ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1994, p. 1883–1889
0066-4804/94/$04.00+0

Vol. 38, No. 9

Characterization of the Chromosomal *aac(6')-Ij* Gene of *Acinetobacter* sp. 13 and the *aac(6')-Ih* Plasmid Gene of *Acinetobacter baumannii*

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Received 17 March 1994/Returned for modification 16 May 1994/Accepted 28 June 1994

The amikacin resistance genes *aac(6')-Ih* of *Acinetobacter baumannii* BM2686 and *aac(6')-Ij* of *Acinetobacter* sp. 13 BM2689 encoding aminoglycoside 6'-*N*-acetyltransferases were characterized. The 441-bp coding sequences predict proteins with calculated masses of 16,698 and 16,677 Da, respectively. Analysis of the deduced amino acid sequences indicated that the proteins belonged to a subfamily of 6'-aminoglycoside acetyltransferase type I enzymes from gram-negative bacteria. The *aac(6')-Ih* gene of BM2686 was located on a 13.7-kb nonconjugative plasmid. The *aac(6')-Ij* gene from BM2689 was not transferable either by conjugation to *Escherichia coli* or *A. baumannii* or by transformation to *Acinetobacter calcoaceticus*. Plasmid DNA from BM2689 did not hybridize with an intragenic *aac(6')-Ij* probe. These results suggest a chromosomal location for this gene. The *aac(6')-Ij* gene was detected by DNA hybridization in all 28 strains of *Acinetobacter* sp. 13 tested but not in other *Acinetobacter* strains, including *A. baumannii*, proteolytic genospecies 4, 6, 14, 15, 16, and 17, and ungrouped strains. The *aac(6')-Ih* and *-Ij* probes did not hybridize in dot blot assays with DNA from members of the families *Enterobacteriaceae* and *Pseudomonadaceae* that produced 6'-*N*-acetyltransferases. These data suggest that the genes are confined to the *Acinetobacter* genus and that the *aac(6')-Ij* gene is species specific and may be used to identify *Acinetobacter* sp. 13.

Members of the *Acinetobacter* genus are ubiquitous microorganisms of the environment and normal inhabitants of the human skin. The *Acinetobacter* genus is delineated by a transformation test (11) and unambiguously identifiable by biochemical criteria (1). Until recently, *Acinetobacter calcoaceticus* was the only recognized species despite genetical heterogeneity of the genus. On the basis of the results of DNA-DNA hybridization experiments, a minimum of 17 DNA groups have been identified (1, 2). Several species have not been named because of the lack of discriminant biochemical characters that can be used in microbiological practice. Most clinical isolates of *Acinetobacter* are *A. baumannii* (formerly *A. calcoaceticus*), which can be easily identified by ability to grow at 44°C. *Acinetobacter* spp. which hydrolyze gelatin and hemolyze horse blood represent approximately 4% of *Acinetobacter* clinical isolates. Among these proteolytic species, *A. haemolyticus* is the most common, followed by *Acinetobacter* sp. 13 (2), which has also been designated *Acinetobacter* sp. 14 (29). Although many isolates of *Acinetobacter* from clinical sources simply reflect colonization, these bacteria are responsible for nosocomial infections in intensive care units. The difficulties associated with *Acinetobacter* infections are in part due to multiresistance to antibiotics. β-Lactams, especially imipenem, and aminoglycosides represent the preferred therapy for *Acinetobacter* infections. Resistance of *Acinetobacter* spp. to imipenem has recently been reported (7, 16, 31), whereas aminoglycoside resistance by enzymic modification is very common in hospital isolates. Epidemiologic studies indicate that most of the aminoglycoside-inactivating enzymes found in members of the family *Enterobacteriaceae* are also present in *Acinetobacter* spp. (25). Resistance to amikacin in *Acinetobacter* spp. is mainly due to production of 3'-aminoglycoside phosphotransferase type VI [APH(3')-VI] and 6'-aminoglycoside acetyltransferase type I [AAC(6')-I]. The APH(3')-VI enzyme is widely distributed in *Acinetobacter* spp. (12, 13) but remains uncommon in members of the families *Enterobacteriaceae* and *Pseudomonadaceae* (15). The five AAC(6')-I enzymes of gram-negative bacteria are encoded by genes designated *aac(6')-Ia*, *-Ib*, *-Ic*, *-If*, and *-Ig*, whereas *aac(6')-Ie*, and *-Ii* are found in gram-positive bacteria and *Enterococcus faecium*, respectively (23). The *aac(6')-Ib* and *-If* genes have been detected in *Acinetobacter* spp. (25, 28), and *aac(6')-Ig* is part of *A. haemolyticus* chromosome (14). We have found *Acinetobacter* strains that produce AAC(6')-I enzymes and do not contain any known *aac(6')-I* sequence (unpublished data). We report the identification of two new genes, *aac(6')-Ih* and *aac(6')-Ij*, from these clinical isolates.

