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Cloning and Characterization of a 3-*N*-Aminoglycoside Acetyltransferase Gene, *aac(3)-Ib*, from *Pseudomonas aeruginosa*

LEE R. SCHWOCHO,[1] CARL P. SCHAFFNER,[2] GEORGE H. MILLER,[1] ROBERTA S. HARE,[1] AND KAREN J. SHAW[1]*

Schering-Plough Research Institute, Kenilworth, New Jersey 07033,[1] and Waksman Institute, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08855[2]

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A novel gene encoding an aminoglycoside 3-*N*-acetyltransferase, which confers resistance to gentamicin, astromicin, and sisomicin, was cloned from *Pseudomonas aeruginosa* Stone 130. Its sequence was determined and found to show considerable similarity to an *aac(3)-I* gene previously cloned from R plasmids from *Enterobacter*, *Pseudomonas*, and *Serratia* spp. We have designated the genes from the R plasmids and this work *aac(3)-Ia* and *aac(3)-Ib*, respectively. The two *aac(3)-I* genes share 74% nucleotide identity, and their deduced protein products are 88% similar. These data suggest that the genes derive from a common ancestor. Homology between the flanking sequences of both *aac(3)-I* genes and other resistance determinants known to reside in integron environments was also observed. Intragenic probes specific for either *aac(3)-Ia* or *aac(3)-Ib* were used in hybridization studies with a series of gentamicin-, astromicin-, and sisomicin-resistant clinical isolates. Of 59 clinical isolates tested, no isolates hybridized with both probes, 30 (51%) hybridized with the *aac(3)-Ia* probe, 12 (20%) hybridized with the *aac(3)-Ib* probe, and 17 (29%) did not hybridize with either probe. These data suggest the existence of at least one other *aac(3)-I* gene.

The three mechanisms by which bacteria become resistant to aminoglycoside antibiotics are (i) target site modification within the bacterium, (ii) diminished uptake, and (iii) enzymatic modification of the drug. For aminoglycosides, enzymatic modification is the principle mechanism by which resistance is conferred. Phosphorylating (APH), adenylylating (ANT), and acetylating (AAC) enzymes compose the three classes of aminoglycoside-modifying enzymes (7, 22). The acetyltransferases are a particularly important class of resistance enzymes because of their ability to inactivate many of the medically useful aminoglycosides, such as gentamicin, tobramycin, amikacin, and netilmicin. Many bacterial aminoglycoside acetyltransferases from both human and animal isolates have been identified, and the sequences of the genes which encode these enzymes have been determined (30). At present, 10 different genes which encompass five distinct resistance patterns belonging to the AAC(3) family have been cloned (30). In addition, four other *aac(3)* genes have been cloned from actinomycete strains (17, 26, 27), but their complete aminoglycoside resistance profiles have not been determined and so they can not be further classified.

Acetylation of the 3-*N* position of gentamicin and sisomicin was first described by using a partially purified enzyme extract from *Pseudomonas aeruginosa* which expressed an AAC(3)-I resistance profile (2). These experiments showed that inactivation of the aminoglycoside occurred by specific acetylation of the 3-amino deoxystreptamine moiety. Additional work showed that astromicin was also a substrate of the AAC(3)-I enzyme (1).

Among aminoglycoside-resistant clinical isolates, 5 to 10% express the AAC(3)-I phenotype (9, 20, 31, 34). Whereas AAC(3)-I has a broad host distribution among members of the family *Enterobacteriaceae*, it appears to be especially prevalent in *Pseudomonas* and *Acinetobacter* strains (9, 20, 29, 31). In these studies, considerable geographical distribution was observed, including several hospitals in Chile, the United States, Argentina, central Europe, and southern Europe. In a study of 394 clinical strains expressing AAC(3)-I, 22% failed to hybridize with an intragenic *aac(3)-Ia*-specific probe, under hybridization conditions which would allow detection of approximately 80% DNA sequence identity (29). These data suggested the existence of a second gene capable of conferring an AAC(3)-I resistance phenotype. We report the cloning of a second gene, *aac(3)-Ib*, isolated from *P. aeruginosa*, which resides in an integron-related background similar to that of the *aac(3)-Ia* gene (34, 39).

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists bacterial strains and plasmids used in the cloning experiments. *P. aeruginosa* Stone 130 is a clinical pathogen first isolated in 1971 from a burn patient in Atlanta, Ga. (37). The pBluescript II plasmids are commercially available cloning vectors (Stratagene, La Jolla, Calif.). *Escherichia coli* DH5α (Bethesda Research Laboratories, Bethesda, Md.) was used as the transformation host and for propagation of plasmids. Strains used for hybridization studies were clinical isolates collected between 1987 and 1991 from the following countries: Argentina, Austria, Belgium, France, Germany, Greece, Italy, Singapore, Spain, the United States, and Venezuela. Strains were grown in LB Schering broth or agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.) with or without antibiotics. Ampicillin (Sigma Chemical Corp., St. Louis, Mo.) and gentamicin (Schering-Plough Research Institute, Kenilworth, N.J.) were used at 100 and 10 μg/ml, respectively. Strain identification was confirmed with the API 20E *Enterobacteriaceae* system (Analytab Products, Plainview, N.Y.).

