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A Novel Gentamicin Resistance Gene in *Enterococcus*

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***Enterococcus gallinarum* SF9117 is a veterinary isolate for which the MIC of gentamicin is 256 μg/ml. Time-kill studies with a combination of ampicillin plus gentamicin failed to show synergism against SF9117. A probe representing *aac(6′)-aph(2″)* did not hybridize to DNA from SF9117. A 3.2-kb fragment from plasmid pYN134 of SF9117 was cloned and conferred resistance to gentamicin in *Escherichia coli* DH5α. Nucleotide sequence analysis revealed the presence of a 918-bp open reading frame whose deduced amino acid sequence had a region with homology to the C-terminal domain of the bifunctional enzyme AAC(6′)-APH(2″). The gene is designated *aph(2″)-Ic*, and its observed phosphotransferase activity is provisionally designated APH(2″)-Ic. An intragenic probe hybridized to the genomic DNA from an *Enterococcus faecium* isolate from the peritoneal fluid of one patient and to the plasmid DNA of an *Enterococcus faecalis* isolate from the blood of another patient. An enterococcal isolate containing this novel resistance gene might not be readily detected in clinical laboratories that use gentamicin at 500 or 2,000 μg/ml for screening for high-level resistance.**

Optimal therapy of serious enterococcal infections usually involves synergistic combinations of an agent interfering with cell wall biosynthesis, such as ampicillin or vancomycin, plus an aminoglycoside. Gentamicin has been the preferred aminoglycoside; however, the increasing prevalence of high-level resistance to gentamicin (as high as 70% in a national survey [15]), which eliminates this synergistic bactericidal effect, has compromised the effectiveness of therapy. Alternatively, the use of streptomycin may be considered for enterococci that are resistant to high concentrations of gentamicin; however, in many isolates with high-level resistance to gentamicin, there is coexisting high-level resistance to streptomycin (9). Currently, high-level gentamicin resistance in enterococci is known to be mediated by one specific gene, *aac(6′)-aph(2″)*, which encodes a bifunctional aminoglycoside-modifying enzyme, AAC(6′)-APH(2″) (14). We describe the characterization of a new gentamicin resistance gene initially found in an *Enterococcus gallinarum* isolate.

(This work was presented in part at the 95th General Meeting of the American Society for Microbiology, Washington, D.C., 21 to 25 May 1995 [4] and the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, Calif., 17 to 20 September 1995 [5].)

MATERIALS AND METHODS

Bacteria, media, antimicrobial susceptibilities and mating and curing procedures. SF9117 is a strain of *E. gallinarum* isolated from an abdominal wound infection in a horse at the University of Pennsylvania. It was identified by using conventional biochemical and motility criteria (12). *Enterococcus faecalis* FA2-2 (8) was used as the plasmid-free recipient strain in mating experiments. *E. faecalis* SF339 and SF350 (23), which are highly resistant to gentamicin, and *E. faecalis* SF245 and *Enterococcus faecium* GE-1 (13), which lack high-level gentamicin resistance, were used as control strains in hybridization experiments with an *aac(6″)-aph(2″)* probe. *E. gallinarum* SF6374, which also lacks high-level gentamicin resistance, was used as a control strain in synergism experiments. *Escherichia coli* DH5α was the host used to maintain recombinant plasmids. *E. coli* was grown in Luria broth, and enterococci were grown in Todd-Hewitt broth (Difco, Detroit, Mich.). Netilmicin, 6′-*N*-ethylnetilmicin, 5-episisomicin, and isepamicin were provided by Schering-Plough Research Institute (Kenilworth, N.J.). Dibekacin was obtained from Meiji (Tokyo, Japan). All other antibiotics were obtained from Sigma Chemical Company (St. Louis, Mo.). Antimicrobial susceptibilities were determined by a standardized broth microdilution method (26); brain heart infusion broth (Difco) was used for enterococci, and Mueller-Hinton broth (Difco) was used for *E. coli*. Screening for high-level gentamicin resistance was performed by using Synergy Quad Plates (Remel, Lenexa, Kans.), which contain gentamicin at 500 μg/ml. Time-kill synergism experiments were performed by previously described methods (18). Cross-streak matings and filter matings were performed as described previously (24). Curing experiments were performed as described previously (7). Thirty-nine consecutive clinical isolates from blood were obtained from William Beaumont Hospital, Royal Oak, Mich., and were used to screen for the presence of the new gentamicin resistance gene.