MATERIALS AND METHODS

Bacterial strains and plasmids. Certain strains and plasmids used are listed in Table 1. *A. baumannii* BM2686 and *Acinetobacter* sp. 13 BM2689 were isolated in 1989 at the Saint Michel Hospital in Paris, France, from a human urine sample and a venous catheter, respectively. Strains 82, 134, 378, 496, 552, 943, 1001, 1158, and 1191 were previously assigned to *Acinetobacter* sp. 13 by DNA-DNA hybridization (2), three additional strains biochemically assigned to this species were from the collection of the Unité des Entérobactéries (Institut Pasteur, Paris), and 15 strains were from the Universität des Saarlandes (30). A total of 32 amikacin-resistant *A. baumannii* clinical isolates (MICs of ≥32 μg/ml), including epidemiologically unrelated strains BM2703, BM2704, and BM2705, which dis-

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TABLE 1. Properties of certain strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
E. coli		
JM83	*ara*Δ(*lac* mutant *proAB*) *rpsL* [Φ80Δ(*lacZ*)*M15*]	33
HB101	*hds* mutant *recA13 ara-14 proA2 lacYI galK2 rpsL20* (*Str*) *xyl-5 mtl-1 supE44*	3
K802N	*hdsR hdsM*$^+$ *gal met supE rpoB nalA*	32
A. calcoaceticus BD413	*trpE27*	11
A. baumannii		
BM2582	Nal^r Rif^r	13
BM2686	Ak^r Km^r Nt^r Tm^r [*aac(6′)-Ih*]	Wild-type strain
Acinetobacter sp. 13 BM2689	Ak^r Km^r Nt^r Tm^r [*aac(6′)-Ij*]	Wild-type strain
Plasmids		
pUC18/19	Tra$^-$ Mob$^-$ Ap^r	33
pKK223-3	Tra$^-$ Mob$^-$ Ap^r	4
pIP1858	Tra$^-$ Ak^r Km^r Nt^r Tm^r [*aac(6′)-Ih*]	From BM2686
pAT479	Tra$^-$ Mob$^-$ Ak^r Ap^r Km^r Nt^r Tm^r [*aac(6′)-Ih*]	pUC18Ω *Hin*dIII 2.3 kb from pIP1858
pAT480	Tra$^-$ Mob$^-$ Ak^r Ap^r Km^r Nt^r Tm^r [*aac(6′)-Ih*]	pKK223-3Ω *Ssp*I-*Dde*I 433 bp from pAT479
pAT481	Tra$^-$ Mob$^-$ Ak^r Ap^r Km^r Nt^r Tm^r [*aac(6′)-Ij*]	pUC19Ω *Pvu*II 2.9 kb from BM2689

[a] Abbreviations: Tra$^+$, self-transferable; Tra$^-$, non-self-transferable; Mob$^-$, nonmobilizable; Ak, amikacin resistance; Ap, ampicillin resistance; Km, kanamycin resistance, Nal, nalidixic acid resistance; Nt, netilmicin resistance; Rif, rifampin resistance; Str, streptomycin resistance; Tm, tobramycin resistance.

played a phenotype compatible with the synthesis of an AAC(6′)-I and did not contain characterized *aac(6′)-I* genes, and 108 *A. baumannii* isolates susceptible to aminoglycosides were from our laboratory collection. Strains 33, 46, 382, 79, 1011, and 942 assigned to species 4, 6, 14, 15, 16, and 17, respectively, and ungrouped strains 80, 631, 640, 930, 944, 1240, and 1271 (2) were from the collection of the Unité des Entérobactéries.

Strain identification and growth conditions. The strains had biochemical properties identical to those which define the *Acinetobacter* genus (1), with identification at the genus level confirmed by the transformation assay (11). Presumptive identification was performed with API 20E strips, and definitive identification was achieved with the carbon source utilization test (1). Hemolysis was recorded after 2 days of growth on tryptocasein soy agar containing 5% (vol/vol) horse blood. Strains were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) or on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). Mueller-Hinton medium supplemented with 0.005% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (IPTG) was used to detect the production of β-galactosidase. Antibiotic susceptibility was determined by disk diffusion on Mueller-Hinton agar. The method of Steers et al. (27), with 10^4 CFU per spot, was used to determine the MICs of aminoglycosides. Incubations were done at 30°C.

