ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 1993, p. 1896–1903
0066-4804/93/091896-08$02.00/0

Vol. 37, No. 9

Characterization of the Chromosomal *aac(6')-Ii* Gene Specific for *Enterococcus faecium*

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Received 29 March 1993/Returned for modification 14 May 1993/Accepted 24 June 1993

Chromosomal gene *aac(6')-Ii* of *Enterococcus faecium* CIP 54-32, encoding a 6'-*N*-aminoglycoside acetyltransferase was characterized. The gene was identified as a coding sequence of 549 bp corresponding to a protein with a calculated mass of 20,666 Da. Analysis of the sequence of the deduced protein suggested that it was the second member of a subfamily of AAC(6')-I enzymes. Insertional inactivation of *aac(6')-Ii* led to aminoglycoside susceptibility of CIP 54-32, suggesting that this gene plays a role in resistance to AAC(6')-I substrates. The gene was detected by DNA hybridization in all 26 strains of *E. faecium* tested but not in 44 other enterococci of 13 species. These data suggest that the *aac(6')-Ii* gene is species specific and may be used to identify *E. faecium*.

The bacterial genus *Enterococcus* includes a minimum of 14 species (19). *Enterococcus faecalis* and *E. faecium* are the most common species causing human disease and represent, respectively, 87 and 9% of clinical enterococcal isolates (24). Some strains of the remaining species are occasionally isolated from humans but are more commonly isolated from plants and animals. Enterococci are increasingly responsible for nosocomial disease, including intra-abdominal infections, urinary tract infections, and bacteremia (19). Treatment of severe enterococcal infections is difficult because of intrinsic resistance to numerous antibiotics. In particular, enterococci are naturally resistant to low levels of aminoglycosides and, unlike streptococci, are relatively resistant to penicillins (19). However, combinations of a penicillin with an aminoglycoside produce synergistic bactericidal effects against enterococci both in vitro and in vivo. As a result, a penicillin combined with an aminoglycoside is the therapy of choice for enterococcal infections (19).

E. faecium is unique among enterococci, since all strains of this species appear to produce a chromosomally encoded 6'-*N*-aminoglycoside acetyltransferase (36). This enzyme catalyzes the acetylation of the 6' amino group of kanamycins A and B, neomycin, netilmicin, sisomicin, and tobramycin (36). In *E. faecium* strains, the rate of modification of these aminoglycosides is not sufficient to confer high-level resistance but results in the loss of synergy between the substrate aminoglycoside and penicillins. Against these strains, only combinations of a penicillin and gentamicin or amikacin remain synergistic (17).

The acquisition of high-level resistance (MICs, >1,000 μg/ml) to virtually all commercially available aminoglycosides has been reported for both *E. faecalis* and *E. faecium* with an increasing frequency. This high-level resistance is mediated by the bifunctional 2"-aminoglycoside phosphotransferase–6'-aminoglycoside acetyltransferase; the corresponding gene is located on self-transferable plasmids or on transposons (6, 8). The gene for the chromosomally encoded AAC(6')-I of *E. faecium* does not hybridize with a probe specific for the *aph(2")-aac(6')* gene (21), indicating that the two acetyltransferases are not closely related.

In this work, we report the cloning, nucleotide sequence, and insertional inactivation of the *E. faecium* CIP 54-32 *aac(6')-Ii* gene, which encodes an enzyme necessary for the expression of resistance to certain aminoglycosides. The distribution of this gene in 14 species of enterococci was studied by nucleic acid hybridization.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *E. faecium* CIP 54-32 was from the Collection of the Institut Pasteur (CIP). Twenty-two clinical isolates of *E. faecium* were collected from five hospitals in France, Spain, the United Kingdom, and the United States. Of these isolates, 11 were highly resistant to gentamicin (MICs, >1,000 μg/ml) and 6 were highly resistant to kanamycin (MICs, >1,000 μg/ml). *E. faecium* ATCC 19434, CIP 54-33, and CIP 103226 were also studied.

Forty-four strains belonging to 13 other enterococcal species were studied. They included the 15 reference strains *E. casseliflavus* ATCC 25788; *E. durans* ATCC 19432; *E. faecalis* ATCC 11700, ATCC 29212, and ATCC 33186; *E. hirae* ATCC 8043, ATCC 9790, NCDO 1258, CDC 976-79, and CDC 1709-79; *E. malodoratus* NCDO 846; *E. mundtii* NCDO 2375; *E. pseudoavium* NCDO 2138; *E. raffinosus* NCTC 12192; and *E. solitarius* NCTC 12193; 6 strains from the bioMérieux Collection (*E. casseliflavus* [1 strain], *E. faecalis* [1 strain], and *E. gallinarum* [4 strains]); and 5 strains from the laboratory collection of L. A. Devriese (*E. avium* D373, *E. casseliflavus* A22, *E. cecorum* K24 and SP152 [5], and *E. columbae* D1 [4]). Eighteen clinical isolates from six hospitals in France, Spain, and the United States were also studied: *E. avium* (1 strain), *E. durans* (3 strains), *E. faecalis* (12 strains), *E. gallinarum* (1 strain), and *E. hirae* (1 strain). Of these isolates, 2 *E. faecalis* isolates were resistant to high levels of kanamycin and 10 were resistant to high levels of gentamicin.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
Strains		
E. coli		
BM694	Nal^r	11
JM83	*ara* Δ(*lac-proAB*) *rspL* [ϕ80 Δ(*lacZ*)*M15*]	37
E. faecium		
CIP 54-32	Wild-type strain; *aac(6')-Ii*	This work
BM4229	Integration of pAT435 into *aac(6')-Ii* of CIP 54-32	This work
Plasmids		
pAT114	Em Km Mob$^+$; integrative vector	34
pAT431	pUC19 Ω*Hin*dIII (3.7-kb fragment from CIP 54-32)	This work
pAT432	pUC19 Ω*Sau*3AI (1.5-kb fragment from pAT431)	This work
pAT433	pUC19 Ω*Kpn*I-*Ssp*I (1.1-kb fragment from pAT432)	This work
pAT434	pUC18 Ω*Sau*3AI-*Eco*RI (0.4-kb fragment from pAT432)	This work
pAT435	pAT114 Ω*Sau*3AI-*Eco*RI (0.4-kb fragment from pAT432)	This work
pUC18 and pUC19	Tra$^-$ Mob$^-$ Ap	37