Preparation of DNA and cloning. Plasmid and chromosomal DNAs were isolated by CsCl-ethidium bromide density gradient centrifugation (8). Plasmid DNA minipreparations were also extracted by a modified alkaline lysis method (25). Methods for restriction endonuclease digestion, agarose gel electrophoresis of DNA, contour-clamped homogeneous electric field electrophoresis of genomic DNA, and electroporation were performed as published previously (2, 11). For detection of DNA-DNA homology, probes were biotin labeled with a nick translation kit (BRL Life Technologies, Gaithersburg, Md.), and DNA was transferred to nitrocellulose by the method of Southern and was exposed to probes for hybridization (12). Nested deletions of cloned DNA were made by using the Erase-a-Base System from Promega (Madison, Wis.). DNA to be sequenced was obtained by a modification of the alkaline lysis method as described in the QIAGEN plasmid handbook (Qiagen, Inc., Chatsworth, Calif.). Gel purification of restriction fragments was performed by using a Qiaex gel purification kit (Qiagen).

DNA sequencing and PCR. The nucleotide sequences of both strands were determined by a modification of the dideoxynucleotide chain termination method with a Sequenase, version 1.0, kit (United States Biochemicals, Cleveland, Ohio) and [α-^{32}P]dATP (16, 22). pBluescript II KS$^+$ (Stratagene Cloning Systems, La Jolla, Calif.) was the plasmid vector used in standard cloning experiments (2). Oligonucleotide primers were synthesized by the DNA Core Facility at the University of Michigan. PCR was performed with a GeneAmp PCR Reagent kit with AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.) (16). Computer analysis was carried out with MacVector software, version 4.5. 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 and the Pileup Multiple Sequence Analysis Program software package of the University of Wisconsin Genetics Computer Group, version 8.1 (10).

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TABLE 1. Susceptibilities of *E. gallinarum* SF9117, *E. gallinarum* NC46 (plasmid-free derivative of SF9117), *E. coli* DH5α (pAM6306), and *E. coli* DH5α to aminoglycosides

Aminoglycoside	MIC (μg/ml)			
	SF9117	NC46	DH5α(pAM6306)	DH5α
Gentamicin	256	≤2	128	0.12
Tobramycin	>1,024	16	32	0.25
6′-*N*-Ethylnetilmicin	128	32	256	2
5-Episisomicin	512	16	128	1
Kanamycin	64	128	32	1
Netilmicin	4	4	8	0.03
Dibekacin	32	16	4	0.5
Amikacin	32	32	2	0.12
Neomycin	16	16	2	0.5
Isepamicin	64	64	1	1
Streptomycin	>2,000	>2,000	1	1

Enzyme assays. Aminoglycoside phosphotransferase activity was determined by the phosphocellulose paper binding assay (with gentamicin at 0.17 mM) as described previously (17), except that reaction mixtures included 0.2 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 7.0), 0.03 M $MgCl_2$, 0.0025 M dithiothreitol, and 0.4 mM [γ-^{32}P]ATP. Aliquots were removed from the reaction mixture at 1, 5, and 15 min for determination of the amount of phosphorylated gentamicin that had bound to the phosphocellulose filters.

Nucleotide sequence accession number. The nucleotide sequence data for the new gentamicin resistance gene are available from GenBank under accession number U51479.

RESULTS AND DISCUSSION

Microbiological characterization of SF9117 and its plasmid, pYN134. The MIC of gentamicin for SF9117 was 256 μg/ml, and the MIC of ampicillin was 8 μg/ml. SF9117 also exhibited high-level resistance to streptomycin (MIC, >2,000 μg/ml). No synergism was exhibited in time-kill studies with SF9117 when ampicillin at 8 μg/ml was combined with gentamicin at 16 μg/ml. Against another *E. gallinarum* strain, strain SF6374 (ampicillin MIC, 8 μg/ml; gentamicin MIC, 16 μg/ml), synergistic killing was shown when ampicillin at 2 μg/ml was combined with gentamicin at 4 μg/ml. SF9117 was killed synergistically when ampicillin at 4 μg/ml was combined with netilmicin at 2 μg/ml, amikacin at 16 μg/ml, or isepamicin at 16 μg/ml. It is interesting that while synergism was seen with netilmicin, there is evidence that the enzyme had some effect on netilmicin, at least for *E. coli* (Table 1). No synergism was shown when ampicillin at 8 μg/ml was combined with amikacin at 8 μg/ml or dibekacin at 8 μg/ml. Plasmid analysis of *E. gallinarum* SF9117 revealed the presence of a single 34-kb plasmid, which was named pYN134. Overnight filter matings with SF9117 as the donor and *E. faecalis* FA2-2 as the recipient, with selection for resistance to gentamicin (100 μg/ml), rifampin, and fusidic acid, resulted in transconjugants at a frequency of 2.0×10^{-3} per recipient CFU. All of the transconjugants screened contained a 34-kb plasmid. A probe made from the 1.5-kb *Alu*I fragment from *E. faecalis* plasmid pSF815A (14), which contains *aac(6′)-aph(2″)*, failed to hybridize to either the chromosomal or plasmid DNA of SF9117.

