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Identical Genes Confer High-Level Resistance to Gentamicin upon *Enterococcus faecalis*, *Enterococcus faecium*, and *Streptococcus agalactiae*

ACHIM KAUFHOLD,[1] ANDREAS PODBIELSKI,[1] THEA HORAUD,[2] AND PATRICIA FERRIERI[3]†*

Institut für Medizinische Mikrobiologie, Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen, D-5100 Aachen, Germany[1]; Laboratoire des Staphylocoques et des Streptocoques, Institut Pasteur, 75724 Paris Cedex 15, France[2]; and Departments of Laboratory Medicine and Pathology and Pediatrics, University of Minnesota Medical School, Minneapolis, Minnesota 55455[3]

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The structural gene coding for the bifunctional aminoglycoside-modifying 6′-acetyltransferase-2″-phosphotransferase (6′AAC-2″APH) enzyme was specifically amplified by the polymerase chain reaction using template DNA of clinical isolates of enterococci (both *Enterococcus faecalis* and *Enterococcus faecium*) and the single high-level gentamicin-resistant *Streptococcus agalactiae* strain identified thus far. The results of the present study demonstrated that the genes encoding this antibiotic resistance trait are highly homologous in these species. In dot blot hybridization assays using nonradioactively labeled oligonucleotide probes, strains with and without high-level gentamicin resistance could be discerned unequivocally. The gene segment encoding 6′-acetylating activity and the gene segment encoding 2″-phosphorylating activity were simultaneously present in all isolates exhibiting high-level gentamicin resistance.

High-level resistance to gentamicin (MIC > 2,000 μg/ml) among clinical isolates of *Enterococcus faecalis* was first reported in 1979 in France (11). In 1983, Mederski-Samoraj and Murray (17) first observed this resistance trait in *E. faecalis* in the United States, and more recently, highly resistant strains have been found in many hospitals throughout the United States and other countries (19). Resistance to gentamicin in both *E. faecalis* and *Staphylococcus aureus* is due to a bifunctional enzyme with 6′-acetyltransferase (6′AAC) and 2″-phosphotransferase (2″APH) activities and the nucleotide sequences for the gene encoding this enzyme have been shown to be identical in the staphylococcal transposon Tn*4001* and the *E. faecalis* plasmid pIP800 (6, 22). Recent studies conducted by Hodel-Christian and Murray (8, 9) confirmed that the resistance determinant in *E. faecalis* resides on a transposon as well. After the first three highly gentamicin-resistant *Enterococcus faecium* isolates were described in 1988 by Eliopoulos et al. (4), the emergence of other multiple-antibiotic-resistant clinical *E. faecium* strains, including 20 isolates showing high-level gentamicin resistance, was investigated at the University of Minnesota Medical School (14). In this study, our results of Southern blot hybridization experiments had suggested the presence of related genetic determinants for high-level gentamicin resistance in *E. faecalis* and *E. faecium*. Most recently, chromosome-mediated high-level gentamicin resistance was discovered in a group B *Streptococcus* isolate and again, DNA-DNA hybridization homology was detected between a 2.4-kb *Hin*dIII chromosomal fragment of *Streptococcus agalactiae* B128 and the gene conferring this resistance trait in *E. faecalis* (3). Since all these data suggested a very similar genetic determinant for high-level gentamicin resistance in various gram-positive cocci, the present study was undertaken to prove this hypothesis. Therefore, an approach using the polymerase chain reaction (PCR) and synthetic oligonucleotide probes was applied.

MATERIALS AND METHODS

Bacterial strains. The strains of *E. faecalis* studied were isolated from various clinical specimens submitted in the first months of 1991 to the Institute of Medical Microbiology at the Rheinisch-Westfälische Technische Hochschule (RWTH) Aachen. All *E. faecium* strains investigated in this study were isolated from February to November 1989 from clinical specimens in the Clinical Microbiology Laboratory of the University of Minnesota Hospital and Clinics and are described in detail in a separate paper (14). The species identification was confirmed by conventional physiological tests as described by Facklam and Collins (5).