DNA preparation and analysis. Total DNA was prepared from *P. aeruginosa* Stone 130 by lysozyme-detergent lysis of overnight cultures followed by RNase and proteinase K treatment, subsequent chlorpane (chloroform-phenol, 25:1) extraction, and ethanol precipitation. Alternatively, total DNA was prepared by overnight preparative cesium chloride ultracentrifugation (18) or by the G NOME Protocol (BIO 101, Inc., La Jolla, Calif.). Plasmid DNA was prepared from transformants by using Qiagen kits according to the manufacturer's instructions (Qiagen Inc., Studio City, Calif.). Restriction enzyme digests were per-

* Corresponding author. Mailing address: Schering-Plough Research Institute, 2015 Galloping Hill Rd. 4700, Kenilworth, NJ 07033. Phone: (908) 298-3154. Fax: (908) 298-3918. Electronic mail address: KAREN.SHAW@spcorp.com.

TABLE 1. Bacterial host strains and plasmids

Strain or plasmid	Relevant properties[a]	Origin or reference
Strains		
P. aeruginosa Stone 130	Gm^r $Astm^r$ $Siso^r$ Sm^r Su^r Tc^r	37
E. coli DH5α	Gm^s $Astm^s$ $Siso^s$	Bethesda Research Laboratories
Plasmids		
pBluescript II KS−	Ap^r colE1 *lacZ*	Stratagene
pBluescript II SK−	Ap^r colE1 *lacZ*	Stratagene
pSCH6001	5.0-kb *Sau*3AI fragment from Stone 130 cloned into pBluescript II SK−; Gm^r $Astm^r$ $Siso^r$ Ap^r	This work
pSCH6002	1.5-kb *Sau*3AI fragment from Stone 130 cloned into pBluescript II SK−; Gm^r $Astm^r$ $Siso^r$ Ap^r	This work
pSCH6003	5.0-kb fragment from pSCH6001 subcloned into pBluescript II KS−; Gm^r $Astm^r$ $Siso^r$ Ap^r	This work
pSCH6006	2.0-kb *Pst*I fragment of pSCH6001 subcloned into pBluescript II KS−; Gm^r $Astm^r$ $Siso^r$ Ap^r	This work
pSCH2006	307-bp *Ava*I-*Hin*dIII fragment internal to *aac(3)-Ia* subcloned into pBluescript II KS−	34

[a] Gm, gentamicin; Astm, astromicin; Siso, sisomicin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; Ap, ampicillin.

formed according to the manufacturer's recommendations (New England Biolabs, Beverly, Mass.). DNA was analyzed by agarose gel electrophoresis in Tris-borate-EDTA buffer.

Cloning strategy. Total DNA from *P. aeruginosa* Stone 130 was partially digested with *Sau*3AI. The partial digests were ligated into the *Bam*HI site of pBluescript II SK− by using T4 DNA ligase (New England Biolabs). After overnight incubation at 9°C, ligation mixtures were used to transform *E. coli* DH5α (4). Transformants containing inserts were identified as white colonies on LB Schering agar containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Stratagene) plus ampicillin and/or gentamicin. MIC or disk diffusion susceptibility testing was performed on transformants according to National Committee for Clinical Laboratory Standards guidelines (23).

DNA sequencing. Double-stranded sequencing was performed by the dideoxy method (28) using the Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio). KS and SK primers (Stratagene) were used to initiate sequencing, and subsequent reactions were primed with 17- to 19-base oligonucleotides synthetically prepared by Research Genetics (Huntsville, Ala.). The Genetics Computer Group (Madison, Wis.) Sequence Analysis Software Package, version 7.3, and the GenBank database (release 88, April 1995) were used. Alignment of nucleotide and protein sequences was performed with the Pileup Multiple Sequence Analysis Program software package of the University of Wisconsin Genetics Computer Group (8).

Hybridization studies. Both dot blot and Southern hybridizations were performed with GeneScreen Plus nylon membranes (NEN Research Products, Boston, Mass.) as previously described (29). Fragments were labeled with ^{32}P by using the Pharmacia (Piscataway, N.J.) oligonucleotide labeling kit according to the manufacturer's instructions. Unbound label was removed by column chromatography on prepackaged Sephadex G-50 columns (5 Prime→3 Prime, Inc., West Chester, Pa.). Probes consisted of a 226-bp intragenic *Sac*II-*Hin*dIII fragment of the *aac(3)-Ib* gene (this work) and a 307-bp intragenic *Ava*I-*Hin*dIII fragment of the *aac(3)-Ia* gene (34) which had been subcloned into pBluescript II KS−, resulting in pSCH2006 (28a).