[a] Abbreviations: Mob, mobilizable; Tra, self-transferable.

All strains were identified as belonging to the genus *Enterococcus* by Gram staining, the absence of catalase, the inability to produce gas, the presence of Lancefield antigen group D, and growth in 40% bile, in 6.5% sodium chloride, in 0.1% methylene blue, and at pH 9.6. Species identification (7) was based on the following tests: potassium tellurite reduction, pigmentation, motility at 30°C, and biochemical tests in API 50CH strips (bioMérieux, Marcy-l'Etoile, France). Plasmids and strains used in cloning and insertional inactivation experiments are listed in Table 1. Strains were grown in brain heart infusion broth and on brain heart infusion agar (Difco Laboratories, Detroit, Mich.). All incubations were performed at 37°C.

Antibiotic susceptibility testing. Antibiotic susceptibility was determined by the disk diffusion technique on Mueller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). The MICs of aminoglycosides were determined on Mueller-Hinton agar with an inoculum of 10^4 CFU per spot by the method of Steers et al. (30).

Aminoglycoside-modifying enzyme assays. Bacterial extracts (S100) were prepared, and inactivation of aminoglycosides was detected by the phosphocellulose paper-binding technique with [1-^{14}C]acetyl coenzyme A (specific activity, 25 μCi/mmol), [γ-^{32}P]ATP (specific activity, 30 Ci/mmol), and [α-^{32}P]ATP (specific activity, 30 Ci/mmol) as described previously (10). The final concentration of the aminoglycoside in the assay mixture was 66.7 μg/ml, and the reaction was allowed to proceed for 30 min at 30°C.

DNA manipulation. Total DNA of enterococci (16) and plasmid DNA (1) were prepared as described previously. Restriction enzyme-generated DNA fragments were analyzed by 0.8% agarose gel electrophoresis. Fragments were extracted from the gels by use of a Gene Clean II kit (Bio 101, Inc., La Jolla, Calif.). For dot blot and Southern hybridizations, DNA was immobilized on Nytran membrane filters (Schleicher & Schuell, Dassel, Germany). Prehybridization and hybridization were carried out for 4 h and overnight, 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. The probe was radiolabeled by use of a nick translation system kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). *E. faecium* CIP 54-32 cells were transformed with plasmid DNA by electroporation (2) by use of a Bio-Rad Gene Pulser. Electrotransformants were selected on brain heart infusion agar containing erythromycin (8 μg/ml).

DNA sequencing and computer analysis of sequence data. Double-stranded DNA sequencing was performed with universal primers (New England Biolabs, Beverly, Mass.). Synthetic oligodeoxyribonucleotides complementary to the sequence of the insert used to complete the DNA sequence were synthesized by the methoxy phosphoramidite method (Unité de Chimie Organique, Institut Pasteur, Paris, France). Nucleotide sequencing was performed by the dideoxynucleotide chain termination method (25) with a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio), modified T7 DNA polymerase, and [α-^{35}S]dATP. Nucleotide and amino acid sequences were analyzed and compared by use of GenBank, EMBL, and Swiss-Prot data bases with Genetics Computer Group software (3) and with the FASTA program (22).

Analysis of plasmid-encoded proteins. The proteins specified by the recombinant plasmids were synthesized in an *E. coli* in vitro transcription-translation system (Amersham Corp., Arlington Heights, Ill.) (38). Proteins were labeled with L-[^{35}S]methionine and electrophoresed in an SDS–15% polyacrylamide gel as described previously (12).

Enzymes and chemicals. Restriction enzymes and T4 DNA ligase were purchased from Amersham. RNase A (bovine pancreas) was obtained from Calbiochem-Behring (La Jolla, Calif.), and SeaKem GTG agarose was obtained from FMC Bioproducts (Rockland, Maine). Radiolabeled compounds were purchased from the Amersham Radiochemical Center (Amersham, England). The antibiotics were provided by the following manufacturers: amikacin, ampicillin, and kanamycin, Bristol Laboratories (Syracuse, N.Y.); gentamicins C1, C1a, and C2, netilmicin, 2′-*N*-ethyl-netilmicin, 6′-*N*-ethyl-netilmicin, and sisomicin, Schering Plough Research Institute (Kenilworth, N.J.); tobramycin, Eli Lilly & Co. (Indianapolis, Ind.); and erythromycin, Roussel-Uclaf (Romainville, France).

