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Characterization of the 6′-*N*-Aminoglycoside Acetyltransferase Gene *aac(6′)-Il* Associated with a *sulI*-Type Integron

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The amikacin resistance gene *aac(6′)-Il* from *Citrobacter freundii* Cf155 encoding an aminoglycoside 6′-*N*-acetyltransferase was characterized. The gene was identified as a coding sequence of 521 bp located downstream from the 5′ conserved segment of an integron. The sequence of this *aac(6′)-Il* gene corresponded to a protein of 173 amino acids which possessed 64.2% identity in a 165-amino-acid overlap with the *aac(6′)-Ia* gene product (F. C. Tenover, D. Filpula, K. L. Phillips, and J. J. Plorde, J. Bacteriol. 170:471–473, 1988). By using PCR, the *aac(6′)-Il* gene could be detected in 8 of 86 gram-negative clinical isolates from two Belgian hospitals, including isolates of *Citrobacter*, *Klebsiella* spp., and *Escherichia coli*. PCR mapping of the *aac(6′)-Il* gene environment in these isolates indicated that the gene was located within a *sulI*-type integron; the insert region is 1,700 bases long and includes two genes cassettes, the second being *ant(3″)-Ib*.

The genes encoding aminoglycoside-modifying enzymes are often located on plasmids or transposons, enabling their rapid dissemination in a wide variety of bacterial species. From studies on the structure and characteristics of transposable elements it is clear that integrons carrying one or more antibiotic resistance-encoding genes, integrated by a site-specific recombination system, play a crucial role in the flux of these genetic determinants (19).

The aminoglycoside 6′-*N*-acetyltransferases, AAC(6′)s, are of particular interest since they can modify a number of clinically important aminoglycosides such as amikacin, gentamicin, netilmicin, and tobramicin. The AAC(6′)-I type confers resistance to amikacin but not to gentamicin, whereas the AAC(6′)-II type acetylates gentamicin but not amikacin. To date different genes, designated *aac(6)-Ia* to *aac(6′)-Ik*, encoding the AAC(6′)-I enzyme have been characterized (2, 5, 9, 10, 14, 16, 18, 23–25). In a recent study on the detection of *aac(6′)* genes in gram-negative isolates from two Belgian hospitals, we found that 25% of the *Citrobacter freundii* isolates contained an as far unknown gene encoding an AAC(6′)-I enzyme (26). Here we report on the characterization of this gene found in *C. freundii* Cf155. We arbitrarily called it *aac(6′)-Il.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The characteristics of the principal bacterial strains and plasmids used in these experiments are listed in Table 1. The reference strains used in the PCR experiments have been described previously (26). In addition, 4 other reference strains were included, i.e., *Staphylococcus aureus* FK422 (F. H. Kayser, Zurich, Switzerland), *Acinetobacter haemolyticus* BM2685, *Acinetobacter baumanii* BM2686, and *Acinetobacter* sp. 13 BM2689 (T. Lambert, Paris, France). Clinical isolates were provided by P. Maes from the St. Jozefkliniek (Ostend, Belgium) and by J. M. Hubrechts from the J. Bracops Hospital (Brussels, Belgium). Culture media were Mueller-Hinton broth and agar (Gibco Europe NV, Ghent, Belgium). If necessary, the media were supplemented with 8 μg of amikacin per ml.

Sensitivity testing. The MICs of amikacin, kanamycin, and ampicillin (Bristol-Myers Squibb, Brussels, Belgium), gentamicin, and netilmicin (Schering-Plough, Brussels, Belgium), gentamicin C1, gentamicin C1a, gentamicin C2, 2′-*N*-ethylnetilmicin, and 6′-*N*-ethylnetilmicin (Schering-Plough, Kenilworth, N.J.), tobramycin (Eli Lilly, Brussels, Belgium), streptomycin (Sigma, Bornem, Belgium), and spectinomycin (Upjohn, Puurs, Belgium) were determined in Mueller-Hinton broth with a microdilution MIC2000 system (Cooke Dynatech, Chantilly, Va.) and an inoculum of 1 μl containing 1.5×10^5 CFU.

