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A New High-Level Gentamicin Resistance Gene, *aph(2″)-Id*, in *Enterococcus* spp.

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***Enterococcus casseliflavus* UC73 is a clinical blood isolate with high-level resistance to gentamicin. DNA preparations from UC73 failed to hybridize with intragenic probes for *aac(6′)-Ie-aph(2″)-Ia* and *aph(2″)-Ic*. A 4-kb fragment from UC73 was cloned and found to confer resistance to gentamicin in *Escherichia coli* DH5α transformants. Nucleotide sequence analysis revealed the presence of a 906-bp open reading frame whose deduced amino acid sequence had a region with homology to the aminoglycoside-modifying enzyme APH(2″)-Ic and to the C-terminal domain of the bifunctional enzyme AAC(6′)-APH(2″). The gene is designated *aph(2″)-Id*, and its observed phosphotransferase activity is designated APH(2″)-Id. A PCR-generated intragenic probe hybridized to the genomic DNA from 17 of 118 enterococcal clinical isolates (108 with high-level gentamicin resistance) from five hospitals. All 17 were vancomycin-resistant *Enterococcus faecium* isolates, and pulsed-field typing revealed three distinct clones. The combination of ampicillin plus either amikacin or neomycin exhibited synergistic killing against *E. casseliflavus* UC73. Screening and interpretation of high-level aminoglycoside resistance in enterococci may need to be modified to include detection of APH(2″)-Id.**

High-level gentamicin resistance (MIC ≥ 2,000 μg/ml) in enterococci is known to be associated with the *aac(6′)-Ie-aph(2″)-Ia* gene, which encodes the bifunctional aminoglycoside-modifying enzyme AAC(6′)-APH(2″) (14). The presence of this gene eliminates the synergism between a cell wall-active agent, such as ampicillin or vancomycin, and virtually all commercially available aminoglycosides—including gentamicin, tobramycin, netilmicin, kanamycin, and amikacin—except streptomycin (17). *aph(2″)-Ic* is a midlevel gentamicin resistance gene (MIC = 256 μg/ml), found less commonly than *aac(6′)-Ie-aph(2″)-Ia* in enterococci, that eliminates the synergism between ampicillin and gentamicin (6). We describe a new high-level gentamicin resistance gene initially found in *Enterococcus casseliflavus* that is distinct from *aac(6′)-Ie-aph(2″)-Ia* and *aph(2″)-Ic*.

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MATERIALS AND METHODS

Bacterial strains, media, and antimicrobial susceptibilities. Enterococci were identified by conventional biochemical criteria (13). UC73 is an *E. casseliflavus* blood isolate from a patient in Chicago, Ill. (20). *Escherichia coli* DH5α was used as the recipient for electroporation and the host to maintain recombinant plasmids. *Enterococcus faecium* GE1 (12) and *Enterococcus faecalis* FA2-2 (7) were the recipient strains in mating experiments. Gentamicin was obtained from Fluka (Buchs, Switzerland). Netilmicin, 6′-*N*-ethylnetilmicin, and 5-episisomicin were a gift from Karen J. Shaw (Schering-Plough Research Institute, Kenilworth, N.J.). All other antimicrobial agents were obtained from Sigma Chemical Company (St. Louis, Mo.). Transformants from electroporation were selected on Luria-Bertani plates containing gentamicin (20 μg/ml). Filter matings were performed as previously described (23). Antimicrobial susceptibilities were determined by a standardized broth microdilution method (28). Tests of synergistic killing were performed at least in triplicate to ensure reproducibility of results and were done by previously described methods (19). Synergism was defined as a $\geq$2-$\log_{10}$ decrease in the number of CFU per milliliter between the combination (ampicillin plus aminoglycoside) and its most active constituent (ampicillin) after 24 h (the aminoglycoside had no effect on the growth curve); the number of surviving organisms in the presence of the combination was $\geq$2-$\log_{10}$ CFU/ml below that in the starting inoculum. One hundred eighteen enterococcal clinical isolates (108 with high-level gentamicin resistance) from five Detroit, Mich., area hospitals were obtained to screen for the presence of the new gene.