Nucleotide sequence accession number. The nucleotide sequence of the insert of pAT432 including the *aac(6')-Ii* gene from *E. faecium* CIP 54-32 has been deposited in the

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

Species (no. of strains)	MIC[a] (μg/ml) of:					
	Amikacin	Gentamicin	Kanamycin	Netilmicin	Sisomicin	Tobramycin
E. faecium CIP 54-32	32	4	128	32	32	64
E. faecium[b] (8)	16–32	4–16	128–1,024	16–128	16–128	64–256
E. faecium[c] (6)	32–128	4–8	>4,096	32–64	32–64	64–128
E. faecium[d] (11)	128–2,048	>4,096	>4,096	128–>4,096	>4,096	>4,096
Enterococcus spp.[e] (28)	16–256	1–32	8–128	1–64	1–64	1–32
Enterococcus spp.[f] (5)	32–512	2–16	>4,096	2–8	4–8	8–16
Enterococcus spp.[g] (11)	512–>4,096	512–>4,096	>4,096	16–512	512–>4,096	2,048–>4,096
E. coli BM694	1	0.5	2	0.5	0.5	1
E. coli BM694/pAT432	128	1	>256	128	128	128
E. faecium BM4229	NT	NT	NT	2	2	4

[a] Determined on Mueller-Hinton agar. NT, not tested; this strain contains insertionally inactivated *aac(6')-Ii* but also the *aphA-3* gene from pAT114.
[b] Strains resistant to low levels of aminoglycosides.
[c] Strains resistant to high levels of kanamycin.
[d] Strains resistant to high levels of gentamicin.
[e] Enterococci resistant to low levels of aminoglycosides (2 *E. avium*, 3 *E. casseliflavus*, 2 *E. cecorum*, 1 *E. columbae*, 3 *E. durans*, 4 *E. faecalis*, 4 *E. gallinarum*, 5 *E. hirae*, 1 *E. malodoratus*, 1 *E. mundtii*, 1 *E. pseudoavium*, and 1 *E. solitarius*).
[f] Enterococci resistant to high levels of kanamycin (1 *E. durans*, 2 *E. faecalis*, 1 *E. raffinosus*, and 1 *E. hirae*).
[g] Enterococci resistant to high levels of gentamicin (10 *E. faecalis* and 1 *E. gallinarum*).

GenBank data library (Los Alamos, N. Mex.) under accession number L12710.

RESULTS

Susceptibility of enterococcal species to aminoglycosides. The MICs of aminoglycosides for enterococci are shown in Table 2. The MICs of netilmicin, sisomicin, and tobramycin for *E. faecium* CIP 54-32 were 8- to 16-fold higher than that of gentamicin; the MIC of kanamycin was 128 μg/ml. This phenotype could be due to the synthesis of a 6'-*N*-aminoglycoside acetyltransferase (36). However, we could not detect any aminoglycoside-acetylating, -phosphorylating, or -nucleotidylating activity in S100 extracts of *E. faecium* CIP 54-32 in the phosphocellulose paper-binding assay. A similar resistance phenotype was observed for the other strains of *E. faecium* that did not display high-level resistance (MICs, <1,000 μg/ml) to kanamycin or gentamicin (Table 2). In the absence of high-level resistance to kanamycin or gentamicin, the MICs of netilmicin, sisomicin, and tobramycin for most enterococcal species other than *E. faecium* did not differ by more than 1 dilution. The MICs of aminoglycosides, except for amikacin and netilmicin, against enterococci that were resistant to high levels of gentamicin were >1,000 μg/ml, as has been observed elsewhere (21).

Cloning of the *aac(6')-Ii* gene of CIP 54-32. Total DNA from *E. faecium* CIP 54-32 and pUC19 DNA digested with *Hin*dIII were mixed, ligated, and introduced by transformation into *Escherichia coli* BM694. Transformants selected on ampicillin (100 μg/ml) and kanamycin (8 μg/ml) were screened for their plasmid content by agarose gel electrophoresis. The smallest hybrid plasmid, pAT431, was found to contain a 3.7-kb *Hin*dIII fragment. For subcloning experiments, the insert of pAT431 was partially digested with *Sau*3AI and cloned into pUC19 cleaved by *Bam*HI to generate pAT432 (Fig. 1). Plasmid pAT432 conferred aminoglycoside resistance in *E. coli* BM694 and contained a 1.5-kb *Sau*3AI insert. *E. coli* BM694/pAT432 was resistant to amikacin, kanamycin, 2'-*N*-ethyl-netilmicin, netilmicin, sisomicin, and tobramycin and susceptible to gentamicin and 6'-*N*-ethyl-netilmicin. This phenotype was consistent with modification of the 6' amino group of aminoglycosides. Analysis by the phosphocellulose paper-binding assay of bacterial extracts indicated that aminoglycoside resistance in *E. coli* BM694/pAT432 was due to an acetylating activity. Kanamycin acetylation was defined as 100%, with gentamicin C1a (110%) and C2 (110%), netilmicin (62%), sisomicin (70%), amikacin (45%), and tobramycin (72%) being acetylated at the percentages indicated; gentamicin C1 was not acetylated. On the basis of the substrate profile, the enzyme encoded by pAT432 is a 6'-*N*-aminoglycoside acetyltransferase. Aminoglycoside phosphotransferase and nucleotidyltransferase activities were not detected.