Assay for aminoglycoside-modifying enzymes. Activity of the aminoglycoside-modifying enzymes was detected in bacterial extracts by the phosphocellulose paper-binding technique (8). The final concentration of aminoglycoside in the assay mixture was 66.7 μg/ml, and the reaction was allowed to proceed for 30 min at 30°C.

Genetic techniques. Conjugation into *Escherichia coli* K802N (20), transformation of *E. coli* HB101 and JM83 (20) and *A. calcoaceticus* BD413 (14), and curing of antibiotic resistance traits with ethidium bromide (14) were performed as described previously. The following antibiotics were used for selection of transcipients at the concentrations indicated: amikacin, 10 μg/ml; ampicillin, 100 μg/ml; nalidixic acid, 50 μg/ml; and tobramycin, 10 μg/ml.

Preparation and analysis of DNA. Total DNA (19) and small- and large-scale (20) preparations of plasmid DNA have been described previously. Electrophoresis was performed in 0.8% agarose gels (Sigma Chemical Co., St. Louis, Mo.) with a Tris-borate buffer system.

DNA techniques. For dot blot and Southern hybridization, DNA was immobilized on Nytran membranes (Schleicher & Schuell, Dassel, Germany). 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) containing 0.5% sodium dodecyl sulfate (SDS) and 0.05% nonfat dry milk (10). The 188-bp *Hin*dIII-*Ava*I, 172-bp *Ssp*I-*Hin*dIII, and 177-bp *Ssp*I-*Cla*I fragments internal to the *aac(6′)-Ig*, *aac(6′)-Ih*, and *aac(6′)-Ij* genes, respectively, were purified by electrophoresis in low-temperature-gelling agarose type VII (Sigma Chemical Co.), extracted (20), and radiolabeled by nick translation (20). The *aac(6′)-Ij* gene was detected after amplification by PCR of a 768-bp fragment with primers 5′-CTCTCGGACCCATGCAGT-3′ and 5′-GATGTTAAATT TAGCTT-3′ as described previously (20).

DNA sequencing. Fragments of the *aac(6′)-I* genes and their flanking regions were cloned into pUC vectors (33). Double-stranded sequencing was carried out by using synthetic oligonucleotides (Unité de Chimie Organique, Institut Pasteur). Sequencing reactions were performed by the dideoxynucleotide chain terminator technique (21). DNA fragments were resolved by electrophoresis on 8% vertical polyacrylamide gels containing 8 M urea.

Computer analysis of sequence data. Nucleotide and amino acid sequences were analyzed and compared by use of GenBank, EMBL and Swiss-Prot databases with Genetics Computer Group software (6) and with the FASTA program (17).

Analysis of plasmid-encoded proteins. The proteins specified by the recombinant plasmids were synthesized in a coupled in vitro transcription-translation system (34). Proteins were labeled with L-[^{35}S]methionine and processed for electro-

TABLE 2. MICs of various aminoglycosides against *Acinetobacter* strains BM2686 and BM2689 and *E. coli* JM83 with and without *aac(6')-I* genes

Strain	aac(6') gene	MIC (μg/ml)			
		Amikacin	Gentamicin	Netilmicin	Tobramycin
A. baumannii BM2686	Ih	32	1	128	4
E. coli JM83		0.5	0.5	0.25	0.5
E. coli JM83(pAT479)	Ih	128	2	256	64
E. coli JM83(pAT480)	Ih	32	0.5	32	16
Acinetobacter sp. 13 BM2589	Ij	4	1	16	8
E. coli JM83(pAT481)	Ij	8	1	32	16

phoresis in an SDS-polyacrylamide gel as described previously (20).