Curing experiments with novobiocin performed with *E. gallinarum* and *E. faecalis* FA2-2(pYN134) (named SF9600; gentamicin MIC, 256 μg/ml) resulted in plasmid-free *E. gallinarum* strains (one of which was designated NC46) that also lost resistance to gentamicin (MIC, ≤2 μg/ml) and plasmid-free *E. faecalis* FA2-2 (MIC, 16 μg/ml). Table 1 lists the MICs of 11 aminoglycosides for SF9117 and NC46. No synergism was exhibited in time-kill studies with *E. faecalis* SF9600 (ampicillin MIC, 1.0 μg/ml) when ampicillin at 1.0 and 2.0 μg/ml was combined with gentamicin at 8 and 64 μg/ml.

Cloning and expression of the gentamicin resistance gene in *E. coli* DH5α. A 6.5-kb *Hin*dIII fragment from pYN134 was cloned into pBluescript II KS$^+$ and was introduced into *E. coli* DH5α by electroporation. The MIC of gentamicin was 128 μg/ml for all transformants. Further subcloning of the insert in pBluescript II KS$^+$ resulted in a plasmid that contained a 3.2-kb *Hin*dIII-*Sst*I fragment and that could confer gentamicin resistance (MIC, 128 μg/ml) in *E. coli* DH5α. This plasmid was named pAM6306. The MICs of 11 aminoglycosides for *E. coli* DH5α with and without pAM6306 are listed in Table 1.

Nucleotide sequencing. Nested deletions from the *Hin*dIII site and the *Sst*I site were made from pAM6306 by using exonuclease III and S1 nuclease. Sequencing was performed in both directions by using a combination of nested deletions and synthesized oligonucleotide primers to fill in the gaps. The nucleotide sequence analysis suggested that a 918-bp open reading frame (ORF) with a G+C content of 44% encoded resistance (Fig. 1). *E. coli* DH5α harboring recombinant plasmids containing this ORF was resistant to gentamicin (MIC, 128 μg/ml); recombinants encompassing only a portion of this ORF failed to confer gentamicin resistance to their hosts (MIC, 0.125 μg/ml).

The deduced amino acid sequence of this ORF was compared with the sequences in the GenBank database. The BLAST program identified homology with the bifunctional enzyme AAC(6′)-APH(2″) (GenBank accession number M13771), a viomycin phosphotransferase (VPH) from *Streptomyces vinaceus* (3) (GenBank accession number X02393), and an unknown hypothetical protein (ORF 9) from *Bacillus subtilis* (GenBank accession number D30808). Results of Gap Analysis comparing the ORF sequence with the sequences of these three proteins were as follows: 53.1% similarity and 24.6% identity with AAC(6′)-APH(2″), 50.9% similarity and 23.3% identity with VPH, and 49.8% similarity and 25.1% identity with ORF 9. Pileup Analysis comparing the ORF sequence with predicted protein sequences from all known aminoglycoside resistance genes found homology specifically with the C-terminal domain, APH(2″)-Ia, of the bifunctional enzyme. Within a 49-amino-acid region of the ORF, there was 56.5% identity with APH(2″)-Ia. This 49-amino-acid region contains motifs 1 and 2, which are conserved among members of the aminoglycoside phosphotransferase family (21). The ORF also shares homology with APH(2″)-Ib, a putative aminoglycoside phosphotransferase from *E. coli* SCH2111602 (19). These data suggest that this gentamicin resistance gene, which we have designated *aph(2″)-Ic*, may encode a putative novel phosphorylating enzyme, APH(2″)-Ic.