Sequence of the insert in pAT432 and identification of the *aac(6')-Ii* gene. The DNA sequence of the 1,485-bp insert in pAT432, determined by use of the strategy displayed in Fig. 1, is shown in Fig. 2. The largest open reading frame,

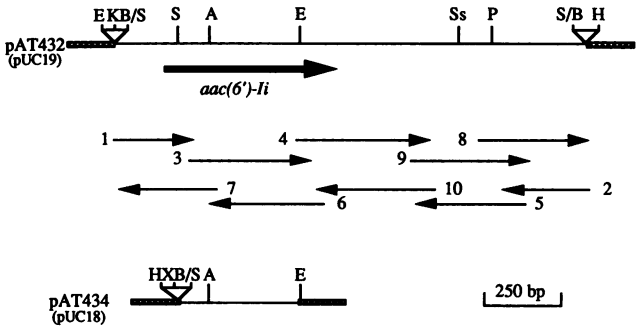

FIG. 1. Partial restriction map of the inserts in pAT432 and pAT434 and sequencing strategy. Thin line, *E. faecium* CIP 54-32 DNA; cross-hatched boxes, pUC DNA. The thick arrow indicates the direction and extent of transcription of the *aac(6')-Ii* gene. Thin arrows indicate the direction and extent of the sequencing reactions with a universal primer (1), a reverse primer (2), and sequence-specific primers (3 to 10): 3, 5'-TACTGAGACTGACTTGG-3' (positions 227 to 242); 4, 5'-CAGAACCTTCGTGAACA-3' (positions 577 to 593); 5, 5'-TGAGGCACGACTAAGTT-3' (positions 1282 to 1266); 6, 5'-GCATTTGGTAAGACACC-3' (positions 651 to 634); 7, 5'-CTCATCTTGGTCTACCG-3' (positions 333 to 227); 8, 5'-GTTA TGGCGGCAGTTCA-3' (positions 1195 to 1211); 9, 5'-GGCTTT-TGGAAGACCAT-3' (positions 947 to 963); and 10, 5'-TCGTGAGG TTTCAAGGA-3' (positions 1016 to 1000). Restriction sites: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*I; S, *Sau*3AI; Ss, *Ssp*I; X, *Xba*I.

