ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Jan. 1993, p. 134–137
0066-4804/93/010134-04$02.00/0

Vol. 37, No. 1

Molecular Characterization of Highly Gentamicin-Resistant *Enterococcus faecalis* Isolates Lacking High-Level Streptomycin Resistance

LEE ANN THAL,[1,2] JOSEPH W. CHOW,[1,3] JAN EVANS PATTERSON,[1,4] MARY BETH PERRI,[1,2] SUSAN DONABEDIAN,[1,2] DON B. CLEWELL,[5] AND MARCUS J. ZERVOS[1,2,3]*

Departments of Internal Medicine[1] and Laboratory Medicine,[4] Yale University School of Medicine, New Haven, Connecticut 06510; William Beaumont Hospital, Royal Oak, Michigan 48073[2]; Wayne State University School of Medicine, Detroit, Michigan 48202[3]; and Departments of Biologics and Materials Sciences and Microbiology and Immunology, University of Michigan, Ann Arbor, Michigan 48109[5]*

Received 24 June 1992/Accepted 1 November 1992

Antimicrobial susceptibilities and DNA contents were analyzed for six clinical isolates of *Enterococcus faecalis* that had high-level resistance to gentamicin (MIC > 2,000 μg/ml) but not streptomycin and were obtained from patients in diverse geographic areas. Contour-clamped homogeneous electric field electrophoresis of genomic DNA showed all isolates to be different strains. Gentamicin resistance was transferred from four isolates to plasmid-free enterococcal recipients in filter matings. Restriction enzyme analysis of transconjugants showed distinct gentamicin resistance plasmids. A probe specific for the gentamicin resistance determinant hybridized to the plasmids of four isolates and to the chromosomes of two isolates. These findings suggest that clonal dissemination is not responsible for the spread of these resistant strains, that resistance determinants occur on different plasmids as well as on the chromosome of *E. faecalis*, and that the genetic determinants of resistance are related.

High-level resistance to gentamicin in enterococci was first reported in France in 1979 (6, 13). The resistance was plasmid mediated, due to aminoglycoside-modifying enzymes, and not associated with high-level resistance to streptomycin. Since the initial reports, high-level gentamicin resistance has been reported worldwide (7, 12–16, 24–32, 34–37). Most isolates exhibiting resistance to high levels of gentamicin are also resistant to all other aminoglycosides. However, isolates with high-level gentamicin resistance which lack resistance to streptomycin have been increasingly reported from diverse geographic areas in the United States, Canada, and France (22, 24, 26, 35–37). High-level gentamicin resistance eliminates in vitro synergism between a cell wall-active antibiotic and an aminoglycoside (16, 22). To evaluate potential treatment alternatives for this resistance trait, we evaluated in vitro antibiotic susceptibility and potential synergism of streptomycin plus cell wall-drug combinations for isolates from diverse locations in the United States and Canada. To determine whether the spread of this resistance is clonal or polyclonal or the result of a single plasmid, plasmid subunit, or a limited number of plasmids, the DNA contents of the isolates was compared.

Six clinical isolates of *Enterococcus faecalis* (from Winnipeg, Canada; Dallas, Tex.; Cleveland, Ohio; Charlottesville, Va.; Stamford, Conn.; and Fayetteville, N.C.) were evaluated. Four isolates (SF339, SF350, SF370, and SF381) were part of an earlier study (26); the MICs of gentamicin and streptomycin for these isolates were >2,000 and ≤125 μg/ml, respectively (26). The fifth (SF3502) was a blood isolate from a patient in Stamford, Conn., with endocarditis who failed therapy with ampicillin and gentamicin but was successfully treated with ampicillin and streptomycin. The sixth isolate (SF4855) was a β-lactamase-producing strain from the urine of a North Carolina patient.