Determination of phosphotransferase activity in *E. coli* DH5α(pAM6306). The crude extract prepared from *E. coli* DH5α (pBluescript II KS$^+$) exhibited no phosphorylation of gentamicin at 1, 5, and 15 min. The range of radioactivity (86 to 95 cpm) was similar to that for a negative control reaction mixture that lacked extract (75 to 86 cpm). When gentamicin was omitted from the reaction mixture, extract from *E. coli* DH5α(pAM6306), which contains the inserted gene, showed no increased radioactivity over time and had a similar range (84 to 105 cpm). When gentamicin was included in the reaction mixture, the extract from *E. coli* DH5α(pAM6306) exhibited increasing radioactivity over time (456, 1,285, and 3,996 cpm at 1, 5, and 15 min, respectively), thus showing that gentamicin phosphotransferase activity is associated with the presence of the *aph(2″)-Ic* gene.

Identification of similar gentamicin resistance determinants in *E. faecium* and *E. faecalis*. The presence of this novel

FIG. 1. Nucleotide sequence of *aph(2″)-Ic* isolated from *E. gallinarum* SF9117. Two potential promoter regions (regions 1 and 2) are shown. RBS-1 and RBS-2 are potential ribosome-binding sites. Asterisks denote start and stop codons of the ORF. Inverted repeats (IR) (one mismatched nucleotide) are designated by arrows. Italics and boldface highlight nucleotide bases of interest.

gentamicin resistance gene was investigated in isolates of *E. faecalis* and *E. faecium* cultured from humans and other animals. An intragenic probe for *aph(2″)-Ic* was generated by PCR with synthetic oligonucleotide primers (5′-TGACTCAG TTCCCAGAT-3′ and 5′-AGCACTGTTCGCACCAAA-3′) and hybridized to filters containing DNAs from the chromosomal and plasmid bands (separated by CsCl gradient centrifugation) from 3 animal and 12 human isolates of *E. faecalis* and *E. faecium* with intermediate-level resistance to gentamicin (MICs, 64 to 512 μg/ml). The probe hybridized to DNAs from the chromosomal (but not the plasmid) bands of an *E. faecium* isolate (SF6313; gentamicin MIC, 256 μg/ml) from the peritoneal fluid of a patient hospitalized in North Carolina (20) and to DNAs from both the chromosomal and plasmid bands of an *E. faecium* isolate (SF9583; gentamicin MIC, 512 μg/ml) from goose liver (from the University of Pennsylvania School of Veterinary Medicine). Neither SF6313 nor SF9583 exhibited synergism in time-kill studies with ampicillin plus gentamicin (data not shown).

Thirty-nine consecutive isolates of enterococci from the blood of separate patients in a community teaching hospital were screened with the intragenic probe, and plasmid DNA from one *E. faecalis* isolate (SF12261) was found to hybridize with the probe under conditions of high stringency (12). In mating studies with SF12261 (gentamicin MIC, 256 μg/ml), the plasmid and gentamicin resistance were found to transfer into *E. faecalis* FA2-2 at a frequency of 10^{-6}/recipient CFU.

Currently, enterococci with high-level resistance to gentamicin are tested with only one other aminoglycoside, streptomycin, since the bifunctional enzyme confers high-level resistance to the other clinically available aminoglycosides (including amikacin, kanamycin, tobramycin, netilmicin, and sisomicin). The novel gentamicin resistance gene reported in this study, *aph(2″)-Ic*, compromises ampicillin-gentamicin synergism but confers a gentamicin MIC of only 256 μg/ml. Strains bearing this gene might not be detected with consistency by conventional screening tests with 500 μg of gentamicin per ml. On the Synergy Quad Plate, between 1 and 15 colonies of SF9117, SF6313, and SF12261 have been seen, compared to the usually confluent growth of enterococcal isolates possessing the bifunctional enzyme (6). When such minimal growth is detected, these strains might be falsely assumed to possess the bifunctional enzyme and thus to be resistant to the synergistic activities of all available aminoglycosides other than streptomycin. In fact, however, they should be susceptible to ampicillin-netilmicin (or amikacin or isepamicin) synergism. If *aph(2″)-Ic* becomes prevalent, clinical microbiology laboratories may need to alter their screening methods for ampicillin-gentamicin synergism to allow for the detection of this resistance gene. For instance, growth might be tested in the presence of gentamicin at 128 μg/ml as well as at 500 or 2,000 μg/ml. Any strain readily detected in the presence of the drug at 128 μg/ml but not at 500 or 2,000 μg/ml might also be tested for susceptibility to intermediate levels of netilmicin, amikacin, and isepamicin. If the strain is susceptible, it may indeed contain *aph(2″)-Ic.*

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