Enzymes and chemicals. T4 DNA ligase, T4 DNA polymerase (for filling in cohesive ends), restriction endonucleases (Amersham, Buckinghamshire, England), and the Sequenase version 2.0 DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio) were used according to the recommendations of the manufacturers. Lysozyme was obtained from Sigma Chemical Co., and RNaseA (bovine pancreas) was obtained from Calbiochem-Behring (La Jolla, Calif.). Nick translation kits were from Bethesda Research Laboratories Inc. (Gaithersburg, Md.); [α-^{32}P]ATP (triethylammonium salt), [α-^{32}P]dCTP, [1-^{14}C]acetyl coenzyme A, [γ-^{32}P]ATP (triethylammonium salt), α-^{35}S-dATP (400 Ci/mmol), and L-[^{35}S]methionine were obtained from the Radiochemical Centre (Amersham); *Taq* DNA polymerase was purchased from Perkin-Elmer Cetus (Norwalk, Conn.). The following antibiotics were provided by the laboratories indicated: amikacin, ampicillin, and kanamycin B, Bristol-Myers Squibb (Princeton, N.J.); polymyxin B, Burroughs Wellcome Co. (Research Triangle Park, N.C.); tobramycin, Eli Lilly & Co. (Indianapolis, Ind.); rifampin, Merrell Dow (Winnersh, United Kingdom); gentamicin, gentamicins C1a, C1, and C2, isepamicin, netilmicin, 2'-*N*-ethylnetilmicin, and 6'-*N*-ethylnetilmicin, Schering-Plough Research Institute (Kenilworth, N.J.); and nalidixic acid, Sterling Winthrop (New York, N.Y.).

Nucleotide sequence accession number. The nucleotide sequences of the *aac(6')-Ih* and *-Ij* genes have been deposited in the GenBank data library (Los Alamos, N.Mex.) under accession no. L29044 and L29045, respectively.

RESULTS AND DISCUSSION

Identification of *Acinetobacter* strains. The 32 strains identified as *A. baumannii* grew at 44°C, did not produce hemolysis on horse blood agar after 24 h at 30°C, and did not hydrolyze gelatin. The 28 strains identified as *Acinetobacter* sp. 13 did not grow at 44°C; produced a clear hemolysis on horse blood agar after 24 h at 37°C; hydrolyzed gelatin; grew on DL-lactate, L-phenylalanine, and phenylacetate, and except for six strains, failed to grow on ethanol. All strains identified as *Acinetobacter* sp. 13 were resistant to polymyxin B (MIC for 50% of the strains, 8 μg/ml; range, 4 to 256 μg/ml) whereas *Acinetobacter* strains from other species were inhibited by 2 μg/ml.

Aminoglycoside resistance of *A. baumannii* BM2686 and *Acinetobacter* sp. 13 BM2689. Disk susceptibility tests indicated that the activity of 2'-*N*-ethylnetilmicin was diminished, compared with that of 6'-*N*-ethylnetilmicin, against the 32 amikacin-resistant *A. baumannii* strains and for 23 of the 28 *Acinetobacter* sp. 13 strains. These two compounds have similar intrinsic activities against aminoglycoside-susceptible strains, and the difference observed can be taken as evidence for production of a 6'-N-acetylating enzyme (24). Consistent with this notion, netilmicin and tobramycin were less efficient than gentamicin against these strains. The MICs (range) of amikacin, gentamicin, netilmicin, and tobramycin for 50% of the 28 isolates of *Acinetobacter* sp. 13 were 8 μg/ml (4 to ≥256 μg/ml), 2 μg/ml (1 to ≥256 μg/ml), 16 μg/ml (2 to ≥256 μg/ml), and 8 μg/ml (2 to ≥256 μg/ml), respectively. Two of these strains, *A. baumannii* BM2686 and *Acinetobacter* sp. 13 BM2689, were selected for further studies. MICs of aminoglycosides for BM2686 and BM2689 are shown in Table 2. Amikacin was less active against these strains than against susceptible strains of *A. baumannii* (MIC for 90% of strains tested, 2 μg/ml).

Aminoglycoside-modifying enzymes in *A. baumannii* BM2686 and *Acinetobacter* sp. 13 BM2689. Extracts of BM2686 and BM2689 were shown to contain aminoglycoside acetyltransferase activity but no phosphotransferase or nucleotidyltransferase activity. Since gentamicins C1a and C2 and 2'-*N*-ethylnetilmicin were modified whereas gentamicin C1 and 6'-*N*-ethylnetilmicin were not, the 6' amino group appears to be the site of acetylation. Amikacin, kanamycin B, isepamicin, tobramycin, and netilmicin, which have a free amino group at the 6' position, were acetylated by the enzymes of BM2686 and BM2689 (data not shown). The two enzymatic profiles were consistent with the presence of AAC(6')-I activities (23). No hybridization was detected between total DNA of BM2686 and BM2689 and probes specific for the *aac(6')-Ia*, *-Ib*, *-Ic*, *-Ie*, *-If*, and *-Ig* genes, under the stringent hybridization conditions described in Materials and Methods.