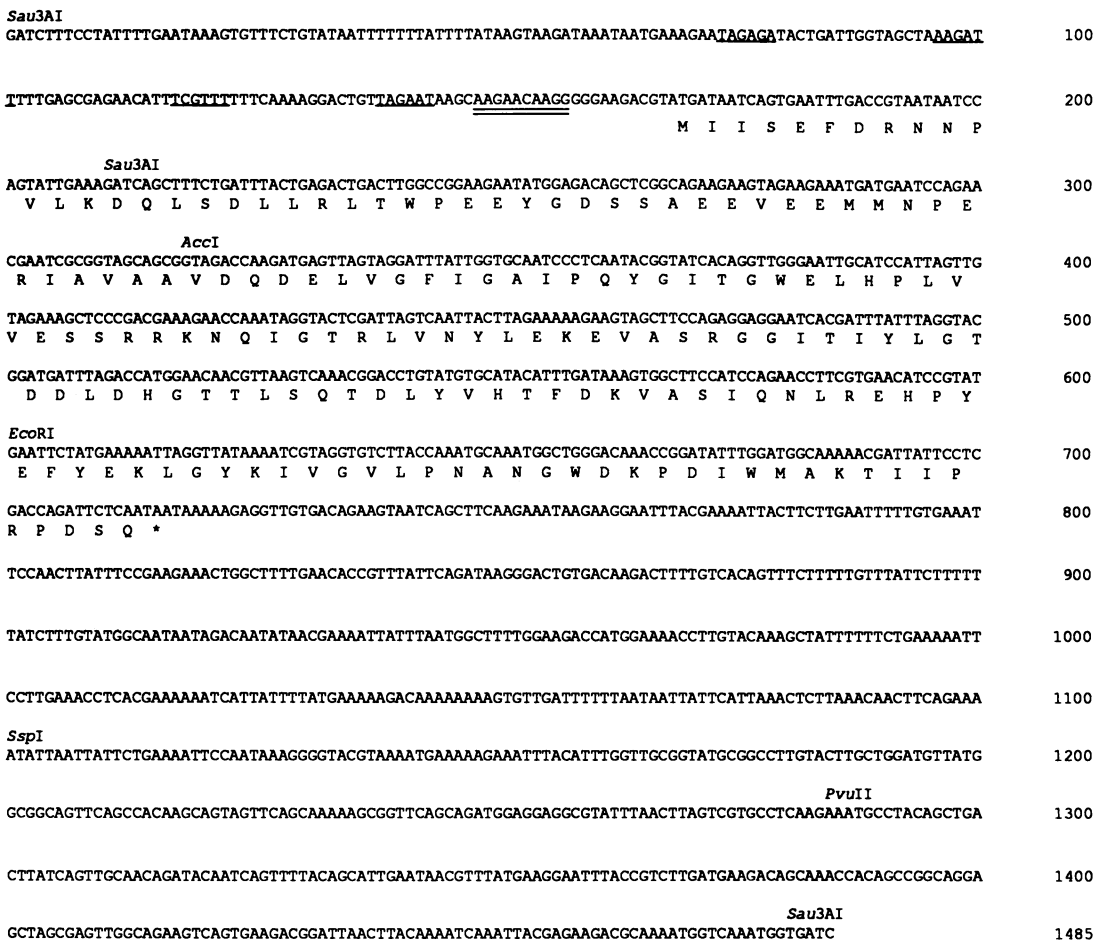

FIG. 2. Nucleotide and deduced amino acid sequences of the 1,485-bp *Sau*3AI insert in pAT432 containing the *aac(6')-Ii* gene. The putative −35 and −10 sequences (two each) are underlined. The putative ribosome binding site is doubly underlined. The TAA stop codon is indicated by an asterisk. Only the restriction sites of the pAT432 insert shown in Fig. 1 are indicated.

spanning 574 nucleotides, was found between the TAA stop codons located at positions 144 and 717. Translation start codon ATG at position 169 was preceded at 10 bp by a ribosome binding site-like sequence, 5′-AAGAACAAGG-3′, complementary at 6 bases (underlined) to the 3′-OH-terminal (5′-UCUUUCCUCC-3′) sequence of *Bacillus subtilis* 16S rRNA (18). Transcriptional promoters can often be found 5′ to the site at which RNA synthesis begins (position +1), within a 40-bp region. Within this region, two consensus sequences (one at −10, consisting of 6 bp, and another at −35, containing 8 to 12 bp) are often found. Analysis of this region revealed two potential −35 motifs, TAGAGA at positions 74 to 79 and TCGTTT at positions 118 to 123, and two potential −10 motifs, AAGATT at positions 96 to 101 and TAGAAT at positions 139 to 144, separated by 16 and 15 nucleotides, respectively (18). The 549-bp sequence designated *aac(6')-Ii*, which extended from the ATG initiation codon at position 169 to the TAA termination codon at position 715, could code for a protein of 182 amino acid residues and with a calculated molecular mass of 20,666 Da. Two 8-bp imperfect inverted repeat sequences separated by 3 bp were present at positions 105 to 123 and overlapped with the potential −35 hexamer TCGTTT. The G+C content of the gene was 41 mol%.

Analysis of the AAC(6′)-Ii enzyme. Plasmid pAT433, constructed by cloning the 1.1-kb *Kpn*I-*Ssp*I fragment of pAT432 into pUC19, conferred resistance to aminoglycosides when introduced into *E. coli* JM83 (data not shown). This plasmid was used as a template in a cell-free coupled transcription-translation system (38). One band of approximately 23,000 Da, a mass that closely approximates the predicted mass of AAC(6′)-Ii, was encoded by pAT433 but not by pUC19 (Fig. 3). Computer analysis of the putative translated product of *aac(6')-Ii* indicated 42.2% identical amino acids and 49.5% conserved amino acid substitutions between AAC(6′)-Ii and AAC(6′)-Ia from *Citrobacter diversus* (31) and *Shigella sonnei*, the latter being recorded only in GenBank (accession number M86913) (Fig. 4). Certain domains were more highly conserved: motif 1 (residues 56 to 78, with 16 identical amino acids), motif 2 (residues 103 to 113, with 8 identical amino acids), and motif 3 (residues 142 to 176, with 25 identical amino acids).

Insertional inactivation of the *aac(6')-Ii* gene. The 391-bp *Sau*3AI-*Eco*RI fragment from plasmid pAT434 (Fig. 1), internal to the *aac(6')-Ii* gene, was cloned into pAT114, a plasmid that cannot replicate in gram-positive bacilli (34), after digestion with *Bam*HI and *Eco*RI. The resulting suicide plasmid, pAT435 (Fig. 5), was introduced into *E. faecium* CIP 54-32 by electrotransformation, and clones expected to result from a single crossover leading to the integration of

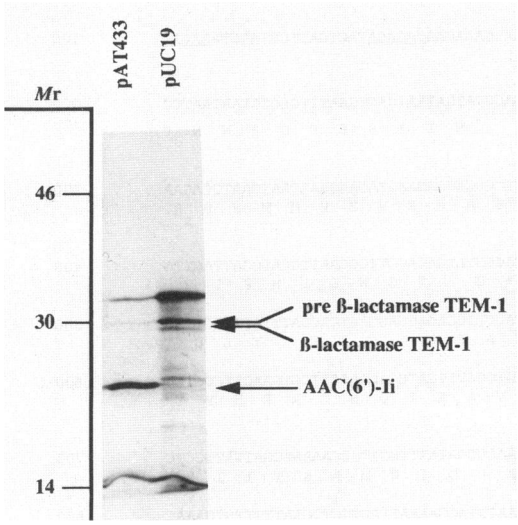

FIG. 3. Autoradiogram of L-[^{35}S]methionine-labeled polypeptides specified in vitro by pAT433 and pUC19. The proteins were electrophoresed in a 15% polyacrylamide gel containing SDS (9). Molecular weights are expressed in thousands. The positions of the AAC(6')-Ii enzyme, β-lactamase, and pre-β-lactamase are indicated.