DNA preparation and cloning. Plasmid DNA minipreparations and total genomic DNA were obtained by a modified alkaline lysis method (27). Restriction endonuclease digestion, agarose gel electrophoresis of DNA, contour-clamped homogeneous electric field (CHEF) electrophoresis of genomic DNA, and electroporation were performed as previously described (3, 10). DNA typing of isolates was done by visual inspection of gel bands by using published criteria (22). For detection of DNA-DNA homology, biotin-labeled probes were prepared as instructed by the manufacturer (GIBCO BRL Life Technologies, Gaithersburg, Md.). The probe for *aac(6′)-Ie-aph(2″)-Ia* was a 1.5-kb *Alu*I fragment from *E. faecalis* plasmid pSF815A (14). The probe for *aph(2″)-Ic* was generated by PCR as previously reported (6). DNA was transferred to MagnaGraph nylon membranes (Micron Separation, Inc., Westboro, Mass.) by the method of Southern and exposed to probes for hybridization (11). DNA to be sequenced was obtained as described in the Qiagen plasmid handbook (Qiagen, Inc., Chatsworth, Calif.). The vector pBluescript II KS+ (Stratagene Cloning Systems, La Jolla, Calif.) was used in standard cloning experiments (3).

DNA sequencing and PCR. Nested deletions of cloned DNA were made by using the Erase-a-Base System from Promega (Madison, Wis.). The nucleotide sequences of both strands were determined by a modification of the dideoxynucleotide chain termination method with a Sequenase kit (version 1.0; United States Biochemical, Cleveland, Ohio) and [α-^{32}P]dATP (Amersham Life Science, Arlington Heights, Ill.) (15, 21). PCR was performed with a GeneAmp PCR Reagent kit with AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.) (15). Computer analysis was performed by using MacVector software, version 6.0, and AssemblyLIGN, version 1.0 (Oxford Molecular Group, Oxford, United Kingdom). The GenBank database was searched by using the BLAST program from the National Center for Biotechnology Information (1). Amino acid sequences were compared by using the Gap Analysis Program from the University of Wisconsin Genetics Computer Group, version 8.1 (9).

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TABLE 1. Susceptibilities of *E. casseliflavus* UC73, *E. coli* NC95, and *E. coli* DH5α(pBluescript II KS+) to aminoglycosides

Aminoglycoside	MIC (μg/ml)		
	UC73	NC95[a]	DH5α (pBluescript II KS+)
Gentamicin	>2,000	128	0.25
Dibekacin	>2,000	64	1
Tobramycin	>2,000	16	0.25
6′-*N*-Ethylnetilmicin	>2,000	32	0.5
Kanamycin	1,024	16	1
5-Episisomicin	1,024	32	0.12
Netilmicin	64	2	0.03
Neomycin	16	0.31	0.31
Amikacin	32	0.25	0.12

[a] DH5α containing pBluescript II KS+ into which a 4-kb fragment containing *aph(2″)-Id* had been cloned.

Enzyme assays. Aminoglycoside phosphotransferase activity was confirmed through a modified phosphocellulose paper binding assay as previously described (6, 18). A substrate was considered to be modified if its radioactive counts were greater than five times those of negative controls (8).

Nucleotide sequence accession number. The nucleotide sequence data for the new high-level gentamicin resistance gene, *aph(2″)-Id*, are available from GenBank under accession no. AF016483.

RESULTS

Microbiological characterization. Aminoglycoside MICs for *E. casseliflavus* UC73 are shown in Table 1. The ampicillin MIC was 1.0 μg/ml, and the streptomycin MIC was 32 μg/ml. Ampicillin at 0.5 μg/ml was used in synergy studies of UC73 because ampicillin alone at 1.0 μg/ml exhibited too much killing at 24 h to easily detect synergistic killing. Synergistic killing was not seen for UC73 in time-kill studies using ampicillin (0.5 μg/ml) and either gentamicin (16 μg/ml) or netilmicin (16 μg/ml). Synergistic killing was exhibited against UC73, however, when ampicillin (0.5 μg/ml) was combined with amikacin (8 and 16 μg/ml) or with neomycin (8 and 16 μg/ml).

The probes for *aac(6′)-Ie-aph(2″)-Ia* and *aph(2″)-Ic* did not hybridize to Southern blots of total cellular DNA from *E. casseliflavus* UC73. Filter matings with UC73 as the donor and *E. faecium* GE1 or *E. faecalis* FA2-2 as the recipient did not result in transfer of gentamicin resistance (frequency, $<10^{-9}$ per recipient CFU). Electroporation of a plasmid preparation from UC73 into competent *E. faecalis* FA2-2 cells also did not result in the selection of gentamicin-resistant transformants.