Cloning of amikacin resistance genes. Plasmid DNA from BM2686 and pUC19 DNA digested with *Hin*dIII were mixed, ligated, and introduced by transformation into *E. coli* JM83. Transformants selected on medium containing ampicillin plus amikacin were screened for their plasmid content by agarose gel electrophoresis of crude bacterial lysates. The smallest recombinant plasmid, pAT479, contained two *Hin*dIII fragments with lengths of 1.2 and 1.1 kb and conferred amikacin resistance on the host by synthesis of an AAC(6')-I. Subcloning experiments indicated that neither of the two fragments conferred amikacin resistance.

Total DNA of BM2689 digested with *Pvu*II and pUC19 DNA linearized by *Sma*I were mixed, ligated, and introduced by transformation into *E. coli* JM83. The smallest recombinant plasmid conferring amikacin resistance, pAT481, contained a 2.9-kb *Pvu*II fragment encoding an AAC(6')-I enzyme. Digestion of pAT481 by *Hin*dIII generated fragments with lengths of 1.9 and 3.7 kb. The 3.7-kb fragment was self ligated, whereas the 1.9-kb fragment was ligated with pUC19 linearized by *Hin*dIII. The resulting plasmids did not confer resistance to amikacin.

Nucleotide sequence of *aac(6')-I* genes and adjacent regions. The sequence of the 1,049-bp fragment adjacent to the 2.3-kb insert of pAT479, derived from the plasmid pIP1858 of

FIG. 1. Comparative analysis of the nucleotide and deduced amino acid sequences of 1,049-bp fragment of pIP1858 containing *aac(6')-Ih* and 773-bp fragment of BM2689 containing *aac(6')-Ij*. The −35 and −10 regions of the proposed promoters are underlined; start codons are indicated in boldface characters; TAA and TGA stop codons are indicated by an asterisk; the putative ribosome binding sites are doubly underlined; restriction sites are underlined with wavy lines. The deduced amino acid sequence of AAC(6')-Ih from pIP1858 is in capital letters. The alternative predicted amino acids from BM2689 are indicated below the corresponding nucleotide sequence.

BM2686, was determined (Fig. 1). The sequenced portion includes 527 bp upstream from the *Hin*dIII site of *aac(6')-Ih* located in the 1.2-kb *Hin*dIII fragment and 522 adjacent bp in the 1.1-kb *Hin*dIII fragment (Fig. 1). A search for stop codons in the three reading frames of each DNA strand identified an open reading frame spanning 519 nucleotides located between the TAA codons at coordinates 268 and 790. Two potential ATG start codons were located at positions 352 and 361. Only the first ATG was preceded by a ribosome binding site-like sequence (GTAG) complementary (underlined) to 4 bases of the 3′-OH terminus of *E. coli* 16S rRNA (5′-GAUCACCUC CUUA-3′). This ribosome binding site was located 7 bp upstream from the translation initiation codon. Analysis of the adjacent upstream region showed three potential −35 and −10 promoter sequences (9): TTGCTA (N-17) TATTAT at positions 232 to 260, TTGATT (N-17) TTTAAT at positions 243 to 271, and TTGTAT (N-18) TTTTGT at positions 252 to 281. Interestingly, plasmid pAT480 constructed by cloning the 433-bp *Ssp*I-*Dde*I fragment at positions 355 to 789 in expression vector pKK223-3, which contains the strong *tac* promoter regulated by the *lac* repressor, conferred aminoglycoside resistance to *E. coli* JM83. This result shows that the three N-terminal amino acids of the protein deduced from the first ATG at position 352 are not required for resistance.

The sequence of 773 adjacent bp of the insert in pAT481 was determined on both strands from each side of the *Hin*dIII site (Fig. 1). A search for stop codons in the three reading frames of each DNA strand identified an open reading frame spanning 495 nucleotides located between the TAA and TGA codons at coordinates 292 and 790, respectively. Three potential ATG start codons were located at positions 325, 352, and 361. Only the second ATG was preceded by a ribosome binding site-like sequence (GAG). This ribosome binding site was located 9 bp upstream from the translation initiation codon. Analysis of the adjacent region showed potential −35 and −10 promoter sequences [TTGGAG (N-15) TATGAT] at positions 255 to 291.

In these open reading frames, the coding sequences starting at position 352 and extending to the termination codons at position 790 could direct synthesis of proteins of 146 amino acids with molecular weights (M_rs) of 16,698 and 16,677, designated AAC(6')-Ih and -Ij, respectively. The base composition (percent G+C) of *aac(6')-Ih* (41.5%) and *aac(6')-Ij* (43.5%) was similar to that of the *Acinetobacter* chromosome (40 to 43%) (1), suggesting that these genes are indigenous to this genus.