PCR studies. Oligonucleotides were obtained from Research Genetics. PCRs were carried out in 100-μl volumes containing 10 μl of 10× Pfu buffer [200 mM Tris-Cl (pH 8.5), 100 mM $(NH_4)SO_4$, 20 mM $MgCl_2$, 1 mg of bovine serum albumin per ml, 1% Triton X-100]; 6 ng of each primer; 2 mM (each) dATP, dCTP, dGTP, and dTTP; 2.5 U of cloned Pfu DNA polymerase enzyme (Stratagene); and 2 ng of template DNA. The reaction mix was overlaid with mineral oil. Cycle times in the thermal cycler were as follows: 1 min of denaturation at 94°C, 2 min of annealing at 55°C, and 6 min of extension at 72°C for a total of 30 cycles. The amplification products were visualized by agarose gel electrophoresis.

Nucleotide sequence accession number. The nucleotide sequence presented in Fig. 1 appears in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession number L06157.

RESULTS

Cloning of the *aac(3)-Ib* gene. Two recombinant plasmids which conferred resistance to both ampicillin and gentamicin were obtained (see Materials and Methods). The plasmids contained 5.0- and 1.5-kb inserts in Bluescript II SK− and were designated pSCH6001 and pSCH6002, respectively (Table 1). The two inserts had overlapping restriction maps, and the corresponding *E. coli* transformants displayed a resistance profile (Gm^r $Siso^r$ $Astm^r$) similar to that of *P. aeruginosa* Stone 130 (Table 2). Initial studies using stringent hybridization conditions (29) demonstrated that the 307-bp intragenic *Ava*I-*Hin*dIII probe from the *aac(3)-Ia* gene did not hybridize with either of the two transformants or *P. aeruginosa* Stone 130 (data not shown).

Restriction analysis of pSCH6001 and pSCH6002 and Southern blotting to compare both plasmids with total DNA from *P. aeruginosa* Stone 130 showed that in addition to regions of overlap, both inserts contained chimeric sequences. These chimeric sequences likely resulted from the ligation of unrelated *Sau*3AI fragments to the common region of *P. aeruginosa* Stone 130, pSCH6001, and pSCH6002 (data not shown). Subcloning of a 2.0-kb *Pst*I fragment from pSCH6001 into the *Pst*I site of pBluescript II KS− generated plasmid pSCH6006 (Fig. 1; Table 1). Transformants harboring this plasmid displayed the same aminoglycoside susceptibility pattern as those harboring the original clone, pSCH6001 (Table 2), indicating that the *Pst*I fragment contained the putative *aac(3)-Ib* gene.

To determine whether expression of the putative *aac(3)-Ib*

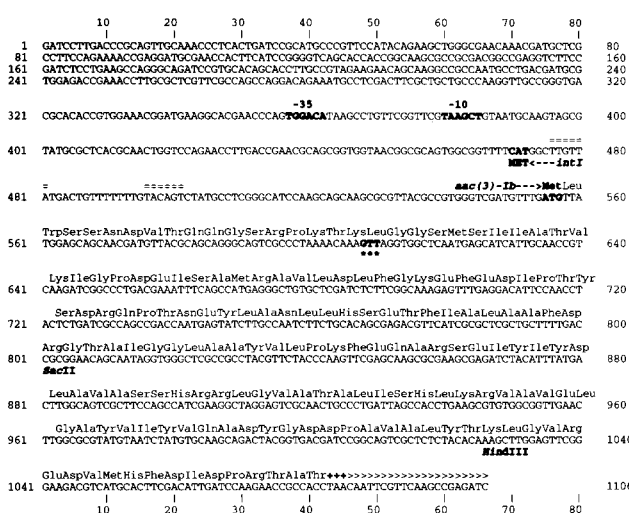

FIG. 1. DNA sequence of the 1,106-bp *Sau*3AI fragment from *P. aeruginosa* Stone 130. The predicted sequence of the AAC(3)-Ib protein is indicated above the sequence of the *aac(3)-Ib* open reading frame. <––, start sites of the *intI* and *aac(3)-Ib* genes; ======, a second potential promoter for *aac(3)-Ib*; +++, *aac(3)-Ib* stop codon; ***, integron recombination site; >>>>, homology with the consensus 59-base element.

