance to amikacin has been uncommon, even when it has been used as almost the sole aminoglycoside in some hospitals (2, 11, 29, 38).

In staphylococcal species, a mechanism for amikacin resistance is modification by 4′-aminoglycoside nucleotidyltransferase [ANT(4′)] (23, 33). This transferase acts on dibekacin, which lacks a 4′ target, at the 4″-hydroxyl, so that the enzyme can also be termed an ANT(4′,4″) (33). Until recently, ANT(4′) activity had not been found in gram-negative pathogens (27), but in 1989, Kettner et al. (20) reported ANT(4′,4″) in two amikacin-susceptible members of the family *Enterobacteriaceae* from Czechoslovakia.

We report here the discovery of amikacin resistance caused by an ANT(4′) that does not modify the 4″-hydroxyl of dibekacin and that is mediated by transmissible plasmids. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and other enteric organisms that produce this novel modifying activity emerged after almost exclusive use of amikacin at a Denver hospital.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. Table 1 lists the relevant characteristics of the standard bacterial strains and plasmids used in this study. Phages B3, D3, E79, G101, M6, and PB1, which are active on *P. aeruginosa* (15), and phage M, which is lytic for strains carrying IncM plasmids (3), were also used.

Selection of aminoglycoside-resistant strains. Clinical isolates were chosen because of aminoglycoside resistance at the Veterans Administration Medical Center, Denver, Colo. Between 1979 and 1981, isolates were characterized as resistant if the zone sizes on Kirby-Bauer disk susceptibility testing were ≤14 mm for amikacin or ≤12 mm for gentamicin and tobramycin. After 1981, resistant isolates were identified by use of an automated machine (autoScan 4; Baxter Healthcare Corp., MicroScan Div., West Sacramento, Calif.) if the MIC was >16 μg/ml for amikacin or >4 μg/ml for gentamicin or tobramycin by using brain heart infusion medium not supplemented with cations.

Media and mating conditions. *P. aeruginosa* strains were grown in nitrate nutrient broth (15). Matings, selection of transconjugants, incompatibility testing, and selective plates were as described previously (17). *E. coli* and other members of the family *Enterobacteriaceae* were grown and mated in L broth as specified previously (31). In crosses with prototrophic donors, rifampin (100 μg/ml) was used for counterselection.

Serological and pyocin typing. Cultures were serotyped by slide agglutination (39) by using O antisera 1-16 obtained from T. L. Pitt, Central Public Health Laboratory, London, United Kingdom. Pyocin typing was done by the technique of Gillies and Govan (12).

Plasmid characterization. Resistance to antibiotics was evaluated by disk diffusion and by growth on antibiotic-containing plates. Testing for resistance to mercuric ion, phenylmercuric acetate, and potassium tellurite was done as described previously (17). Inhibition of phage propagation in

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference
E. coli		
J53-2	F^- *met pro* Rif^r	24
C600	F^- *lacY leu thi thr*	24
P. aeruginosa		
PAO38 Rif^r	FP^- *leu* Rif^r	17
PAO303	FP^- *arg*	17
Plasmids		
pIBI25	Ap mcr	IBI[b]
pMLC28	Cm mcr	B. Seed
pTZ18R	Ap mcr	USB[c]
$pUBH_2$	ANT(4')-I clone	8
R135	Gm Sm Su Tc Hg IncM	14
R3108	Sm Su Tc Hg Pmr Ter IncP-2	17

[a] Abbreviations: Ap, resistance to ampicillin; Cm, resistance to chloramphenicol, Gm, resistance to gentamicin; Sm, resistance to streptomycin; Su, resistance to sulfonamide; Tc, resistance to tetracycline; Hg, resistance to mercuric chloride; Pmr, resistance to phenylmercuric acetate; Ter, resistance to potassium tellurite; mcr, multiple cloning site polylinker in *lacZα*; Inc, incompatibility group.

[b] IBI, International Biotechnologies Inc., New Haven, Conn.

[c] USB, United States Biochemical Corp., Cleveland, Ohio.