The alignment of *aac(6')-Ih* and *-Ij* showed 78% identity (Fig. 1). Comparison of the flanking regions indicated that a stretch of 46 bp upstream from the initiation codons was highly conserved (74% identity) and that the 73-bp sequenced fragment downstream from the termination codons was also conserved (59% identity). By contrast, the alignment of these sequences with *aac(6')-Ig* from *A. haemolyticus* showed that despite the high degree of similarity between *aac(6')-Ig* and *aac(6')-Ih* (71% identity), or *aac(6')-Ig* and *aac(6')-Ij* (70% identity), the flanking regions of *aac(6')-Ig* did not display significant homology with the corresponding regions of *aac(6')-Ih* and *-Ij* (data not shown).

Analysis of AAC(6')-Ih and -Ij proteins. The proteins specified by plasmids pUC18, pAT479, and pAT481 were characterized in an *E. coli* in vitro-coupled transcription-translation system. A band with a molecular weight of approximately 16,000 which should correspond to the resistance proteins was encoded by the recombinant plasmids but not by the vector (data not shown). This apparent M_r was in good agreement with those of 16,698 and 16,677 calculated from the predicted amino acid sequences and was closely related to those of the AAC(6')-Ic, -If, and -Ig proteins. Computer analysis indicated

TABLE 3. Sequence identity between the deduced amino acid sequences of 6'-*N*-acetyltransferases[a]

Gene	% Identity to:				
	aac(6')-Id	*aac(6')-If*	*aac(6')-Ig*	*aac(6')-Ih*	*aac(6')-Ij*
aac(6')-Ic	45.4	44.8	40.5	39.0	40.4
aac(6')-Id		45	47.6	47.6	45.0
aac(6')-If			47	45.9	43.2
aac(6')-Ig				69.2	69.2
aac(6')-Ih					82.2

[a] Identity between pairs of sequences was derived from the alignment in Fig. 2. The genes are *aac(6')-Ic* from *S. marcescens* (24), *aac(6')-Id* from *K. pneumoniae* (22), *aac(6')-If* from *Enterobacter cloacae* (28), and *aac(6')-Ig* from *A. haemolyticus* (14).

homology between proteins AAC(6')-Ih and -Ij and among the AAC(6')-Ic, -Id, -If, and -Ig enzymes (Table 3). The phylogenetic relationship of AAC(6') enzymes has been studied, and three subfamilies were distinguished on the basis of amino acid similarity (23). The first subfamily comprises AAC(6')-Ia from *Citrobacter diversus* and AAC(6')-Ii from *E. faecium* (5, 23); the second and largest consists of AAC(6')-Ib from *Serratia marcescens*, AAC(6')-IIa and -IIb from *Pseudomonas aeruginosa*, and AAC(6')-Ie, the amino-terminal portion of the AAC(6')-APH(2") bifunctional enzyme from gram-positive cocci. The third subfamily includes AAC(6')-Ic from *S. marcescens*, AAC(6')-Id from *Klebsiella pneumoniae*, AAC(6')-If from *Enterobacter cloacae*, and AAC(6')-Ig from *A. haemolyticus*, which exhibit more than 62% similarity (14). AAC(6')-Ih and Ij were found to be members of the latter subfamily (Fig. 2). The *aac(6')-Ic*, *-Ig*, and *-Ii* genes are part of the host chromosome and are species specific (5, 14, 24, 26).

Genetic location of the *aac(6')-Ih* and *-Ij* genes. All attempts to transfer amikacin resistance by conjugation from *A. baumannii* BM2686 to *A. baumannii* BM2582 and *E. coli* K802N

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AAC(6')-Ic      MIVICDHDNLDAWLALRTALWPSGSPEDHRAEMREILASPHITA--FMARGLDGA  53
AAC(6')-Id      MIEACHSVECPGWLQLRFLLWPQDSADEHLAEMAIFVAEPNRFA-QFIAYDEANK  54
AAC(6')-If      MDEASLSM----WVGLRSQLWPDHSYEDHILDSQHILSCPDKYV-SFLAINNQSQ  50
AAC(6')-Ig  MNIKPASEASLKD----WLELRNKLWSDS-EASHLQEMHQLLAE--KYALQLLAYSD-HQ  52
AAC(6')-Ih  MNIMPISESQLSD----WLALRCLLWPDH-EDVHLQEMRQLITQ--AIRLQLLAYTDTQQ  53
AAC(6')-Ij  MNIMPVSESLMAD----WLGLRKLLWPDH-DEAHLQEMQRLLQQ--TQSLQLLAYSDTQQ  53
                + ++  +     ++ ++ +++++++ + +++++  +++ +   + +++++ +  +