TABLE 2. Susceptibilities of *P. aeruginosa* and *E. coli* strains to selected aminoglycosides

Organism	MIC (μg/ml) of[a]:								
	GM	ASTM	SISO	TOB	AMK	ISP	NTL	APRM	NEO
P. aeruginosa Stone 130	>64	>512	>64	1	4	4	8	16	64
E. coli									
DH5α	≤1	≤4	0.5	≤0.5	≤1	≤1	≤1	≤4	≤1
DH5α/pSCH6001	32	>512	64	≤0.5	≤1	≤1	≤1	≤4	≤1
DH5α/pSCH6002	>64	>512	>64	2	4	≤1	8	≤4	1
DH5α/pSCH6003	16	512	16	≤0.5	2	≤1	≤1	≤4	≤1
DH5α/pSCH6006	4	128	8	≤0.5	≤1	≤1	≤1	≤4	≤1

[a] GM, gentamicin; ASTM, astromicin; SISO, sisomicin; TOB, tobramycin; AMK, amikacin; ISP, isepamicin; NTL, netilmicin; APRM, apramycin; NEO, neomycin.

gene was mediated by its own promoter or by the *lacZ* promoter of the pBluescript II SK− vector, the insert from pSCH6001 was excised and religated into the companion vector pBluescript II KS−, in which the orientation of the multiple cloning sites is reversed with respect to the *lacZ* promoter. The reversed orientation of the insert in pSCH6003 was confirmed by restriction enzyme analysis (Table 1). Both pSCH6001 and pSCH6003 conferred similar aminoglycoside resistance levels, suggesting that the insert sequence contains a functional promoter (Table 2).

DNA sequence analysis. The nucleotide sequence of the 2.0-kb *Pst*I fragment present in pSCH6006 was determined. The analysis of this sequence confirmed that chimeric sequences were present on this clone and that the chimeric sequences began at *Sau*3AI sites (GATC), as predicted. The nonchimeric 1.1-kb portion of this sequence which encodes the *aac(3)-Ib* gene and flanking sequence is shown in Fig. 1. PCR analysis confirmed that the sequence presented in Fig. 1 corresponds to the DNA configuration present in *P. aeruginosa* Stone 130 (see below). A homology search of this sequence with the GenBank database identified an open reading frame from nucleotide 555 to 1082 with 74% identity to the *aac(3)-Ia* gene (*aacC1*) (34, 39). These data suggest that this open reading frame encodes the *aac(3)-Ib* gene and that the *aac(3)-Ia* and *aac(3)-Ib* genes derive from a common ancestor.

The GenBank search also showed that the region composed of residues 1 to 610 is nearly identical to the 5′ conserved element of an integron previously described (3, 11) (Fig. 1). This region encodes part of the integrase gene (*intI*), which is transcribed in the opposite direction to the putative *aac(3)-Ib* gene (Fig. 1). The *aac(3)-Ia* gene is one of the many resistance genes that have been shown to be present in an integron environment (32, 34, 39). The integron has been shown to catalyze the integration of resistance gene cassettes by site-specific recombination and to promote expression of these genes from a common promoter (*Pant*) in the 5′ conserved region (5, 32). The *Pant* promoter is observed between nucleotides 357 and 362 (TGGACA; −35 region) and 380 and 385 (TAAGCT; −10 region) (Fig. 1). Except for two nucleotide substitutions (G for C in the −35 region and G for A in the −10 region) this promoter matches the promoter described for the *aac(3)-Ia* gene (34, 39). This variant sequence has been observed previously and has been shown to have a 20-fold decrease in promoter strength relative to the TTGACA···TAAACT version (6, 16). A second potential promoter region, which extends between nucleotides 476 and 481 (TTGTTA; −35 region) and 496 and 501 (TACAGT; −10 region) and corresponds to the P2 promoter for *aac(6′)-Ia*, *oxaI*, and *ant(3″)-Ia* from R538-1, has been described previously (34). Since it lacks the GGG sequence abutting the −10 region and has a suboptimal 14-bp spacing between the −35 and −10 regions (Fig. 1), it is unlikely that this promoter is functional (16).

Figure 2 shows the alignment of the 5′ flanking sequences of the *aac(3)-Ia* and *aac(3)-Ib* genes, beginning at position 143 of the *aac(3)-Ia* gene (34) and position 498 of the *aac(3)-Ib* gene (Fig. 1). The initiation codon for the *aac(3)-Ia* gene is within a 19-bp direct repeat (34) (nucleotides 209 to 227 and 228 to 246) observed only once in the *aac(3)-Ib* sequence (Fig. 2). However, the first 5 nucleotides of this repeat is duplicated in both the *aac(3)-Ia* and *aac(3)-Ib* sequences. This region is part of the integron 5′ conserved sequence. In addition, the sequence G**ATG**TTA, which contains the initiation codon (boldface), is repeated three times in the *aac(3)-Ia* sequence and twice in the *aac(3)-Ib* sequence (Fig. 2). The unique recombination site into which many resistance genes have been inserted has been identified (24, 33, 38), and the crossover point for this recombination event has been localized to a GTT triplet (10). This triplet corresponds to nucleotides 272 to 274 of the *aac(3)-Ia* sequence and nucleotides 608 to 610 of the *aac(3)-Ib* sequence (Fig. 2).