P. aeruginosa was assayed by a qualitative spot test (17). Phage M susceptibility was tested on R plates (28). Plasmids were sized by agarose gel electrophoresis (36) in comparison with standard plasmids with known sizes.

Aminoglycoside susceptibility. Aminoglycoside MICs were determined by a microtiter technique in unsupplemented Mueller-Hinton (Difco Laboratories, Detroit, Mich.) broth (34). In addition to amikacin, dibekacin, gentamicin, neomycin, netilmicin, sisomicin, and tobramycin, test aminoglycosides included isepamicin (Sch 21420; HAPA-gentamicin B), Sch 27598 (5-epi-isepamicin), Sch 21561 (2'-*N*-ethyl-netilmicin), Sch 21562 (6'-*N*-ethyl-netilmicin), Sch 21768 (2',3'-dideoxy-gentamicin B), Sch 22591 (5-epi-sisomicin), and fortimicin. A biochemical mechanism was assigned to a particular aminoglycoside resistance pattern (AGRP) as described in detail previously (27, 34). Selected MICs were also determined in Mueller-Hinton broth supplemented with 25 μg of $MgCl_2$ per ml and 50 μg of $CaCl_2$ per ml.

Aminoglycoside-modifying enzyme assay. Modifying activity was assayed with the phosphocellulose paper-binding assay (13) by using [U-^{14}C]ATP (New England Nuclear, Boston, Mass.) or [2-^{3}H]ATP (Radiochemical Centre, Amersham, England) as the radioactive cofactor. In Boston, cells were grown overnight in L broth, harvested by centrifugation, and disrupted by sonication. The supernatant from centrifugation at 4,500 × *g* for 15 min was used for assay. The assay was performed in a volume of 35 μl at 30°C for 30 min in 19 mM Tris maleate buffer (pH 7.8) containing 12 mM $MgCl_2$, 114 mM NH_4Cl, 0.5 mM dithiothreitol, 148 μM ATP, and 2 μg of aminoglycoside substrate. Controls for nonspecific binding of each substrate contained a comparable extract of R^- cells. In Zurich, osmotic shock extracts (32) were partially purified by centrifugation at 150,000 × *g* in a Beckman L8-70 ultracentrifuge. Enzymatic activity was mainly contained in a jellylike, loosely bound sediment which was dissolved in 0.5 mM $MgCl_2$ containing 0.8 mM dithiothreitol. Modifying activity was assayed at several pHs as described previously (33) by using Tris hydrochloride buffer between pHs 7.5 and 9.0 and glycine-NaOH buffer between pHs 8.5 and 10.0. Results are expressed at the optimal pH for a particular substrate.

Aminoglycoside inactivation was evaluated by bioassay, as described previously (32).

Hybridization techniques. A probe for the ANT(4')-I gene was prepared by *Hae*III digestion of $pUBH_2$ (8) and labeled with ^{32}P by nick translation. Hybridization was carried out by the dot blot technique as described previously (8).

Construction of recombinant plasmids. Plasmid DNA was purified by cesium chloride-ethidium bromide gradient centrifugation (24). For cloning with PAO38(pMG77), genomic DNA was used (1). The DNA was digested with restriction endonucleases (New England BioLabs, Inc., Beverly, Mass., or International Biotechnologies Inc., New Haven, Conn.), religated by using T4 DNA ligase, and transformed into *E. coli* strains (24) by selecting for resistance to amikacin. Inserted DNA was sized by redigesting recombinant plasmids with the endonuclease(s) that was used for cloning.

TABLE 2. Relation of amikacin usage and aminoglycoside resistance

Phase	No. of mo	% Amikacin use[a]	Total no. of isolates studied	% Resistant to:		
				Amikacin	Gentamicin	Tobramycin
Base-line period (7/79–11/79)[b]	5	15	823	1.8	12.2	8.7
Amikacin phase (12/79–10/84)	59	97	8,190	3.7	7.2	6.7
Postamikacin phase						
11/84–6/85	8	32	1,232	4.3	11.4	9.3
1986[c]	12	35	2,118	5.0	6.2	8.9
1987	12	4	2,091	2.6	5.4	5.1
1988	12	2.4	1,649	2.9	6.4	4.7

[a] Expressed in terms of patient-days.