AAC(6')-Ic  FVAFAEVALRYDYVNGCESSPVAFLEGIYTAERARRQGWAARLIAQVQEWAKQQGCSELA  113
AAC(6')-Id  PLGFVEAALRSDYVNGTNSSPVAFLEGVYVLPEARRRGIAHALVGAVEIWARNRACTEFA  114
AAC(6')-If  AIAFADAAVRHDYVNGCESSPVVYLEGIFVIPEQRGHGVAKLLVAAVQLWGVAKGCTEMA  110
AAC(6')-Ig  AIAMLEASIRFEYVNGTETSPVGFLEGIYVLPAHRRSGVATMLIRQAEVWAKQFSCTEFA  112
AAC(6')-Ih  AIAMLEASIRYEYVNGTQTSPVAFLEGIFVLPEYRRSGIATGLVQQVEIWAKQFACTEFA  113
AAC(6')-Ij  AIAMLEASIRYEYVNGTQTSPVAFLEGIYVLPDYRRSGIATHLVQQVEAWAKPFGCIEFA  113
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AAC(6')-Ic  SDTDIANLDSQRLHAALGFAETERVVFYRKTLG                          146
AAC(6')-Id  SDASTDNPESHRFHQSLGFKETERVVYFRKHLAPE                        149
AAC(6')-If  SDAALDNHISYQMHQALGFEETERVVFFRKRIAG                         144
AAC(6')-Ig  SDAALDNVISHAMHRSLGFQETEKVVYFSKKID                          145
AAC(6')-Ih  SDAALDNQISHAMHQALGFHETERVVYFKKNIG                          146
AAC(6')-Ij  SDAALDNRISHAMHQALGFHETERVVYFKKHIG                          146
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FIG. 2. Alignment of deduced amino acid sequences of AAC(6') enzymes. Sequences are those of the AAC(6')-Ic from *S. marcescens* (24), AAC(6')-Id from *K. pneumoniae* (22), AAC(6')-If from *Enterobacter cloacae* (28), and AAC(6')-Ig from *A. haemolyticus* (14). Dashes represent gaps introduced to optimize similarity. •, identical amino acid in six sequences; +, identical amino acid in at least three sequences. Conserved motifs are underlined.

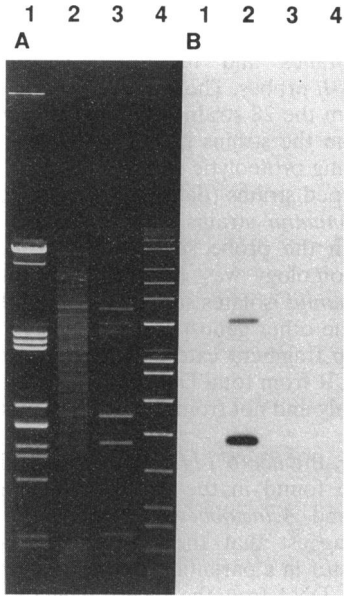

FIG. 3. Analysis of total and plasmid DNA of *Acinetobacter* sp. 13 BM2689 by agarose gel electrophoresis (A) and hybridization (B). DNA was digested with *Hin*dIII, and the resulting fragments were separated by electrophoresis in a 0.8% agarose gel, transferred to a Nytran filter, and hybridized to the in vitro ^{32}P-labeled *aac(6')-Ij* probe. Lanes: 1, bacteriophage λ DNA digested by *Pst*I was used as an internal standard; 2, total DNA of *Acinetobacter* sp. 13 BM2689 digested with *Hin*dIII; 3, plasmid DNA of BM2689; 4, size marker Raoul (Appligene).

were unsuccessful. Amikacin resistant clones of *A. calcoaceticus* BD413 were obtained after transformation with BM2686 plasmid DNA, whereas transformation of *E. coli* HB101 failed. Taken together, these data indicate that amikacin resistance in BM2686 was mediated by a nonconjugative plasmid that did not replicate in *E. coli*. Analysis by agarose gel electrophoresis of plasmid DNA from BM2686 indicated that this strain harbored two plasmids with sizes of 17.5 and 13.7 kb, respectively (data not shown). Digestion by *Hin*dIII of the 13.7-kb plasmid, designated pIP1858, generated eight fragments with sizes of 3.7, 2.2, 2.1, 1.5, 1.4, 1.1, 0.9, and 0.8 kb, which were also present in *A. baumannii* BM2703, BM2704, and BM2705 and *A. calcoaceticus* amikacin-resistant transformants (data not shown). Southern hybridization with the 173-bp *Ssp*I-*Hin*dIII fragment internal to *aac(6')-Ih* showed that this portion of the gene was part of the 1.4-kb fragment of pIP1858 (data not shown).