Genes within an integron environment have been shown to have a conserved 3′ 59-bp element (3). The 3′ flanking sequence of the *aac(3)-Ib* gene has homology with the 59-bp element of the integron (3, 10) (Fig. 1).

PCR mapping of the *aac(3)-Ib* gene. In order to determine whether the *aac(3)-Ib* gene was present on an intact integron, several oligonucleotides were employed in PCR experiments utilizing *P. aeruginosa* Stone 130 DNA as a template. The oligonucleotides listed in Table 3 were used in the combinations shown in Fig. 3 to precisely map the environment of the

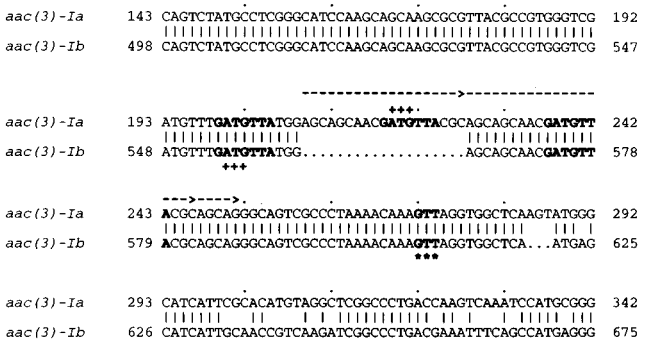

FIG. 2. Nucleotide sequence alignment of the 5′ flanking regions of the *aac(3)-Ia* and *aac(3)-Ib* genes showing the 19-bp direct repeat present in *aac(3)-Ia* (−−>) but absent in *aac(3)-Ib*. Symbols: ***, integron integration site; +++, initiation codon. The *aac(3)-Ia* sequence is numbered according to the system of Tenover et al. (34), and the *aac(3)-Ib* sequence is numbered according to Fig. 1.

TABLE 3. Oligonucleotides utilized in PCR analysis

Primer and source or characteristic	Nucleotide sequence (5′ to 3′)	Direction	Accession no.
A, *intI*	CTACCTCTCACTAGTGAGGGGCGG	→	X15852
B, *intI*	GCCCTTGCCCTCCCGCACCATG	→	X15852
C, *intI*	GCCTCGACTTCGCTGCTGCCC	→	X15852
D, *intI*	GGGCAGCAGCGAAGTCGAGGC	←	X15852
E, *aac(3)-Ib*	GCGGAACAGCAATAGGTGG	→	L06157
F, *aac(3)-Ib*	CCACCTATTGCTGTTCCGC	←	L06157
G, 59-bp box	AAGCCGGAGCGCTTTGCGGC	←	X15852
H, *qacEΔ1*	AAGCTTTTGCCCATGAAGCAACCA	←	X15370
I, *sulI*	GAATGCCGAACACCGTCACC	←	X15370
J, *glmS*-like	GCGCTTCTGCACCTGGCCGATG	→	L06157
K, 3′ flanking region	CATGCTCGAACAGCTCGGCG	←	L06157

gene. Primer A begins with the termination codon of the *intI* gene (antisense strand); primers B, C, and D are from sequences within the *intI* gene; primers E and F are complementary sequences from within the *aac(3)-Ib* gene; primer G is from the end and 3′ flanking sequence of the 59-bp box distal to the *aac(3)-Ia* gene; primer H begins at the second *Hin*dIII site within the *qacEΔ1* gene (antisense strand); and primer I is from the start of the *sulI* gene (antisense strand) (Table 3). Since a large portion of the *intI* gene was present on the *aac(3)-Ib* clone, it was possible to predict the exact sizes of the PCR products expected if the entire integrase gene was present upstream of the *aac(3′)-Ib* gene in *P. aeruginosa* Stone 130. Both the *sulI* gene (encoding resistance to sulfonamide) and *qacEΔ1* (encoding resistance to ethidium bromide and quaternary amines) are present downstream of many genes in an integron environment. Since *P. aeruginosa* Stone 130 is resistant to sulfonamide (15), primers H and I were used to test whether this arrangement was present distal to the *aac(3)-Ib* gene. The 17 primer combinations shown in Fig. 3B were utilized in PCRs, and all yielded PCR products of the predicted sizes upon amplification. Results of representative PCR amplifications are shown (Fig. 3A). The resulting structure of the *aac(3)-Ib* gene in *P. aeruginosa* Stone 130 as determined by PCR is shown in Fig. 3C. Primers specific to the sequences present on the 2.0-bp *Pst*I fragment of pSCH6006 which were predicted to be chimeric were also designed. These sequences included a region proximal to *aac(3)-Ib* on the *Pst*I fragment which had 60% nucleotide identity with the chromosomal *E. coli* glucosamine synthetase gene (*glmS*) (36) and a region distal to *aac(3)-Ib* on the *Pst*I fragment with no homology to any sequence in GenBank (Table 3). No PCR product was detected when these primers and the appropriate *aac(3)-Ib* primers (primer combinations J plus F and E plus K) were used in PCR experiments with *P. aeruginosa* Stone 130 DNA as a template (data not shown). These data are consistent with the conclusion that pSCH6006 contains chimeric sequences and Fig. 3C represents the true configuration of the *aac(3)-Ib* gene in *P. aeruginosa* Stone 130.