[b] Resistance data for 1979 to 1985 are from assays done at Bristol Laboratories. Numbers in parentheses indicate month/year.

[c] Resistance data for 1986 to 1988 are from assays done at the hospital microbiology laboratory.

RESULTS

Appearance of amikacin resistance. In the late 1970s, gentamicin was the predominant aminoglycoside used at the Veterans Administration Medical Center in Denver. By 1979, however, over 12% of clinically significant gram-negative isolates were gentamicin resistant (Table 2). Consequently, amikacin was substituted for gentamicin, and between December 1979 and October 1984, amikacin constituted 97% (expressed in terms of patient-days) of the aminoglycoside in use at the hospital. Resistance to gentamicin decreased, but resistance to amikacin increased from 1.8 to 3.7% for the gram-negative isolates. Gentamicin was again made available, and amikacin resistance declined as its usage diminished, although never to the 1979 level (Table 2).

Aminoglycoside-resistant strains were assigned a particular AGRP based on susceptibility to a panel of test antibiotics. Beginning in 1981 (Table 3), an unusual AGRP appeared in isolates of *P. aeruginosa* characterized by resistance to amikacin, tobramycin, and isepamicin, while susceptibility to gentamicin, netilmicin, sisomicin, and other test aminoglycosides was unchanged. Since this AGRP was previously recognized only in *Staphylococcus* strains producing ANT(4'), a new variety of gram-negative aminoglycoside-modifying enzyme [ANT(4')-II] was suspected. Beginning in 1984, the same pattern appeared in isolates of enteric gram-negative organisms, including *E. coli*, *Citrobacter* spp., *Klebsiella* spp., and *Serratia* spp., always in combination with other resistance mechanisms (Table 3).

TABLE 3. Appearance of the ANT(4')-II resistance pattern

Organism and resistance pattern[a]	No. of isolates collected in:					
	1981	1982	1983	1984	1985	1986
Pseudomonas spp.						
ANT(4')-II		2	27	30	10	14
ANT(4')-II + ANT(2")	1	3			2	1
ANT(4')-II + AAC(6')-II				1	1	
Total no. examined	10	11	38	39	24	21
Members of the family *Enterobacteriaceae*						
ANT(4') + ANT(2')				2	13	37
ANT(4') + ANT(2') + AAC(6')					5	12
Total no. examined	17	18	6	21	59	84

[a] The resistance mechanism was determined by the aminoglycoside resistance pattern.

Epidemiology of resistance. Seven *P. aeruginosa* strains with this AGRP were studied in detail. As shown in Table 4, they were isolated from cultures of blood, sputum, urine, or wound specimens from patients on three different wards during a 5-month period in 1984. Serological and pyocin typing indicated that more than cross-colonization with a single resistant organism was involved. Strains 003 and 101 had indistinguishable serological and pyocin types, as did strains 102 and 706; but these pairs could be distinguished from each other and from strains 005, 103, and 702, each of which gave a unique typing reaction. Nearly all the patients had been treated with amikacin, as well as with other antibiotics, for an average of 5.3 antibiotics per patient. Most of the patients were debilitated or compromised hosts. Each had been hospitalized for at least 2 weeks and had spent time in a medical or surgical intensive care unit. Eight of the nine patients had received cimetidine.

Transfer of aminoglycoside resistance in *P. aeruginosa*. The strains listed in Table 4 were mated with *P. aeruginosa* PAO38 Rif^r by using rifampin for counterselection and selecting for transfer of tobramycin resistance. Strain 005 transferred resistance, at a frequency of 10^{-4} per donor, after overnight mating. Transconjugants resistant to tobramycin were also resistant to amikacin, kanamycin, Hg^{2+}, and tellurite (Table 5). They inhibited the propagation of phages B3, D3, E79, G101, M6, and PB1 and on agarose gel electrophoresis contained a large plasmid, about 450 kb in size, termed pMG77. These characteristics are typical of plasmids of the IncP-2 group in *P. aeruginosa* (17). To confirm this assignment, pMG77 was introduced into strain PAO303 containing the IncP-2 plasmid R3108 by mating. Transconjugants lost sulfonamide resistance encoded by R3108, indicating that the plasmids were incompatible and, hence, that pMG77 indeed belonged to the IncP-2 group. Some of the other *P. aeruginosa* strains in Table 4 were also resistant to tellurite and contained plasmids of about 450 kb, but no other strain was able to transfer aminoglycoside resistance.