The MICs of gentamicin for all enterococcal isolates were determined by a standard agar dilution technique employing Mueller-Hinton agar (18). Besides 24 *E. faecalis* and 11 *E. faecium* strains exhibiting high-level gentamicin resistance (MIC > 2,000 μg/ml), several strains lacking this resistance trait were included in the study (6 *E. faecalis* strains, 6 *E. faecium* strains, 1 *S. agalactiae* strain [74-360; from the strain collection of P.F.], and 1 *Streptococcus pyogenes* strain [3-3/317, reference strain obtained from the National Institute of Public Health, Center of Epidemiology and Microbiology, Prague, Czechoslovakia]). *S. agalactiae* B128 has been described recently (3). *Escherichia coli* JM109, harboring the recombinant plasmid pSF815A that carries the gene specifying the bifunctional 6′AAC-2″APH enzyme in *E. faecalis*, was provided by J. J. Ferretti (6).

Nucleic acid techniques. DNA was extracted from strain B128 as described earlier (3). Whole-cell DNA from all other strains used in this study was prepared by the protocol of Huang et al. (12) from 30-ml bacterial cultures in brain heart infusion broth. pSF815A was prepared by the method originally described by Birnboim and Doly (1). DNA was blotted

* Corresponding author.
† Reprint requests: University of Minnesota Hospital and Clinics, Box 134, Mayo Memorial Building, 420 Delaware St. S.E., Minneapolis, MN 55455.

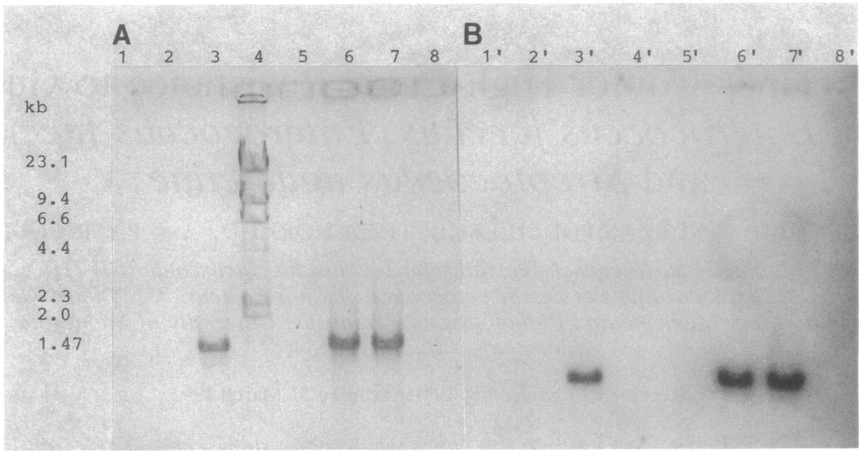

FIG. 1. (A) Representative gel electrophoretogram of PCR products after specific amplification of the entire high-level gentamicin resistance gene. *Hin*dIII-digested bacteriophage lambda DNA served as a molecular size marker (lane 4). For a negative control, every PCR assay included a reaction without added target DNA (lane 8). The following target DNA was used: *S. pyogenes* 3-3/317 (lane 1), *E. faecium* M3737 without high-level gentamicin resistance (lane 2), *E. faecium* M3586 with high-level gentamicin resistance (lane 3), *S. agalactiae* 74-360 without high-level gentamicin resistance (lane 5), and *S. agalactiae* B128 with high-level gentamicin resistance (lane 6). pSF815A served as the positive control (lane 7). (B) Southern blot of the same gel shown in panel A after hybridization with the digoxigenin-labeled AAC probe.

in 20-μg amounts onto Biodyne B membrane (Pall BioSupport, Dreieich, Germany) by conventional methods (23). For Southern blots, the DNA was transferred from agarose gels to the above-mentioned nylon membrane by vacuum (Vacu-Aid; Hybaid Ltd., Teddington, United Kingdom).

Sequences for oligonucleotides were selected using the published DNA sequence for the gentamicin resistance gene in *E. faecalis* (6). The design of optimized oligonucleotide probes as well as PCR primers was accomplished by the aid of PC GENE (IntelliGenetics, Mountain View, Calif.) and OLIGO (National Biosciences, Plymouth, Minn.) software.

The oligonucleotides were synthesized on a Beckman 200 A DNA synthesizer (Beckman Instruments, Munich, Germany). Further preparation of the oligonucleotides including the labeling of the probe with digoxigenin-dUTP (Boehringer GmbH, Mannheim, Germany) and hybridization assays were performed as previously described (20). The chemiluminescent substrate 3-(2′-spiroadamantane)-4-methoxy(3″-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD) (Boehringer) was used for visualization.