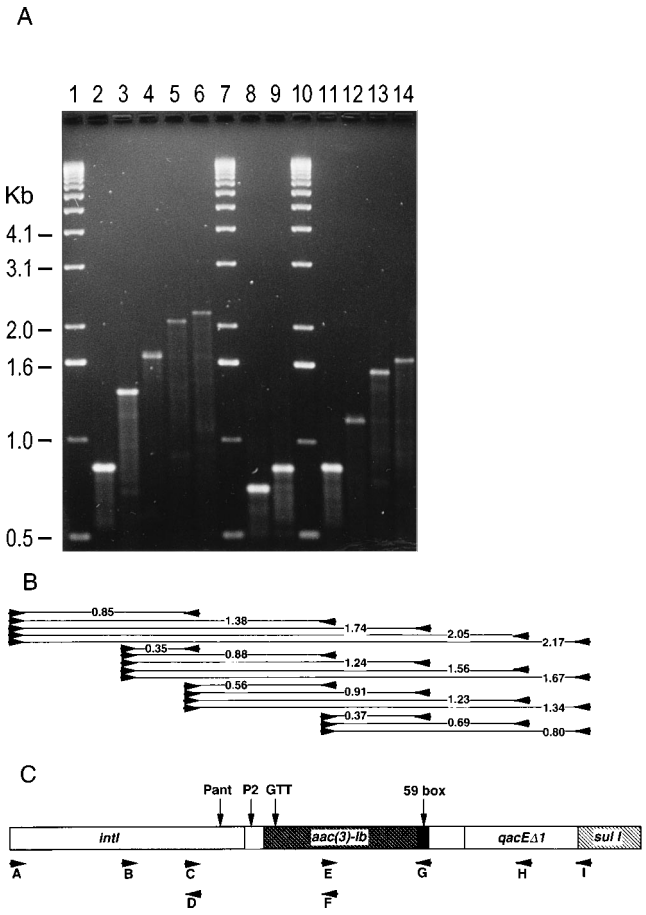

FIG. 3. PCR amplification of *P. aeruginosa* Stone 130 DNA using primers from *aac(3)-Ib* and the integron (Table 3). The PCR products were separated by 0.9% agarose gel electrophoresis. (A) Lane 1, size markers; lane 2, primers A and D; lane 3, primers A and F; lane 4, primers A and G; lane 5, primers A and H; lane 6, primers A and I; lane 7, size markers; lane 8, primers E and H; lane 9, primers E and I; lane 10, size markers; lane 11, primers B and F; lane 12, primers B and G; lane 13, primers B and H; lane 14, primers B and I. (B) Predicted sizes in kilobases of PCR products obtained by using 17 primer pairs. (C) Structure of the *aac(3)-Ib* gene and its environment in *P. aeruginosa* Stone 130.

Comparisons of the AAC(3)-Ia and AAC(3)-Ib proteins. The proteins encoded by at least two genes located in an integron environment have amino-terminal fusions to sequences encoded by the 5′ conserved region. The N-terminal amino acid sequence of AAC(3)-Ia has been determined (14), and the first 19 amino acids is encoded by integron DNA (Fig. 4). Similarly, the ANT(3″)-Ia protein may also contain an amino-terminal fusion (3, 13). If translation of the *aac(3)-Ib* gene begins at the corresponding integron-encoded ATG at positions 555 to 557 (Fig. 1), then the amino acid sequences of AAC(3)-Ia and AAC(3)-Ib would be 76% identical (Fig. 4). The two proteins would differ in length by a single amino acid: serine 22 of AAC(3)-Ia is absent in AAC(3)-Ib, resulting in proteins of 177 and 176 amino acids, respectively (Fig. 4). The predicted molecular mass of each protein is approximately 19 kDa.