TABLE 5. Properties of ANT(4')-II plasmids

Donor	Plasmid	Size (kb)	Properties[a]
P. aeruginosa 005	pMG77	ca. 450	Ak Km Tm Hg Ter IncP-2
K. pneumoniae 110	pMG220	68	Ak Gm Km Su Tm IncM
K. pneumoniae 120	pMG221	72	Ak Gm Km Sm Su Tm Tp IncM
E. coli 116	pMG222	68	Ak Ap Gm Km Su Tm IncM

[a] Abbreviations are as described in footnote *a* of Table 1, plus resistance to amikacin (Ak), kanamycin (Km), tobramycin (Tm), and trimethoprim (Tp).

Aminoglycoside-modifying activity. By the phosphocellulose paper-binding assay, each of the *P. aeruginosa* strains in Table 4 adenylylated amikacin, as did PAO38(pMG77), but PAO38 without the plasmid did not.

Amikacin has seven hydroxyl groups that are potentially capable of adenylylation at positions 2', 3', 4', 5, 2", 4", and 6". To identify the site of modification, a range of substrates was tested (Table 6). Activity with Sch 21768, which lacks 2'- and 3'-hydroxyl groups, ruled out these sites. Lack of activity with dibekacin and other substrates with 5-, 2"-, 4"-, and 6"-hydroxyl groups excluded these sites. As shown in Table 6, activity as a substrate correlated perfectly with the presence of a 4'-hydroxyl group, confirming the enzyme as an ANT(4').

Relationship to staphylococcal ANT(4'). The relationship of the gram-negative determinant for ANT(4') to the ANT(4') found in *Staphylococcus* spp. was investigated by using a 600-bp *Hae*III fragment from $pUBH_2$. No hybridization was found with *P. aeruginosa* or enteric amikacin-resistant bacteria producing the adenylyltransferase, under conditions at which ≥75% similarity would have been detected. Hence, the designation ANT(4')-II is proposed for the new transferase.

Transfer of aminoglycoside resistance from the *Enterobac-*

TABLE 4. Characteristics of strains with an ANT(4')-II resistance pattern

Strain	Isolation date (mo/day/yr)	MIC (μg/ml)[a]			Source of isolate	Patient's hospital ward	Prior no. of days patient was hospitalized	Prior amikacin use
		Ak	Gm	Tm				
P. aeruginosa								
003	3/29/84	>256	16	256	Wound	5W	212	Yes
005	4/17/84	256	8	256	Blood	5N	26	Yes
101	5/15/84	>256	32	>256	Sputum	5N	61	Yes
102	5/17/84	>256	32	>256	Urine	5W	16	Yes
706	6/14/84	>256	32	>256	Sputum	5W	20	No
103	6/16/84	256	16	>256	Sputum	5N	106	Yes
702	7/15/84	>256	32	>256	Urine	6W	20	Yes
E. coli 116	8/08/86	256	64	128	Urine	5NE	43	Yes
K. pneumoniae 110	7/24/86	256	64	128	Urine	3N	21	No

[a] MICs were determined in cation-supplemented Mueller-Hinton medium. Abbreviations: Ak, amikacin; Gm, gentamicin; Tm, tobramycin.

TABLE 6. Aminoglycoside adenylylating activity of PAO38(pMG77)

Substrate	% Adenylylation relative to that of amikacin[a]	Presence of 4'-hydroxyl
Amikacin	100	+
Isepamicin	145	+
Sch 21768	97	+
Neomycin	90	+
Gentamicin B	60	+
Kanamycin	30	+
Tobramycin	13	+
Sisomicin	>5	−
Dibekacin	>5	−
Gentamicin C1	>5	−
Sch 22591	>5	−
Netilmicin	>5	−

[a] Butirosin, ribostamycin, paromomycin, and lividomycin, all with 4'-hydroxyl groups, were also actively modified.