pAT435 into *aac(6')-Ii* were selected on erythromycin. One clone, BM4229, was compared with CIP 54-32 by Southern hybridization (Fig. 6) with the 391-bp *Sau*3AI-*Eco*RI fragment used as a probe (Fig. 1) and an *aphA-3* probe specific for pAT114 (15). The *aac(6')-Ii* probe hybridized to a 3.7-kb *Hin*dIII fragment of CIP 54-32, whereas no signal was observed with the *aphA-3* probe. The *aac(6')-Ii* probe hybridized to 2.0- and 6.7-kb *Hin*dIII fragments of BM4229, whereas the *aphA-3* probe hybridized only to the 6.7-kb *Hin*dIII fragment. These results indicate that 5.8-kb plasmid pAT435 had been integrated into the *aac(6')-Ii* gene. Determination of aminoglycoside MICs for CIP 54-32 and BM4229 (Table 2) indicated that insertional inactivation of *aac(6')-Ii* abolished resistance to aminoglycosides that are substrates for AAC(6')-Ii.

Distribution of the *aac(6')-Ii* gene in enterococci. The 391-bp *Sau*3AI-*Eco*RI fragment from plasmid pAT434, internal to the *aac(6')-Ii* gene, was used as a probe (Fig. 1). This specific probe hybridized to the DNAs of all 26 strains of *E. faecium* tested by dot blot hybridization under high-stringency conditions (Table 2). No hybridization was observed with any of the 44 other enterococcal strains belonging to 13

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AAC(6')Ia  MNYQIVNIAECSNYQLEAANILTEAFNDLGNNSWPDMTSATKEVKECIESPNLCFGLLINNSLVG   65
AAC(6')Ii  MIISEFDRNNPVLKDQLSDLLRLTWPEEYGDSSAEEVEEMMNPERIAVAAVDQDELVG   58

AAC(6')Ia  WIGLRPMYKET-WELHPLVVRPDYQNKGIGKILLKELENRAREQGIIGIALGTDDEYYRTSLSLI   129
AAC(6')Ii  FIGAIPQYGITGWELHPLVVESSRRKNQIGTRLVNYLEKEVASRGGITIYLGTDDLDHGTTLS-Q   122

AAC(6')Ia  TITEDNIFDSIKNIKNINKHPYEFYQKNGYYIVGIIPNANGKNKPDIWMWKSLIKE   185
AAC(6')Ii  TDLYVHTFDKVASIQNLREHPYEFYEKLGYKIVGVLPNANGWDKPDIWMAKTIIPRPDSQ   182
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FIG. 4. Alignment of the deduced amino acid sequences of AAC(6')-Ii from *E. faecium* CIP 54-32 and of AAC(6')-Ia from *C. diversus* (31). Dashes represent gaps introduced to optimize similarity. Asterisks indicate identical amino acids, and colons indicate conserved amino acids. Regions of high similarity with other AAC(6') enzymes are underlined (14, 28). For classification as conservative substitutions, chemically similar amino acids were grouped as follows: RK, LFPMVI, STQNC, H, ED, and Y.

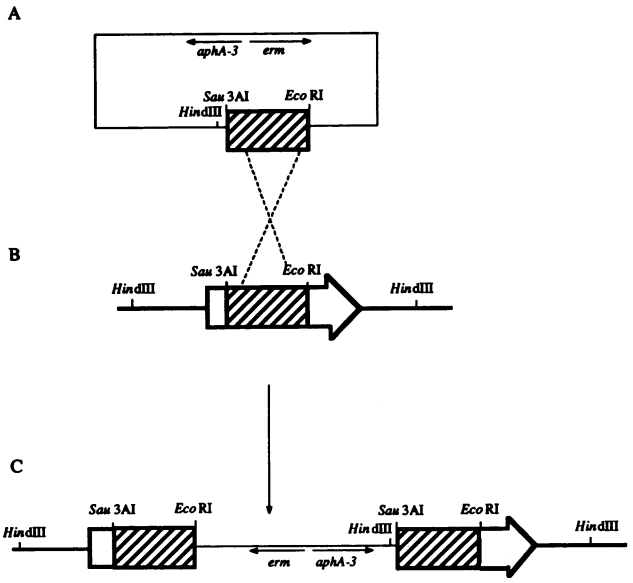

FIG. 5. Insertional inactivation of *aac(6')-Ii*. The *aac(6')-Ii* gene is represented by a large open arrow; the internal 391-bp *Sau*3AI-*Eco*RI fragment is hatched. Thin line, pAT114 DNA; thick lines, CIP 54-32 chromosomal DNA; thin horizontal arrows, antibiotic resistance genes: *aphA-3*, gene encoding 3'-aminoglycoside phosphotransferase; *erm*, gene encoding erythromycin methyltransferase. (A) Plasmid pAT435. (B) *aac(6')-Ii* region in CIP 54-32 chromosomal DNA. (C) *aac(6')-Ii* region after integration of pAT435.