Aminoglycoside-modifying enzyme assays. Bacterial extracts (S100) were prepared, and the substrate profile of the aminoglycoside-acetylating enzymes was determined by the phosphocellulose paper-binding radioassay by using different antibiotics as described before (26). Radiolabeled [^{14}C]acetyl coenzyme A (0.15 mM; specific activity, 10 Ci/mol) was obtained from Amersham International, Ghent, Belgium. Acetylation of 1 nmol of substrate was equivalent to 16,500 dpm in a 30-min assay at 35°C.

Preparation and analysis of DNA. Plasmid DNA was prepared by using the Qiagen plasmid kit according to the manufacturer's instructions (Qiagen Inc., Studio City, Calif.). DNA was digested with restriction enzymes according to the manufacturer's instructions (Boehringer Mannheim, Brussels, Belgium), and the fragments were separated on 0.7% agarose (Bio-Rad Laboratories, Nazareth, Belgium) gels in Tris acetate-EDTA buffer (pH 8). The fragments were extracted by use of Prep-A Gene (Bio-Rad Laboratories).

Genetic techniques. All plasmids were introduced into electrocompetent bacteria by electroporation in a chilled cuvette (0.2-cm electrode gap) with a Genepulser (Bio-Rad Laboratories, Hercules, Calif.). Electroporation conditions were set at 1,400 V, 1,000 Ω, and 25 μFd. Electrocompetent *Escherichia coli* MC1061 and DH5α were prepared by following the instructions of the manufacturer (Bio-Rad Laboratories). The cells were transferred to 1 ml of SOB broth (15), and the mixture was incubated for 1 h at 37°C. Dilutions were plated onto Mueller-Hinton agar containing 100 μg of ampicillin per ml and 8 μg of amikacin per ml. The plates were incubated for 24 to 48 h at 37°C. To confirm the presence and to determine the size of the donor plasmid in the transformants, the plasmid was isolated and digested with the appropriate restriction endonucleases (*Eco*RI and *Hin*dIII) and was screened for the insert by gel electrophoresis.

DNA sequencing. Double-stranded sequencing was performed by using a *Taq* fluorescent dye-primer cycle sequencing kit (Amersham Belgium SA/NV, Ghent, Belgium). All oligonucleotides used as primers for sequencing were prepared by R&D Systems Europe Ltd. (Abingdon, United Kingdom). The Vistra DNA Sequencer 725 (Amersham Belgium SA/NV) was used for the electrophoretic separation, detection, and analysis of fluorescence-tagged DNA molecules.

PCR studies. Published sets of primers specific for genes *aac(6′)-Ia*, *-Ib*, and *-If*, *aac(6′)-IIa* (26), and *aac(3)-Ia* (27) were used in this study. The characteristics of the other primer sequences are presented in Table 2. Their specificities were tested with DNA extracts from reference strains as described previously (26). Oligonucleotide primers were synthesized by Eurogentec (Seraing, Belgium).

DNA was prepared following the InstaGene Purification Matrix (Bio-Rad Laboratories). The PCR amplifications were carried out in 50-μl volumes by following the Gibco protocol and by using the complete Gibco set of *Taq* DNA polymerase and buffer (N.V. Life Technologies, Merelbeke, Belgium). After denaturation at 94°C for 3 min, the samples were subjected to 30 amplification cycles in a thermocycler (Trio-Thermoblock; Biometra, Tampa, Fla.). Each cycle consisted of 45 s at 94°C (denaturation), 30 s at 60°C (annealing), and 90 to 120 s at 72°C (extension). An aliquot (10 μl) of each sample was analyzed by 0.9 to 2% agarose gel electrophoresis and was visualized by ethidium bromide staining.