Cloning and expression of the gentamicin resistance gene. Partial *Sau*3AI digestions of total genomic DNA from UC73 were ligated to pBluescript II KS+ digested with *Bam*HI. After electroporation of the ligated products, selection for gentamicin-resistant transformants yielded an *E. coli* DH5α derivative that contained a 7.5-kb cloned fragment. Further subcloning resulted in a gentamicin-resistant transformant, named NC95, which contained a 4-kb cloned fragment. Aminoglycoside MICs for NC95 and DH5α(pBluescript II KS+) are shown in Table 1.

Nucleotide sequencing of the gentamicin resistance gene. Nucleotide sequencing revealed the presence of only one open reading frame (ORF) whose predicted amino acid sequence showed homology with aminoglycoside-modifying enzymes (Fig. 1). Only recombinants that contained the entire ORF (906 bp), and not recombinants that contained a part of the ORF, were gentamicin resistant. Gap analysis revealed 54.4% similarity and 31.1% identity of APH(2″)-Id with AAC(6′)-APH(2″) and 48.6% similarity and 28% identity with APH(2″)-Ic. This gentamicin resistance gene was designated *aph(2″)-Id.*

Determination of phosphotransferase activity. The crude extract prepared from negative-control *E. coli* DH5α(pBluescript II KS+) showed no phosphorylation of gentamicin over time (86, 95, and 95 cpm at 1, 5, and 15 min, respectively), whereas the reaction mixture containing extract from NC95 showed a significant increase in radioactivity observed over time (2,795, 6,215, and 6,415 cpm at 1, 5, and 15 min, respectively), thus showing that gentamicin phosphotransferase activity, designated APH(2″)-Id, is associated with the presence of *aph(2″)-Id.*

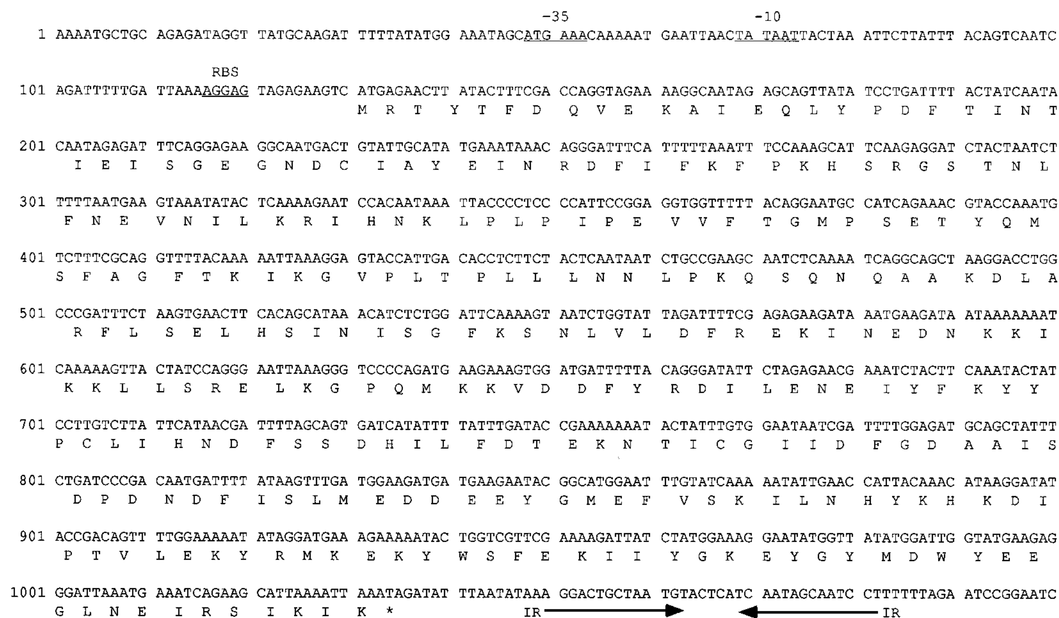

FIG. 1. Nucleotide sequence of *aph(2″)-Id* from *E. casseliflavus* UC73. The potential −35 and −10 sites and the potential ribosome binding site (RBS) are underlined. The stop codon of the ORF is designated by an asterisk. Inverted repeats (IR) (two mismatched nucleotides) are designated by arrows.