Two oligonucleotide probes were constructed. The first probe (5′ CTT CAT CTT CCC AAG GCT CT 3′, hereafter referred to as the AAC probe) corresponds to the gene encoding 6′-acetylating activity of 6′AAC-2″APH, while a second probe (5′ TTC TTT TCT ACC ATT TTC GA 3′, hereafter referred to as the APH probe) corresponds to the portion of the structural gene encoding 2″-phosphorylating activity of 6′AAC-2″APH.

Hybridizations and washings were carried out at 42°C. For PCR, amplification primer 1 (5′ ATA TGA TTA TGA AAA AGG TGA 3′) and primer 2 (5′ ATA ATC AAT CTT TAT AAG TCC 3′ on the complementary strand) flank a 1,472-bp fragment comprising the entire structural gene. The PCR reaction mixture contained PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM $MgCl_2$, 0.01% gelatin), 200 mM of each of the four deoxynucleoside triphosphates, 1.0 mM of each primer, 1 μg of template DNA, and 2.5 U of *Taq* polymerase (Boehringer). The mixture was overlaid with 100 μl of mineral oil. Samples were subjected to 27 cycles at 94°C for 1 min (DNA denaturation), 44°C for 1 min (annealing of primers), and 72°C for 2.5 min (extension of DNA), using an automated Techne PHC-1 thermal heating block. Generally recommended precautions were taken to avoid cross contamination between samples (16). PCR products were analyzed on 1% agarose gels. For sequencing, the PCR product was first purified with a Centricon-100 concentrator (Amicon, Witten, Germany), and the dideoxy-chain termination method (24) was performed using the T7 sequencing kit according to the instructions of the manufacturer (Pharmacia, Freiburg, Germany), applying modifications with regard to sequencing PCR products as described by Kusukawa et al. (15).

RESULTS

Using the described set of primers in the PCR, we were able to amplify a DNA fragment of the expected size in all investigated isolates showing high-level gentamicin resistance. pSF815A served as the positive control, and a control containing all the components of the PCR procedure except the template DNA was included in each experiment. Among our clinical isolates of *E. faecalis*, whole-cell DNA from 24 arbitrarily chosen strains exhibiting high-level gentamicin resistance were subjected to this analysis, and a single 1.47-kb DNA fragment was detected by agarose gel electrophoresis. An identical DNA segment was also amplified in 11 *E. faecium* strains and it is noteworthy that the group B streptococcal isolate B128 showed a PCR product of the same size (Fig. 1A). In contrast, all negative control strains (i.e., isolates lacking high-level gentamicin resistance), including several strains of *E. faecalis* and *E. faecium* and one *S. agalactiae* (strain 74-360) and one *S. pyogenes* (strain 3-3/317) strain, did not show the amplified DNA target sequence. In subsequent Southern blot hybridization experiments, both nonradioactively labeled oligonucleotide probes that corresponded to internal sequences of the resistance gene in *E. faecalis* hybridized strongly and specifically with all amplified DNA fragments (Fig. 1B). Further proof that the amplified DNA fragment actually represented the gentamicin resistance gene was established by direct sequencing of 80 nucleotides from the 5′ terminus of the PCR product of one high-level resistant *E. faecium* isolate (strain

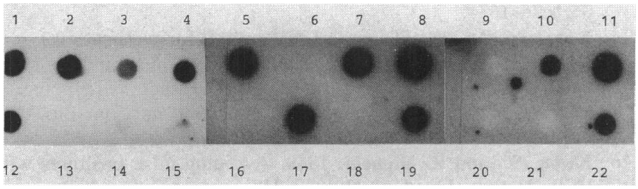

FIG. 2. Representative dot blots of whole-cell DNA extracted from several strains with and without high-level gentamicin resistance and hybridization with the digoxigenin-labeled AAC probe. Dots 1, 8, and 11, pSF815A (positive control). The following dots represent DNA from isolates with high-level gentamicin resistance: dots 2, 3, 4, 12, and 22 (different strains of *E. faecium*), dots 5, 7, 17, and 19 (different strains of *E. faecalis*), and dot 10 (*S. agalactiae* B128). The following dots represent DNA from strains without high-level gentamicin resistance: dots 13, 14, 15, and 21 (different strains of *E. faecium*), dots 6, 16, and 18 (several strains of *E. faecalis*), dot 9 (*S. agalactiae* 74-360), and dot 20 (*S. pyogenes* 3-3/317).

M3586). There was a disagreement of only one base in comparison to the nucleotide sequence published by Ferretti et al. (6).