Computer analysis. Computer-aided analysis of the nucleic acid and deduced amino acid sequences was performed with the Genetics Computer Group (GCG) Program (3) of the Belgian Embnet Node.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Origin
Strains		
C. freundii Cf155	Ak, Gm, Nt, Sm, Tm	St. Jozefkliniek
E. coli MC1061	Sm	Stratagene
E. coli DH5α		Stratagene
Plasmids		
pBluescribe M13+	Ap	Stratagene
pIPB16	3.2-kb *Sau*3A1 fragment from Cf155 into pBluescribe M13+; Ak, Nt, Sm, Tm	This study
pIPB16/5	1.2-kb *Bam*HI-*Kpn*I fragment of pIPB16 into pBluescribe M13+; Ak, Nt, Tm	This study

[a] Ak, amikacin resistance; Ap, ampicillin resistance; Gm, gentamicin resistance; Nt, netilmicin resistance; Sm, streptomycin resistance; Tm, tobramycin resistance.

Phylogenetic tree. The phylogenetic tree was constructed with the help of GCG software. The sequences were aligned with the PileUp program (3). A Jukes-Cantor (8) distance matrix was computed with distances. The tree was constructed with GrowTree by the neighbor-joining method (21) and was plotted with the PHYLIP program Drawtree (4).

Nucleotide sequence accession number. The nucleotide sequence of the *aac(6′)-Il* gene of *C. freundii* Cf155 has been deposited in the EMBL data library under accession number Z54241.

RESULTS AND DISCUSSION

Aminoglycoside resistance in *C. freundii* Cf155. By MIC determination, *C. freundii* Cf155 was revealed to be resistant to amikacin, gentamicin, netilmicin, tobramycin, 2′-*N*-ethylnetilmicin, streptomycin, and spectinomycin and susceptible to 6′-*N*-ethylnetilmicin (Table 3). The phosphocellulose paper-binding radioassay performed with the extract of strain Cf155 indicated that amikacin, gentamicin C1a and C2, 2′-*N*-ethylnetilmicin, and tobramycin were acetylated, whereas apramycin, lividomycin, and paromomycin were not. Both the susceptibility pattern and the enzymatic modification profile are compatible with a type I aminoglycoside 6′-*N*-acetyltransferase. On the other hand, low degrees of acetylation of gentamicin C1 and 6′-*N*-ethylnetilmicin were observed (31 and 26%, respectively, compared to a 100% acetylation of amikacin), indicating the possible presence of a second enzyme, i.e., AAC(3). Despite the presence of an AAC(6′)-I activity, the strain did not give a positive PCR signal with the various sets of primers specific for the already known *aac(6′)* genes (Table 2), suggesting the presence of an unknown *aac(6′)-I* gene.

Cloning of the amikacin resistance gene. Plasmid DNA from strain Cf155 was partially digested with *Sau*3AI. The fragments were ligated to the *Bam*HI site of pBluescribe M13$^+$ and were introduced by electroporation into *E. coli* MC1061. The electrotransformants selected on ampicillin plus amikacin were screened for insert content by agarose gel electrophoresis. The smallest plasmid (pIPB16) containing a 3.2-kb insert was chosen. Its digestion by *Eco*RI and *Hin*dIII enzymes generated two fragments of 1.8 and 1.4 kb. Subsequent subcloning of an internal *Bam*HI-*Kpn*I fragment from plasmid pIPB16 resulted in plasmid pIPB16/5. This plasmid contained a 1.2-kb insert which conferred amikacin resistance to *E. coli* DH5α.