different species (Table 2 and data not shown). Southern blot hybridization was performed with *Acc*I-digested total DNAs of 11 *E. faecium* strains (Fig. 7). There is a single *Acc*I site within the *aac(6')-Ii* gene (Fig. 1). The probe hybridized to 5- and 1.2-kb *Acc*I fragments of CIP 54-32 DNA (Fig. 7, lane 2). The latter fragment corresponds to the 1.2-kb *Acc*I fragment of the insert in pAT431. For the remaining 10 *E. faecium* strains, three hybridization profiles each consisting of two bands, could be distinguished: 7 and 2.75 kb (lane 1), 5 and 1.5 kb (lane 3), and 5 and 1.3 kb (lanes 4 to 11). The last

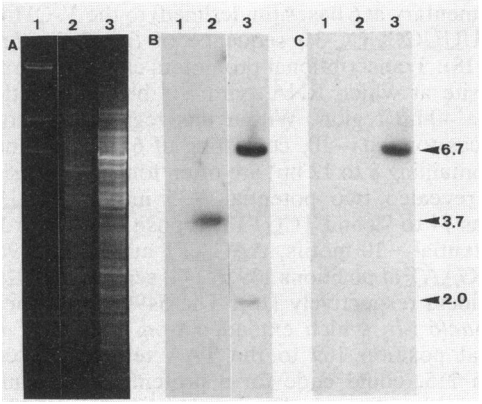

FIG. 6. Southern blot analysis of the integration of pAT435 into the *aac(6')-Ii* gene of CIP 54-32. (A) Total DNAs from CIP 54-32 (lane 2) and BM4229 (lane 3) were digested with *Hin*dIII. Bacteriophage λ DNA digested with *Hin*dIII was used as an internal standard (lane 1). Hybridization was performed with the *aac(6')-Ii* (B) or the *aphA-3* (C) probe. Molecular sizes are indicated in kilobases.

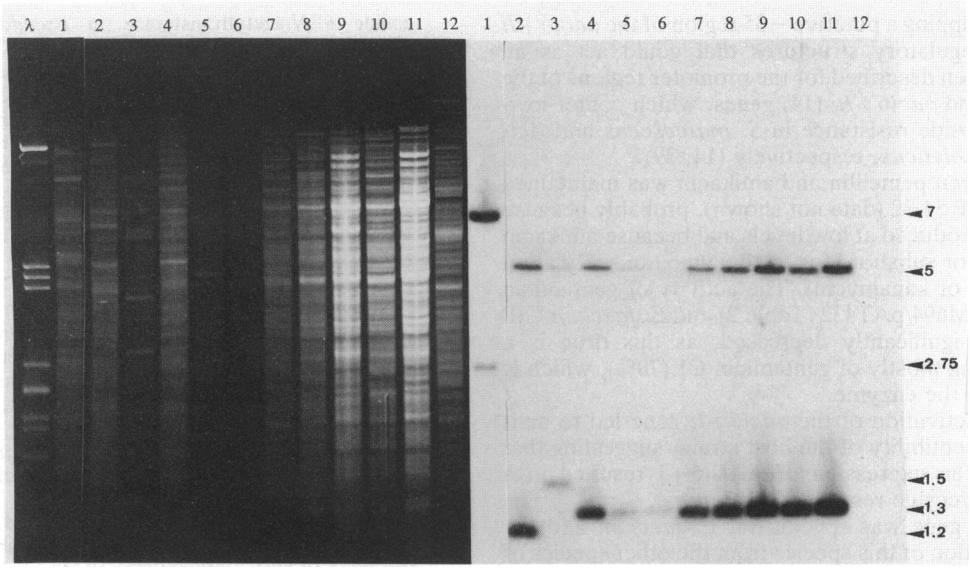

FIG. 7. Analysis of total DNAs by agarose gel electrophoresis (left) and by hybridization (right). Total DNAs were digested with *Acc*I, and the resulting fragments were separated by electrophoresis in an 0.8% agarose gel, transferred to a Nytran filter, and hybridized to the in vitro ^{32}P-labeled *aac(6')-Ii* probe. Bacteriophage λ DNA digested with *Pst*I was used as an internal standard (lane λ). Lanes: 1 to 11, DNAs of *E. faecium* strains; 12, DNA of an *E. faecalis* strain. Molecular sizes are indicated in kilobases.

profile was found for eight strains isolated from four different hospitals located in two countries. There was no correlation between hybridization profiles and aminoglycoside MICs.