Identification of the new gentamicin resistance gene in *E. faecium*. An 849-bp intragenic probe for *aph(2″)-Id* was generated by PCR with synthetic oligonucleotide primers (5′-GACCAGGTAGAAAAGGCAATAGAGCAG-3′ and 5′-ATACCAATCCATATAACCATATTCCTT-3′). The probe hybridized to the Southern blots of total cellular DNA from 17 of 118 enterococcal clinical isolates. These 118 isolates were composed of 108 with high-level resistance to gentamicin (78 *E. faecalis* isolates, 28 *E. faecium* isolates, 1 *E. raffinosus* isolate, and 1 *E. gallinarum* isolate) and 10 that were gentamicin sensitive (1 *E. faecalis* isolate and 9 *E. faecium* isolates). Forty of the 118 isolates were vancomycin resistant (6 *E. faecalis* isolates, 33 *E. faecium* isolates, and 1 *E. gallinarum* isolate). All 17 isolates positive for the *aph(2″)-Id* probe were vancomycin-resistant *E. faecium* and came from 16 patients in four of the five hospitals. The DNA from these 17 *E. faecium* isolates did not hybridize to the *aac(6′)-Ie-aph(2″)-Ia* probe. CHEF gel electrophoresis showed three distinct strain types (data not shown). The probe also hybridized to the DNA on the CHEF gel from all 17 isolates. The 17 *E. faecium* isolates were ampicillin resistant (MIC range, 64 to 256 μg/ml). The amikacin MICs ranged from 256 to 512 μg/ml, and the netilmicin MICs ranged from 512 to ≥2,000 μg/ml. In contrast to results for *E. casseliflavus* UC73, tests of synergism performed on the two isolates (NC103 and SF13485) for which the ampicillin MICs were the lowest (64 μg/ml), in which ampicillin was used at 64 μg/ml and amikacin was used at 32 μg/ml, showed less than a 2-$\log_{10}$ difference in killing with the combination compared to the most active agent alone (average 1.34- and 0.65-$\log_{10}$ difference, respectively).

DISCUSSION

To date, high-level gentamicin resistance in enterococci has been associated only with the presence of the *aac(6′)-Ie-aph(2″)-Ia* gene. Therefore, clinical laboratories test enterococcal isolates for high-level aminoglycoside resistance by using only gentamicin and streptomycin, since enterococci resistant to gentamicin are assumed to be resistant to the other clinically available aminoglycosides, except streptomycin. Our small survey showed that 16% (17 of 108) of enterococci with high-level gentamicin resistance possess *aph(2″)-Id* and not *aac(6′)-Ie-aph(2″)-Ia*. If these results are confirmed in more-extensive surveys, clinical laboratories may need to add amikacin to their screening protocols, since *aph(2″)-Id* does not confer high-level resistance to amikacin and ampicillin-amikacin synergistic killing of *E. casseliflavus* UC73, which contains *aph(2″)-Id*, has been shown. *E. faecium* NC103 and *E. faecium* SF13485 both contain *aph(2″)-Id*, but the ampicillin and amikacin MICs for these strains were high, which may explain why they were not killed as effectively by ampicillin-amikacin as *E. casseliflavus* UC73 was. The high amikacin MICs (256 to 512 μg/ml) for the 17 *E. faecium* isolates containing *aph(2″)-Id* may be due to the presence of another aminoglycoside resistance gene(s). Although an enterococcus for which the amikacin MIC is <256 μg/ml might contain *aph(2″)-Id* and be susceptible to killing by ampicillin-amikacin, a low amikacin MIC does not always imply susceptibility to synergism. Enterococci that possess the aminoglycoside resistance gene *aph(3′)-IIIa*, *aac(6′)-Ie-aph(2″)-Ia*, or *ant(4′)-Ia* are resistant to ampicillin-amikacin synergism but may not have high-level resistance to amikacin (MICs as low as 64 to 256 μg/ml) (2, 4, 5, 16, 17, 24). Tests for synergistic killing may thus prove to be a useful adjunct to confirm the utility of amikacin in combination therapy for isolates thought to contain *aph(2″)-Id*. Alternatives might include the use of probes or PCR to test for the presence of *aph(2″)-Id* and for the absence of the other three aminoglycoside resistance genes.

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