TABLE 2. Oligonucleotides used for PCR amplification

Gene[a]	Accession no.	Position in sequence	Nucleotide sequence (5′ to 3′)	Primer symbol[b]
aac(6′)-Ic	M94066	1742–1764	CTACGATTACGTCAACGGCTGC	
		1871–1850	TTGCTTCGCCCACTCCTGCACC	
aac(6′)-Id	X12618	941–962	TGGCTGCAACTTCGTTTTCTCC	
		1289–1269	GGTGAAATCTATGGCTTTCCG	
aac(6′)-Ie	M13771	1868–1888	ACATTATACAGAGCCTTGGG	
		2222–2201	CTCGTGTAATTCATGTTCTGGC	
aac(6′)-Ig	L09246	685–703	CCTATTCCGATCACCAAGC	
		860–842	TTGTTTTGCCCACACTTCG	
aac(6′)-Ih	L29044	486–504	ATTGGCTTATACCGACACC	
		721–700	GGCTGATCTGATTATCCAACGC	
aac(6′)-Ii	L12710	248–269	AAGAATATGGAGACAGCTCGGC	
		546–524	ACAGGTCCGTTTGACTTAACG	
aac(6′)-Ij	L29045	332–354	CATGACGAGGCACATTTACAGG	
		629–608	GGCTAATACGATTATCGAGGGC	
aac(6′)-Il	Z54241	629–647	TGCTTGGGAATATGTCTGG	B
		1037–1016	TTGTTGGGCTGTTCTTCCTAGC	C
aac(6′)-IIb	L06163	878–899	CGCTTGTTGATTTGCTGTTCGC	
		1003–982	TTGAAACGACCTTGACCTTCCG	
5′ cs	M73819	1190–1206	GGCATCCAAGCAGCAAG	A
3′ cs	M73819	1342–1326	AAGCAGACTTGACCTGA	F
ant(3″)-Ia	X02340	430–448	TCGACTCAACTATCAGAGG	D
		674–654	ACAATGGTGACTTCTACAGCG	E
qacEΔ1	X15370	208–226	GTTATCGCAATAGTTGGCG	G
		435–413	AGCTTTTGCCCATGAAGCAACC	H
sulI	M73819	2265–2283	GATTTTTCTTGAGCCCCGC	I
		2419–2399	TGGACCCAGATCCTTTACAGG	J

[a] 5′ cs and 3′ cs, 5′ and 3′ conserved segments, respectively; *ant*, aminoglycoside nucleotidyltransferase gene; *sul*, sulfonamide resistance gene; *qacEΔI*, gene for resistance to ammonium compounds.

[b] Oligonucleotides primers also used in PCR mapping (see Fig. 3).

TABLE 3. Susceptibilities of bacterial strains to selected aminoglycosides[a]

Strain (plasmid)	MIC (μg/ml)							
	Ak	Gm	Nt	Tm	2′Net	6′Net	Sm	Sp
C. freundii Cf155	64	8	128	64	≥256	2	32	≥256
E. coli MC1061	2	0.5	≤0.25	1	1	0.5	≥1,024	16
E. coli MC1061(pIPB16)	64	≤0.25	32	32	≥256	0.5	≥1,024	8
E. coli DH5α	1	≤0.25	≤0.25	≤0.25	≤0.25	≤0.25	16	8
E. coli DH5α(pIPB16/5)	64	≤0.25	32	32	64	≤0.25	16	8
C. freundii Cf76	32	8	32	128	≥256	1	256	≥256
C. freundii Cf79	64	1	128	128	≥256	4	≥1,024	≥256
C. freundii Cf152	128	16	32	128	≥256	2	256	≥256
C. freundii Cf158	128	0.5	128	128	≥256	4	≥1,024	≥256
E. coli Ec474	128	1	128	128	≥256	2	512	≥256
K. oxytoca Ko151	64	≤0.25	32	128	≥256	2	256	≥256
K. oxytoca Ko159	128	1	128	128	≥256	16	512	≥256
K. pneumoniae Kp260	128	0.5	128	128	≥256	8	512	≥256

[a] Ak, amikacin; Gm, gentamicin; Nt, netilmicin; Sm, streptomycin; Sp, spectinomycin; Tm, tobramycin; 2′Net, 2′-*N*-ethylnetilmicin; 6′Net, 6′-*N*-ethylnetilmicin.