TABLE 7. Substrate spectrum of ANT(4')-II produced by *E. coli* containing pMG235

Substrate	Optimum pH (test buffer)[a]	^{3}H (cpm)[b]	MIC (μg/ml)	Inactivation
Ribostamicin	8.0 (Tl)	13,613	≥128	+
Amikacin	8.5 (Tl)	11,593	128	+
Lividomycin B	8.5 (Tl)	6,358	32	+
Kanamycin B	9.0 (Tl)	3,667	128	+
Tobramycin	8.5 (Tl)	1,363	64	+
Dibekacin	8.5 (Gl)	0	8	−
Gentamicin C1	8.5 (Gl)	0	4	−
Gentamicin C1a	8.5 (Gl)	0	2	−
Gentamicin C2	8.5 (Gl)	0	4	−

[a] Abbreviations: Tl, Tris hydrochloride buffer; Gl, glycine-NaOH buffer.
[b] Counts per minute as a result of nonspecific binding of [^{3}H]ATP were subtracted.

teriaceae. Amikacin resistance was transferred from two resistant *K. pneumoniae* strains and one resistant *E. coli* strain to *E. coli* J53 Rif^r. Each recipient contained a plasmid of 68 to 72 kb that determined resistance to several antimicrobial agents, in addition to amikacin (Table 5). All plasmids determined resistance to gentamicin as well as amikacin, kanamycin, and tobramycin with an AGRP indicative of ANT(2") as well as ANT(4') activity. The plasmids were assigned to IncM based on their incompatibilities with IncM plasmid R135 and the susceptibilities of their hosts to donor-specific phage M (3).

Cloning of the amikacin resistance determinant. To separate the aminoglycoside resistance activities, plasmids pMG221 and pMG222 were isolated and treated with *Bam*HI, *Eco*RI, or *Hin*dIII endonuclease. The resultant fragments were ligated with similarly cleaved plasmid pMLC28 and transformed into *E. coli* C600, selecting for resistance to amikacin.

From pMG221, amikacin resistance was cloned on a 4.8-kb *Hin*dIII fragment, while resistance from pMG222 was cloned on a 3.7-kb *Hin*dIII insert. By subcloning to pIBI25, resistance from pMG221 was localized to a 2.5-kb *Hin*dIII-*Kpn*I insert and resistance from pMG222 was localized to a 2.3-kb *Hin*dIII-*Pst*I fragment. These recombinant plasmids determined resistance to amikacin, kanamycin, and tobramycin but not to gentamicin and, hence, had a resistance profile typical of ANT(4') modification.

Whole-cell DNA from *P. aeruginosa* carrying pMG77 was similarly digested with various restriction endonucleases and was used to clone the amikacin resistance gene into an *E. coli* vector. In Zurich, amikacin resistance was cloned onto a 2.8-kb *Pst*I fragment with pTZ18R used as a vector. In Boston, amikacin resistance was obtained on a 3.1-kb *Hin*dIII fragment in pMLC28 and subcloned into pIBI25 as a 1.6-kb *Hin*dIII-*Kpn*I insert. Restriction mapping and hybridization studies indicated that this 1.6-kb fragment was contained within the 2.8-kb *Pst*I insert. Furthermore, a probe derived from the 2.8-kb *Pst*I fragment of pMG77 hybridized with the 2.3-kb *Hin*dIII-*Pst*I fragment from pMG222, suggesting a close relationship between the ANT(4')-II genes found in *P. aeruginosa* and *E. coli*.

Substrate spectrum of cloned ANT(4')-II. Table 7 shows the substrate spectrum of adenylyltransferase produced by *E. coli* carrying the recombinant plasmid, termed pMG235, containing the 1.6-kb insert from pMG77. Amikacin, kanamycin B, and tobramycin were active substrates, while dibekacin and gentamicin C components were not modified, thus confirming the specificity of the enzyme for the 4'-hydroxyl group. Those substrates that were modified were also inactivated, and the MIC was increased only for those aminoglycosides that were inactivated by the enzyme. Assay of *E. coli* expressing the cloned pMG222 gene gave similar results.