Attempts to transfer amikacin resistance by conjugation from *Acinetobacter* sp. 13 BM2689 to *A. baumannii* BM2582 and *E. coli* K802N were unsuccessful. Transformation of *A. calcoaceticus* BD413 with plasmid or total DNA from BM2689 with selection for amikacin resistance was also unsuccessful. No loss of amikacin resistance from BM2689 was obtained after treatment with ethidium bromide. Total and plasmid DNA prepared from this strain was digested with *Hin*dIII. The resulting fragments were separated by agarose gel electrophoresis, transferred to a filter, and hybridized with the 177-bp *Ssp*I-*Cla*I fragment internal to the *aac(6')-Ij*. The probe which had a *Hin*dIII site hybridized with the 2.4- and 6.5-kb fragments from the total DNA preparation whereas no hybridization was observed with digested plasmid DNA (Fig. 3). On the basis of these observations, the *aac(6')-Ij* gene was tentatively assigned to a chromosomal location.

Distribution of the *aac(6')-Ij* gene in *Acinetobacter* strains. Total DNA from 180 *Acinetobacter* strains was spotted on Nytran membranes and hybridized with the intragenic *aac(6')-Ih* and *-Ij* probes. The *aac(6')-Ij* probe hybridized with total DNA from the 28 strains of *Acinetobacter* sp. 13 but not with DNA from the strains belonging to other *Acinetobacter* species, including proteolytic genospecies 4, 6, 14, 15, 16, and 17 and ungrouped strains (data not shown). Total DNA from the 32 *A. baumannii* strains with an AAC(6')-I phenotype hybridized with the probe specific for the *aac(6')-Ih* gene, whereas no homology was detected with total DNA from either *A. baumannii* isolates susceptible to amikacin or strains belonging to the other genospecies. Consistent with this finding, the 768-bp fragment external to the *aac(6')-Ij* gene was detected by PCR from total DNA of the 28 strains of *Acinetobacter* sp. 13 only and not from the 152 remaining strains (data not shown).

Interestingly, the *aac(6')-Ig* and *-Ij* genes, which were 70% identical, were found in the closely related genospecies *A. haemolyticus* and *Acinetobacter* sp. 13 (2, 29), respectively. These data suggest that these genes may derive from a common ancestor in a parental *Acinetobacter* sp. According to this hypothesis, DNA from the ancestral *aac(6')* gene and from the parental *Acinetobacter* genome would have followed a similar evolutionary pathway. In this scheme, the presence on a plasmid of the *aac(6')-Ih* gene, which is closely related to *aac(6')-Ij* (78% identity), may be the result of a transposition event from the *Acinetobacter* chromosome into an indigenous replicon. It has been reported that plasmids from *Acinetobacter* spp. do not replicate in members of *Enterobacteriaceae* (12). This observation could account for the fact that the *aac(6')-Ih* gene is confined so far to *Acinetobacter* spp.

Because of lack of specificity of biochemical characteristics, genospecies 13 to 17 of proteolytic *Acinetobacter* were not named. Growth on Mueller-Hinton agar containing 2 μg of polymyxin B per ml, which is unusual for members of the *Acinetobacter* genus (30, 31), and detection of the *aac(6')-Ij* gene either by hybridization under stringent conditions or by PCR with specific primers may be convenient tools to delineate *Acinetobacter* sp. 13. The *aac(6')-Ij* gene represents an additional example of a species-specific aminoglycoside resistance gene (5, 14, 18, 24, 26). The biological significance of these genes remains unknown. It has been proposed that they detoxify the antibiotic in the aminoglycoside-producing microorganisms (23) or that they are involved in cellular metabolism. Recently, peptidoglycan, which has structural similarity with aminoglycosides, has been suggested as the possible original substrate for these enzymes (18). The various levels of aminoglycoside resistance in strains of *Acinetobacter* sp. 13 and the susceptibility of certain isolates in which the *aac(6')-Ij* gene was detected by hybridization or by PCR suggest a genetic control of enzyme synthesis. A regulation mechanism has recently been demonstrated for production of AAC(2')-Ia in *Providencia stuartii* (18) and AAC(6')-Ic in *S. marcescens* (24). A putative control of AAC(6')-Ij production in *Acinetobacter* sp. 13 remains to be studied.

ACKNOWLEDGMENTS

We thank P. Bouvet and W. Traub for the gifts of strains and M. Arthur for help with the writing of the manuscript.

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