Acquisition of pIPB16 by *E. coli* MC1061 and acquisition of pIPB16/5 by *E. coli* DH5α resulted in an important increase in the MICs of amikacin, netilmicin, and tobramicin. Furthermore, both strains were resistant to 2′-*N*-ethylnetilmicin and susceptible to 6′-*N*-ethylnetilmicin (Table 3). It should be noted that the extracts from *E. coli* MC106(pIPB16) and *E. coli* DH5α(pIPB16/5) contained AAC(6′)-I activity. Indeed, amikacin, gentamicin C1a, and 2′-*N*-ethylnetilmicin were readily modified, whereas gentamicin C1 and 6′-*N*-ethylnetilmicin were not. Gentamicin C2 was acetylated at a lower degree (70%). These data indicate that the *aac(6′)-I* gene, arbitrarily designated the *aac(6′)-Il* gene, was expressed in *E. coli* MC1061 and *E. coli* DH5α.

DNA sequence analysis. The nucleotide sequences of both strains of the 1,153-bp insert of pIPB16/5 containing the *aac(6′)-Il* gene were determined (Fig. 1). The analysis of the sequence indicated the presence of two open reading frames (ORFs) with opposite polarity. The comparative analysis of this nucleotide sequence with previously characterized sequences from the GenBank database revealed that a stretch of 426 nucleotides of the 5′ flanking sequence of the 1,153-bp insert was similar to sequences found in the 5′ conserved segment of integrons, i.e., a recombination system responsible for integration of antibiotic resistance genes. The first 285 bp encodes parts of the DNA integrase, which is transcribed in the direction opposite that for ORF2. The putative DNA integrase promoter P (TTGCTG . . . N17 . . . TAGACT) is on the complementary strand between nucleotides 340 and 312. The sequence between nucleotides 358 and 426 is known as the *aatI* site into which the gene cassettes are inserted (13). It constitutes the junction between the 5′ conserved segment and the gene cassette from motif GTTGGGC (positions 419 to 426) to motif GTTAAAC (positions 1132 to 1138). Motifs related to GTTGGGC and GTTAAAC, designated the seven-base core site, were located at the boundaries of the gene cassettes. A search of the GenBank database for sequences homologous to this sequence identified a second ORF spanning 521 nucleotides from the start codon ATG at position 530 to the stop codon TGA at position 1051 with 69% identity (in a 482-nucleotide overlap) to the *aac(6′)-Ia* gene (23). These data suggest that this ORF corresponds to the *aac(6′)-Il* gene. Analysis of the adjacent region preceding ORF2 showed two potential −35 and −10 promoters: TGGACA . . . N17 . . . TAA GCT at positions 171 to 199 (promoter P1) and TTGTTA . . . N14 . . . TACAGT at positions 289 to 309 (promoter P2). Promoter P2 could not be functional since there is only a 14-bp spacing between the −35 and −10 regions. Therefore, promoter P1 would be the putative promoter, despite being a weak version of a promoter (11). The ribosome-binding site is located 6 bp upstream (positions 518 to 524) from the translation initiation codon of *aac(6′)-Il.*

Figure 1 shows the alignment of the nucleotide sequence of the 1,153-bp insert of pIPB16/5 containing the *aac(6′)-Il* and the sequence of the cloned 1,186-bp fragment from pBWH100 of *Citrobacter diversus* harboring the *aac(6′)-Ia* gene (23). The sequences of the 5′ conserved segment from the *int* gene in the two fragments are nearly identical (95%). The start and stop codons of the *aac(6′)-Ia* are located at nucleotides 476 and 1033, respectively.

At the 3′ end of the *aac(6′)-Il* gene cassette, we have not found the consensus sequence of the 59-base element specific for the gene functioning as a DNA integrase recognition site (6). However, we found a 109-bp sequence (positions 1029 to 1138) with an approximate 20-base similarity at each end that can be related to the consensus sequence. It is now recognized that the 59-base-element family also includes longer imperfect repeats (up to 110 bp) showing similarity with the consensus sequence at their extremities. Therefore, the aforementioned sequence can be withheld as an imperfect 59-bp (109-bp) base element. Downstream of the *aac(6′)-Il* gene cassette the beginning of another gene cassette with the same orientation and a possible ATG start codon at position 1143 bp was found. The high degree of homology found between this sequence (position 1132 to 1152) and the beginning of the *aadA1* gene cassette found in Tn*7* (20) would indicate the presence of an *ant(3″)-I* gene.