DISCUSSION

We have cloned in *E. coli* and determined the sequence of the structural gene for the AAC(6')-I enzyme of *E. faecium* CIP 54-32. Acetylation of amikacin and gentamicins C1a and C2 but not of gentamicin C1 by bacterial extracts of *E. coli* BM694/pAT432 indicated that this acetyltransferase belongs to the AAC(6')-I family (23) and was therefore named AAC(6')-Ii. By contrast, members of the AAC(6')-II family of enzymes modify all gentamicin C compounds but not amikacin (23). To date, nine *aac(6')-I* genes have been cloned, and eight have been sequenced. Two *aac(6')-II* genes have also been cloned and sequenced. On the basis of a comparison of deduced amino acid sequences, three AAC(6') subfamilies can be distinguished (28). The first described and largest subfamily is composed of three groups: AAC(6')-Ib from *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Serratia marcescens* (9, 20, 33); AAC(6')-Ie, which is a portion of the amino-terminal domain of the bifunctional 2″-aminoglycoside phosphotransferase–6′-aminoglycoside acetyltransferase enzyme from *E. faecalis* (8); and AAC(6')-IIa and AAC(6')-IIb from *P. aeruginosa* (27, 28). The second described subfamily comprises AAC(6')-Ic from *S. marcescens* (29), AAC(6')-Id from *K. pneumoniae* (26), AAC(6')-If from *Enterobacter cloacae* (32), and AAC(6')-Ig and AAC(6')-Ih from *Acinetobacter* spp. (13, 14). The third described subfamily includes a single member, AAC(6')-Ia from *C. diversus* (31) and *S. sonnei* (GenBank accession number: M86913). The AAC(6')-Ia enzyme is distantly related to enzymes of the other subfamilies; only AAC(6')-Ii displayed significant similarity to AAC(6')-Ia.

The base composition of the chromosomal *aac(6')-Ii* gene (41% G+C) is similar to that of the *E. faecium* genome (38.3 to 39%). The G+C content of *aac(6')-Ia* (32%) is significantly different from those of the *C. diversus* (51%) and *S. sonnei* chromosomes (49 to 53%), in which it is part of a Tn*21*-like transposon. Taken together, these observations suggest that the *aac(6')-Ia* gene could have originated in *Enterococcus* spp. and represents an additional example of the transfer of genetic information from gram-positive cocci to gram-negative bacilli (35).

A hydrophobic region consisting of the pentapeptide LHPLV at positions 73 to 77 and composed of three hydrophobic amino acids, two leucines and a valine, is located in the central portion of the AAC(6')-Ii and AAC(6')-Ia enzymes. Similar central hydrophobic portions have been found in the other AAC(6') enzymes and proposed to be the aminoglycoside binding domain (28). The ability to modify amikacin [AAC(6')-Ib] or gentamicin [AAC(6')-IIa] is thought to be due to the presence of a leucine or a serine as the fourth residue of this pentapeptide, respectively (23). In AAC(6')-Ia and AAC(6')-Ii, the fourth residue of the LHPLV pentapeptide is a leucine (underlined), which might be responsible for the ability of the enzyme to modify amikacin but not gentamicin. Another large region at positions 85 to 98 of AAC(6')-Ii (Fig. 4) is relatively conserved in the other AAC(6') proteins (14, 28). Within this motif, a leucine residue at position 91 is conserved in the nine other AAC(6') enzymes, except for AAC(6')-Ie (14, 28). It has been demonstrated that a leucine-to-serine substitution at this site in AAC(6')-Ic leads to a specific loss of amikacin resistance, suggesting that this region may be involved in substrate binding (28).

In *E. faecium* CIP 54-32, the expression of the AAC(6')-I enzyme resulted in a specific aminoglycoside resistance phenotype (Table 2) and the lack of synergism between penicillin and kanamycin, netilmicin, sisomicin, or tobramycin (data not shown). We could not, however, detect enzyme activity in cell lysates prepared from this strain. This result is in agreement with the notion that *E. faecium* strains generally produce low levels of AAC(6')-I that are barely detectable unless highly productive variants are selected (19, 36). The low level of AAC(6')-I production in *E. faecium* could be explained by the presence of inverted repeat

sequences overlapping a putative −35 region of the *aac(6')-Ii* gene. Similar regulatory structures that could act as an operator have been described for the promoter regions of the *aac(6')-Ic* (29) and *aac(6')-Ig* (14) genes, which confer low-level aminoglycoside resistance in *S. marcescens* and *Acinetobacter haemolyticus*, respectively (14, 29).

Synergy between penicillin and amikacin was maintained in *E. faecium* CIP 54-32 (data not shown), probably because AAC(6')-I was produced at low levels and because amikacin is a relatively poor substrate for the enzyme (an acetylation rate 45% of that of kanamycin). The activity of gentamicin against *E. coli* BM694/pAT432 (Table 2) and *E. faecium* CIP 54-32 was not significantly decreased, as this drug is a mixture consisting mostly of gentamicin C1 (70%), which is not substrate for the enzyme.

Insertional inactivation of the *aac(6')-Ii* gene led to aminoglycoside susceptibility of the host strain, suggesting that in *E. faecium*, the expression of AAC(6')-I resulted in a specific aminoglycoside resistance phenotype.

The *aac(6')-Ii* gene was specific for *E. faecium* and enabled discrimination of this species from the other species of enterococci. Thus, the *aac(6')-Ii* probe could be helpful in discriminating species that differ only by a few physiological characters, such as *E. casseliflavus*, *E. faecium*, and *E. gallinarum* (7). Southern hybridization experiments indicated that the resistance gene was not always located in the same *E. faecium* genomic environment. In certain cases, heterogeneity of a hybridization profile could be used in epidemiologic studies based on restriction fragment length polymorphisms.

ACKNOWLEDGMENTS

We thank F. Baquero, L. Devriese, J. P. Gayral, T. Horaud, and G. Prats for the gifts of strains, C. Carlier and E. Derlot for technical assistance, and A. Smith for critical reading of the manuscript.

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