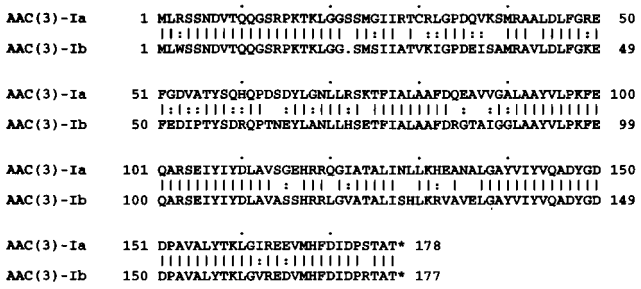

FIG. 4. Alignment of AAC(3)-Ia and AAC(3)-Ib deduced peptides. Vertical lines indicate identical amino acids; double dots indicate conserved amino acid substitutions in the similarity groups (C and Y; D and E; K and R; F, L, W, and Y; and I, L, V, and M).

Distribution of the *aac(3)-Ib* gene among clinical isolates. To determine the frequency and distribution of the *aac(3)-Ib* gene among clinical isolates, DNA hybridization studies were performed. Inspection of the DNA sequence showed unique *Hin*dIII and *Sac*I restriction sites generating a 231-bp fragment which was internal to the *aac(3)-Ib* gene (Fig. 1). Similarly, a 307-bp *Ava*I-*Hin*dIII fragment internal to the *aac(3)-Ia* gene was previously identified (34). These two DNA fragments were isolated and used as probes in hybridization studies of previously defined clinical isolates harboring the known resistance determinants *aac(3)-Ia*, *aac(3)-Ib*, *aac(3)-IIa*, *aac(3)-IIb*, *aac(3)-IIIa*, *aac(3)-IIIb*, *aac(3)-VIa*, *aac(6′)-Ia*, *aac(6′)-Ib*, *aac(6′)-Ic*, *aac(6′)-IIa*, *aac(6′)-IIb*, *aph(3′)-Ia*, *aph(3′)-IIa*, *ant(2″)-Ia*, *ant(3″)-Ia*, and *aac(6′)-aph(2″)* (30). Hybridization was obtained only with the strain from which the probe was derived (data not shown). In order to determine the relative frequency and distribution of each *aac(3)-I* gene, both probes were used in hybridization studies with a geographically diverse collection of 59 clinical isolates previously shown to express an AAC(3)-I resistance profile (Gm^r $Astm^r$) (21). Thirty (51%) of the 59 strains tested hybridized with the *aac(3)-Ia* probe, 12 (20%) hybridized with the *aac(3)-Ib* probe, 17 (29%) did not hybridize with either probe, and no clinical isolates hybridized with both probes (Table 4). Of the 12 strains that hybridized with the *aac(3)-Ib* probe, 11 were *Pseudomonas fluorescens* and 1 was *Acinetobacter calcoaceticus*. In contrast, the *aac(3)-Ia* probe hybridized with strains of the following organisms: *A. calcoaceticus* (seven strains), *Enterobacter* spp. (four strains), *E. coli* (one strain), *Klebsiella* spp. (three strains), *Morganella morganii* (one strain), *Proteus mirabilis* (three strains), *Providencia stuartii* (two strains), *Pseudomonas* spp. (seven strains), and *Serratia marcescens* (two strains) (Table 4). These data suggest that *aac(3)-Ia* is more widely distributed among different species than *aac(3)-Ib*. The 17 strains that did not hybridize with either probe consisted of nine *A. calcoaceticus* strains, six *P. fluorescens* strains, one *P. mirabilis* strain, and one *S. marcescens* strain. The lack of hybridization indicates that at least one other *aac(3)-I* gene is present in these strains.

TABLE 4. Hybridization of clinical isolates with the *aac(3)-Ia* and *aac(3)-Ib* probes

Bacterial species	No. of strains hybridizing with probe for:		Total no. of strains
	aac(3)-Ia	*aac(3)-Ib*	
A. calcoaceticus	7	1	17
Enterobacter aerogenes	1	0	1
Enterobacter cloacae	2	0	2
Enterobacter sakazakii	1	0	1
E. coli	1	0	1
Klebsiella pneumoniae	2	0	2
Klebsiella oxytoca	1	0	1
M. morganii	1	0	1
P. mirabilis	3	0	4
P. stuartii	2	0	2
P. fluorescens	5	11	22
Pseudomonas spp.	2	0	2
S. marcescens	2	0	3
Total	30	12	59

DNAs from six of the strains which hybridized with the *aac(3)-Ib* probe, including five *Pseudomonas* strains from the United States, Austria, and Belgium and one *A. calcoaceticus* strain from Italy, were used as a template in PCRs utilizing primer sets A plus F and E plus I (Table 3; Fig. 3C). The resulting amplification products spanned the *intI–aac(3)-Ib* and *aac(3)-Ib–sulI* regions, respectively. One *Pseudomonas* strain from Austria showed the same configuration as *P. aeruginosa* Stone 130. Two *Pseudomonas* strains from Austria and the *A. calcoaceticus* strain from Italy showed approximately 700-bp insertions with the *intI–aac(3)-Ib* primer set, suggesting that these strains contained other genes inserted proximally to *aac(3)-Ib* in the integron (data not shown). The other two strains tested showed patterns that were difficult to interpret, and this suggested possible rearrangements in *aac(3)-Ib* flanking sequences.