Thus, the presence of the *int* gene, *aatI*, the cassette junctions, and the imperfect 59-bp (109-bp) base element provides support for the fact that the *aac(6′)-Il* gene in *C. freundii* Cf155 is localized within an integron.

Comparison of the AAC(6′) family of enzymes. The protein AAC(6′)-Il encoded by the *aac(6′)-Il* gene consists of 173 amino acids and has a predicted molecular size of 19,777 Da. The amino acid sequence of this protein has been compared to the amino acid sequences of the AAC(6′) enzyme family. The deduced phylogeny of these sequences suggests that all the proteins can be classified into three subfamilies (Fig. 2). The first family consists of AAC(6′)-Ib, -IIa, -IIb, and -Ie, the amino-terminal portion of the bifunctional enzyme of gram-positive cocci. The second subfamily comprises AAC(6′)-Ig, -Ik, -Ih, -Ij, -Id, -If, and -Ic. The third subfamily contains the AAC(6′)-Ii protein (*Enterococcus faecium*), the AAC(6′)-Ia

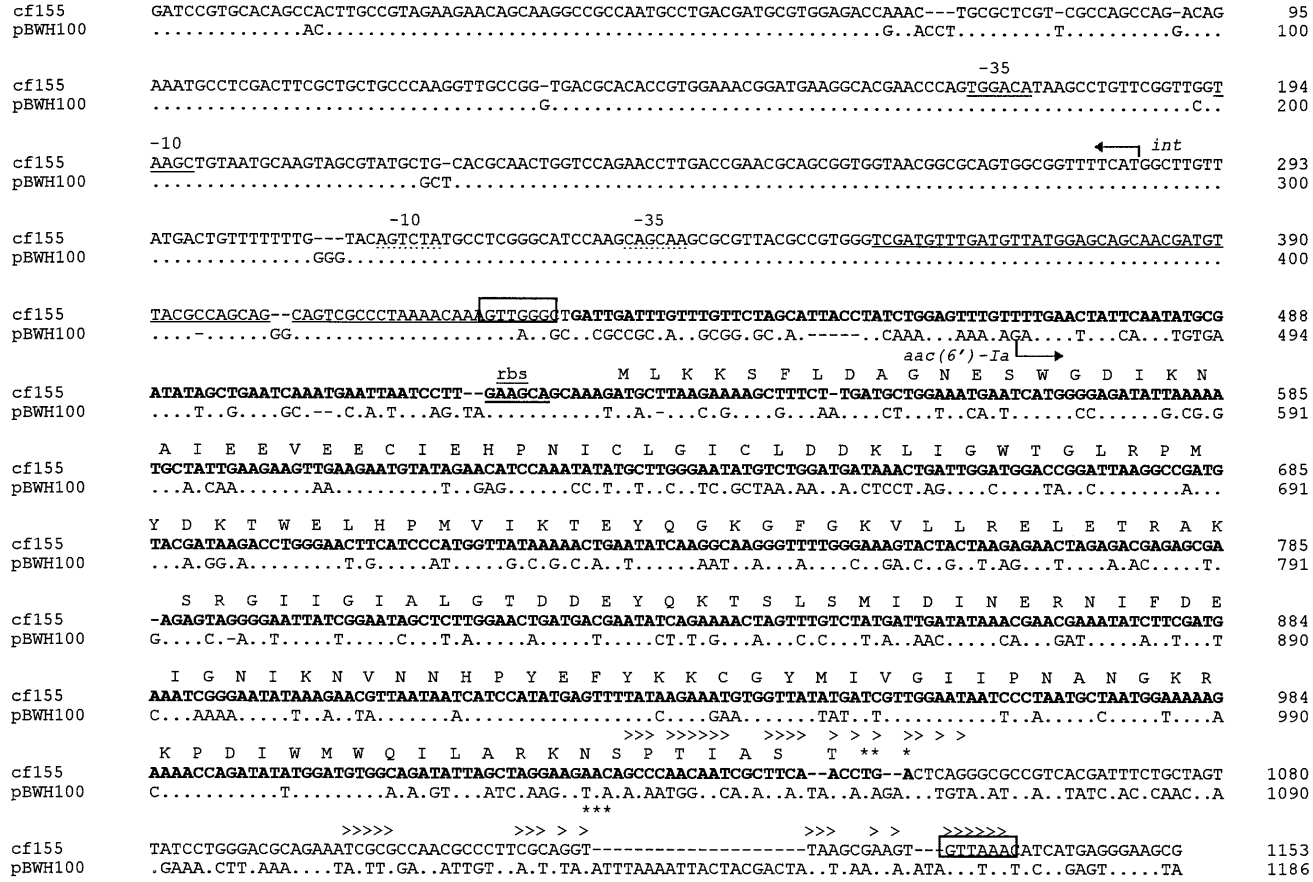

FIG. 1. Comparative analysis of the nucleotide sequences of the 1,153-bp fragment of *C. freundii* Cf155 containing the *aac(6′)-Il* gene and of the 1,186-bp fragment of pBWH100 containing the *aac(6′)-Ia* gene (23). A dot depicts identical nucleotides in the two sequences, and dashes indicate gaps introduced to optimize the alignment. The start codons of the *aac(6′)-Ia* gene and the integrase gene which is reading from the complementary strand are indicated by the arrows. The −10 and −35 sequences of the putative *int* promoter are underlined with dotted lines and are also located