DISCUSSION

At the Denver Veterans Administration Medical Center, use of amikacin as the principal aminoglycoside for 59 months was followed by an increase in amikacin resistance. In addition to the ANT(4')-II described here, *P. aeruginosa* strains were isolated that produced a phosphotransferase that was active on amikacin and an acetyltransferase that was active on netilmicin and other aminoglycosides (unpublished data). At other institutions, substitution of amikacin for alternative aminoglycosides has not generally led to an increased prevalence of resistance (2, 11, 29, 38), but exceptions have also been reported (7, 10, 25, 40). In Denver, the setting was especially conducive for the emergence of antibiotic resistance. The patient population was elderly and debilitated and had a variety of impairments of host defenses. Many patients were admitted from other hospitals or nursing homes. Hospitalization was prolonged, and the patients tended to have prior and multiple antibiotic treatments. Frequent cimetidine usage may have contributed to antibiotic resistance by favoring gastric colonization with gram-negative bacteria (9). The nearly exclusive use of amikacin (97% of patient-days) also created optimal selective conditions favoring the emergence and maintenance of resistance.

The patients from whom resistant strains were isolated were at high risk for nosocomial infections. They were debilitated, were or had recently been in an intensive care unit, and had received multiple antibiotics, usually including amikacin. The ANT(4') phenotype first appeared in isolates of *P. aeruginosa* and, subsequently, in *E. coli*, *Citrobacter* spp., *Klebsiella* spp., and *Serratia* spp. A transmissible plasmid encoding ANT(4') activity was found in only one of seven *P. aeruginosa* isolates tested. This type of plasmid (IncP-2) is not transmissible to *E. coli* or other members of the family *Enterobacteriaceae* (15), yet hybridization studies (including those with a subsequently developed intragenic probe) indicated a close relationship between the ANT(4') determinants from *P. aeruginosa* and the *Enterobacteriaceae*. The plasmids from *E. coli* or *K. pneumoniae* that

determined ANT(4') belonged to the same IncM group, but differed somewhat in resistance markers and in restriction sites near the ANT(4') gene. These findings are consistent with dissemination of the ANT(4') gene by transposition, but a mobile genetic element has not yet been demonstrated experimentally.

In *P. aeruginosa* and other gram-negative pathogens, enzymes conferring resistance to amikacin have included several varieties of 6'-aminoglycoside acetyltransferase (16, 18, 19, 37), 3-aminoglycoside acetyltransferase V (5, 21), and certain 3'-aminoglycoside phosphotransferases (10, 22). Other 3'-aminoglycoside phosphotransferases (6, 30) and ANT(2")-II (4) can modify amikacin in vitro but apparently do so at slower rates, such that amikacin resistance is not augmented in vivo. Impaired permeability to amikacin is also an important resistance mechanism in *P. aeruginosa* (25, 26). In *Staphylococcus aureus* and *Staphylococcus epidermidis*, ANT(4',4") provides resistance to amikacin (23, 33). In prior surveys of more than 2,000 aminoglycoside-resistant gram-negative bacteria by the AGRP technique, no isolates with an ANT(4') profile were detected (27, 34), but one *P. aeruginosa* strain with this mechanism was mentioned by Young et al. (40), and Kettner et al. (20) recently reported plasmid-mediated ANT(4') activity in two aminoglycoside-resistant but amikacin-susceptible isolates of *Citrobacter freundii* and *Serratia marcescens* from Czechoslovakia. This ANT(4') enzyme was active with dibekacin, like the ANT(4', 4") from staphylococci but unlike the ANT(4') from Denver. The ANT(4') gene from Denver also failed to hybridize with a staphylococcal ANT(4') probe. An ANT(4') without activity on dibekacin has previously been found in *Bacillus brevis* (35). Further studies will be needed to establish how the *Bacillus* and gram-negative ANT(4') enzymes are related, but already it is evident that more than one type of ANT(4') has appeared in gram-negative pathogens.

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