When whole-cell DNA from the same strains was blotted in 20-μg amounts onto nylon membranes and hybridizations were carried out with the AAC and APH probes, unequivocal results were also obtained in all cases. Again, all high-level gentamicin-resistant isolates of *E. faecalis* (24 strains), *E. faecium* (11 strains) and *S. agalactiae* B128 showed a distinct, positive hybridization signal, whereas no false-positive result was obtained with any of several control strains (Fig. 2).

DISCUSSION

Since high-level gentamicin resistance correlates with loss of synergistic bactericidal activity between cell wall-active drugs and all currently available aminoglycosides except streptomycin, the prevalence of highly gentamicin-resistant strains of *E. faecalis* in diverse geographic areas is of clinical concern. Moreover, in France 45% of *E. faecalis* strains highly resistant to gentamicin are also highly resistant to streptomycin (10). The *E. faecalis* strains investigated in the present study are the first isolates exhibiting high-level gentamicin resistance reported from Germany. Although still rarely encountered, high-level gentamicin resistance has been observed in *E. faecium* as well (4, 14). Chromosome-borne high-level gentamicin resistance in the single group B streptococcal strain isolated so far in France also resulted in a lack of bactericidal synergy between ampicillin or vancomycin and aminoglycosides, since this strain is highly resistant to all clinically available aminoglycosides (3). In contrast, high-level resistance to gentamicin in enterococci is usually mediated by conjugative and nonconjugative plasmids that may be physically heterogeneous (14, 19, 26). However, a recent article presented evidence that the gentamicin resistance gene in *E. faecalis* can be integrated into the bacterial chromosome as well (21).

The bifunctional enzyme 6′AAC-2″APH has been detected in *E. faecalis* (6), *E. faecium* (4), *S. aureus* (25), and coagulase-negative staphylococci (7, 25), suggesting either direct gene exchange between these species or a common ancestral origin for gentamicin resistance. Recently published data indicate the involvement of a transpositional genetic element (Tn*5281*) encoding gentamicin resistance in *E. faecalis* similar to the staphylococcal transposons Tn*4001* and Tn*4031* (9).

Although the precedent reports in this field revealed strong evidence for the genetic relatedness of high-level gentamicin resistance, the results of our communication present additional proof that the resistance genes of *E. faecalis*, *E. faecium*, and *S. agalactiae* are highly homologous. This was shown by the method of amplifying the resistance gene by PCR. The specificity of the reaction was verified by hybridization with two different oligonucleotide probes and by the simple approach of direct sequencing of a part of the PCR product without laborious cloning procedures. The sequencing should merely be regarded as a fingerprint that demonstrates the overall identity of the genes, whereas minor mutations within the structural gene of the different species obviously cannot be excluded. Additional preliminary work in our laboratories, using different sets of PCR primers derived from the published sequences outside of the structural gene, showed that the regions surrounding the genetic determinant of gentamicin resistance are different in *E. faecalis* and *E. faecium* (data not shown), suggesting an integration of these genes into different transposons.

In our hybridization experiments, all investigated strains showing high-level gentamicin resistance were positive with the AAC probe and the APH probe, indicating the presence of both segments of the 6′AAC-2″APH resistance determinant. This observation is in agreement with the results of a recent study conducted by Huycke et al. (13). Huycke et al. used radiolabeled DNA fragments of the two portions of the fusion gene as probes and found positive signals in whole-colony blot hybridizations for both genes in 67 of 68 gentamicin-resistant enterococci. In contrast to Huycke et al. (13), thus far we have not encountered a gentamicin-susceptible strain harboring one or both segments of the gene, suggesting that their finding (two positive hybridizations among 122 strains lacking high-level gentamicin resistance) is an apparently rare event.

In unpublished experiments, we have recently evaluated a simpler and more rapid technique for the isolation of bacterial DNA from gram-positive cocci applying a protocol described by Bollet et al. (2). The extracted DNA was found to be equally suitable for the PCR procedure as well as for the dot blot hybridization assay. In ongoing investigations, we are also employing whole-colony blot hybridizations that we and others (13, 14) have shown to be valuable when screening for high-level gentamicin resistance of a larger number of enterococcal isolates is desired.

The sensitivity and specificity of the synthetic nonradioactively labeled oligonucleotide probes in dot blot hybridization experiments demonstrated that this approach is a useful tool for epidemiologic studies and could be adapted for application in the diagnostic clinical laboratory. Further, the results of this study demonstrate the usefulness of our approach for future studies elucidating the genetic identity of antibiotic resistance determinants.

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