on the complementary strand. The nucleotide sequence of the *aac(6′)-Il* cassette is indicated in boldface capital letters, and the deduced amino acid sequence of AAC(6′)-Il is in capital letters, indicated above the corresponding triplets of *aac(6′)-Il* (positions 530 to 1051). Putative −35 and −10 promoter sequences of *aac(6′)-Il* are underlined (promoter P1). The proposed ribosome-binding site of *aac(6′)-Il* is presented by rbs. The *aatI* site (13) is double underlined, and the seven-base core sites (6) are boxed. The bases identical to the consensus 59-base element (6) are presented by the symbol >. The stop codons are indicated by a triple asterisk.

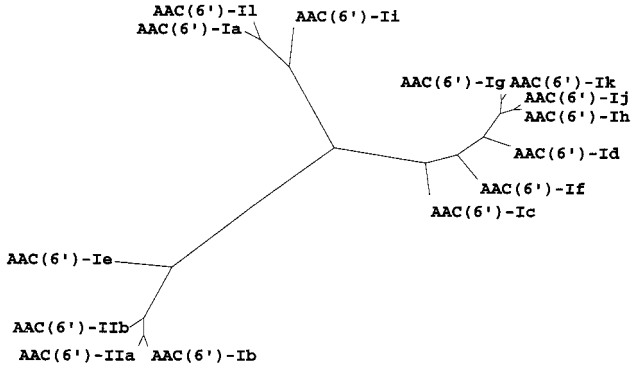

FIG. 2. The phylogenetic tree of the AAC(6′) family of enzymes. The following 6′-*N*-aminoglycoside acetyltransferase sequences can be found in data banks by the indicated accession numbers and in the published references: AAC(6′)-Ia, M18967 (23); AAC(6′)-Ib, M23634 (25); AAC(6′)-Ic, M94066 (18); AAC(6′)-Id, X12618 (16); AAC(6′)-Ie, M13771 (5); AAC(6′)-If, X55353 (24); AAC(6′)-Ig, L09246 (10); AAC(6′)-Ih, L29044 (9); AAC(6′)-Ii, L12710 (2); AAC(6′)-Ij, L29045 (9); AAC(6′)-Ik, L29510 (14); AAC(6′)-Il, Z54241 (this study); AAC(6′)-IIa, M29695, (17); AAC(6′)-IIb, L06163 (unpublished data).

protein (*C. diversus*), and the new AAC(6′)-Il protein (*C. freundii*). The alignment of AAC(6′)-Ia and AAC(6′)-Il confirms the close relatedness of the two proteins. Indeed, their amino acid sequences present 64.2% identity and 77.6% similarity with a 165-amino-acid overlap.