DISCUSSION

We have cloned and sequenced a novel 3-*N*-acetyltransferase gene, *aac(3)-Ib*, conferring resistance to gentamicin, sisomicin, and astromicin to the clinical pathogen *P. aeruginosa* Stone 130 (37). The DNA sequence of the *aac(3)-Ib* gene is 74% identical to that of another acetyltransferase gene, *aac(3)-Ia* (*aacC1*), which encodes an identical AAC(3)-I resistance profile. This gene was cloned from the R plasmids pJR88, R1033 (Tn*1696*), and pUO901 originating from *Enterobacter*, *Pseudomonas*, and *Serratia* species, respectively (34, 35, 39). The DNA sequences flanking the *aac(3)-Ia* and *aac(3)-Ib* genes are homologous to flanking regions of other resistance genes associated with an integron (3, 10, 32).

P. aeruginosa Stone 130 was previously shown to contain the plasmid pMG2, which encodes resistance to streptomycin, sulfonamide, and Hg^{2+}, in addition to gentamicin (15). In this study, experiments involving timed mating between *P. aeruginosa* Stone 130 and a susceptible recipient showed that resistances to streptomycin, sulfonamide, Hg^{2+}, and gentamicin were cotransferred. The PCR data presented here show that the *aac(3)-Ib* gene and the *sulI* gene, which encodes sulfonamide resistance, are closely linked on an integron element. Of six other clinical isolates examined which carried the *aac(3)-Ib* gene, at least four showed clear evidence of an intact integron environment containing both *intI* and *sulI*.

The *mer* gene, which encodes resistance to Hg^{2+}, has been frequently found on Tn*21*-like elements containing the integron (19). If pMG2 contains a Tn*21*-like element, *mer* may be located approximately 8 kb distal to *sulI* (19). Given that streptomycin resistance, commonly encoded by the *ant(3″)-Ia* (*aadA*) gene, is frequently found in an integron environment, it would not have been surprising to find this gene between *intI* and *sulI*. Since PCR experiments demonstrated that the order of the genes is *intI–aac(3)-Ib–qacEΔ1–sulI*, this is clearly not the case. However, prior mapping experiments (15) suggest

that the streptomycin resistance gene is likely to be nearby on the pMG2 plasmid.

The two AAC(3)-I proteins are 76% identical and 88% similar in amino acid composition. The AAC(3)-I enzymes are only approximately 49% similar to the other 3-*N*-acetyltransferases in the family. However, some conservation of residues which are invariant among the other AAC(3) enzymes is observed in the AAC(3)-I proteins (30). This information may be important for assigning potential functional roles to specific amino acids in the enzymes within the AAC(3) group. Interestingly, some homology among ribosomal acetyltransferase (Rim I), streptothricin acetyltransferase (SAT-1), and the AAC(3)-Ia protein (12, 25, 39) has been reported. This region of homology, also present in the AAC(3)-Ib protein, is limited to approximately 24 amino acids found in the central and C-terminal regions and may represent the acetyl coenzyme A binding site or catalytic domain of these enzymes (see also reference 30).

The relative frequencies and distributions of the two *aac(3)-I* genes were determined by using *aac(3)-Ia* and *aac(3)-Ib* probes in DNA hybridization studies of 59 strains expressing an AAC(3)-I phenotype. The *aac(3)-Ia* gene predominated both in frequency and in the diversity of the organisms in which it was found. These results are consistent with those of an earlier study of 394 strains expressing the AAC(3)-I phenotype, in which 78% of the strains tested hybridized with the *aac(3)-Ia* probe (29). A more recent study of 321 gram-negative isolates expressing an AAC(3)-I resistance profile showed that 85% of the strains hybridized with the *aac(3)-Ia* probe and only 6.2% of the strains hybridized with the *aac(3)-Ib* probe (21). The majority of the *aac(3)-Ib* probe-positive strains (65%) in that study were *Acinetobacter* and *Pseudomonas* strains. In all of these studies, a significant percentage of the strains (9 to 29%) did not hybridize with either probe, suggesting the existence of at least a third *aac(3)-I* gene. Both *aac(3)-I* genes were detected in isolates from the United States and Europe, and one strain from the Far East (Singapore) also hybridized with the *aac(3)-Ia* probe. These data demonstrate the global dissemination of both genes.

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