Distribution of the *aac(6′)-Il* gene in clinical isolates. PCR studies were performed to determine the frequency and distribution of the *aac(6′)-Il* gene in clinical isolates obtained from two hospitals. A specific set of primers was developed to detect this new *aac(6′)-Il* gene (Table 2). The *aac(6′)-Il* gene could be detected in 8 of 86 clinical isolates; all the positive isolates originated from the St. Jozefkliniek. The gene was revealed to be present in isolates of *C. freundii*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, and *E. coli.*

For six isolates MICs (Table 3) and modification profiles were found to be comparable to those for the transformant harboring pIPB16/5. The two remaining isolates, i.e., *C. freundii* Cf76 and *C. freundii* Cf152, were resistant to gentamicin and also acetylated, albeit at a low degree, the compounds gentamicin C1 and 6′-*N*-ethylnetilmicin. In this respect, they resembled *C. freundii* Cf155. Indeed, PCR results demonstrated the presence of a second gene which was revealed to be *aac(3)-Ia*. Furthermore, PCR revealed the presence of the *ant(3″)-Ib gene*

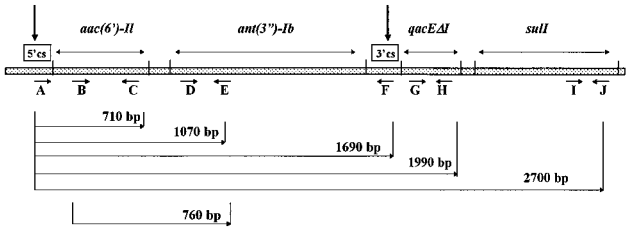

FIG. 3. Structure of the *sulI*-associated integron containing the *aac(6′)-Il* gene, obtained by PCR analysis. Primers A, B, C, D, E, F, G, H, I, and J are described in Table 2. Arrows indicated the primer orientation, but are not to scale. 5′ cs and 3′ cs, 5′ and 3′ conserved segments, respectively.

in all clinical isolates, resulting in streptomycin and spectinomycin resistance.

PCR mapping of *aac(6′)-Il* gene. Plasmid DNAs from the eight clinical isolates were investigated by PCR in order to map the environment of the *aac(6′)-Il* gene. The oligonucleotide primers A, B, C, D, E, F, G, H, I, and J used in these PCR experiments are listed in Table 2. Amplification with the primers specific for the 5′ and 3′ conserved segments of an integron (1) showed that all these isolates possessed an integron(s). Exploration of the conserved 3′ region with the primers for the *sulI* (1) and *qacEΔI* (12) genes enabled us to define these integrons as *sulI*-associated integrons (type I). Furthermore, PCR results indicated the presence of the *ant(3″)-I* gene in all isolates. Because of their close nucleotide sequence homology (89%), the *ant(3″)-Ia* gene (7) and the *ant(3″)-Ib* gene (22) could not be distinguished by PCR.

PCR mapping results obtained by using the combination of sets of primers A, B, C, E, F, H, and J in six representative amplifications are depicted in Fig. 3. The data indicated that in all clinical isolates the gene was located within a *sulI*-type integron; the insert region was 1,700 bases long and harbored the *aac(6′)-Il* gene as well as the *ant(3″)-I* gene (7). The data are consistent with the published sequences of the genes studied in these experiments. Analysis of the data indicates that the space available for the *ant(3″)-I* gene measures 750 bp, meaning that the gene present is the *ant(3″)-Ib* gene rather than the *ant(3″)-Ia* gene, which is 900 bp in length. In the gene cassettes, *aac(6′)-Il* and *ant(3″)-Ib* are inserted in the same orientation with respect to their coding regions and are expressed from a common promoter, promoter P1, localized in the 5′ conserved segment of the integron.

Interestingly, we have found an integron with the same type of genetic organization in different species of the family *Enterobacteriaceae* isolated in one hospital. These findings provide support for the expanding role of the integron in the dissemination of resistance genes.

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