Isolates were speciated by using biochemical reactions as outlined by Facklam and Collins (8) and were evaluated for β-lactamase production with nitrocefin (Glaxo Laboratories, Middlesex, England). High-level aminoglycoside resistance was defined as a MIC of ≥2,000 μg/ml. Antimicrobial susceptibilities were determined by microdilution in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) supplemented with $CaCl_2$ and $MgCl_2$ at 50 and 25 mg/liter, respectively (23). Time-kill synergy experiments were conducted with penicillin or ampicillin-sulbactam (Pfizer Roerig Pharmaceuticals, New York, N.Y.) in combination with streptomycin. Antibiotic concentrations were subinhibitory and did not exceed achievable serum levels for all time-kill experiments. Sulbactam was used in a 1:2 ratio with ampicillin. Synergy was defined as a reduction in bacterial growth of ≥2 $\log_{10}$ CFU/ml after 24 h of incubation with antibiotics in combination compared with the effect of the agents alone. Filter and cross streak matings were performed to determine the transferability of resistance markers (1, 11). *E. faecalis* FA2-2 and JH2-2 were used as plasmid-free recipient strains. Transfer frequencies were expressed as the number of transconjugants per recipient cell present at the time of plating on selective media. Plasmid DNA was isolated by a previously described method (5). DNA was analyzed by agarose gel electrophoresis, and restriction enzyme digestion of plasmid DNA with *Eco*RI and *Hin*dIII was performed according to the manufacturer's specifications. Contour-clamped homogeneous electric field (CHEF) electrophoresis of genomic DNA digested with *Sma*I was conducted by published methods to determine the relatedness of strains (20, 33). A probe specific for the bifunctional 6′-aminoglycoside acetyltransferase 2″-phosphotransferase enzyme in *E. faecalis*, constructed by Ferretti et al., was used (10) to determine the relatedness of the gentamicin resistance de-

* Corresponding author.

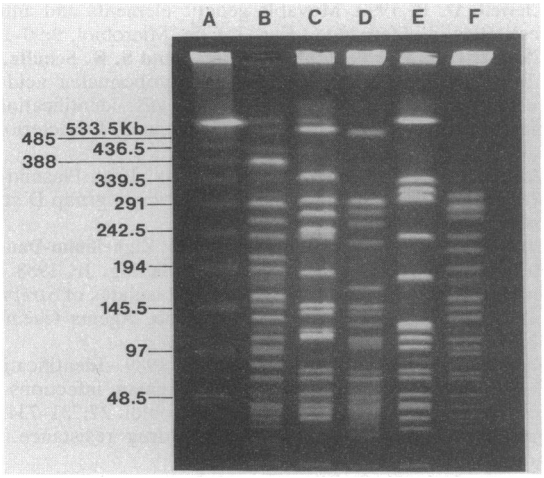

FIG. 1. CHEF electrophoresis of genomic DNA digested with *Sma*I. Lanes: A, bacteriophage lambda ladder; B, SF339; C, SF350; D, SF370; E, SF381; F, SF3502.

terminants. DNA was transferred to nitrocellulose by Southern blot hybridization. The probe was biotin labeled by nick translation (Bethesda Research Laboratories, Gaithersburg, Md.). Hybridization and visualization were done with Blugene according to the manufacturer's recommendation (Bethesda Research Laboratories).

Ampicillin MICs were 0.5 to 1.0 μg/ml and penicillin MICs were 4.0 μg/ml for all six isolates. For SF3502 and SF4855, gentamicin MICs were >2,000 μg/ml and streptomycin MICs were 125 and 62.5 μg/ml, respectively. Synergistic bactericidal killing in vitro with the combination of penicillin plus streptomycin was found in the five non-β-lactamase-producing strains and with the combination of ampicillin-sulbactam plus streptomycin in the β-lactamase-producing isolate (SF4855).

CHEF electrophoresis of chromosomal DNA showed all isolates to be distinct (Fig. 1). All donor strains (clinical isolates) had different plasmid patterns in agarose electrophoretic gels and contained zero to three plasmids. Strain SF4855 was plasmid free. SF350 contains three plasmids; pYN120 is a 43-kb conjugative macrolide-lincosamide-streptogramin B plasmid. This plasmid transfers into JH2-2 recipients at a frequency of 10^{-2} and mediates only macrolide-lincosamide-streptogramin B resistance. The functions of the other plasmids are cryptic. Properties of gentamicin resistance plasmids pYN111, pYN112, and pYN113 are shown in Table 1. Three isolates (SF370, SF381, and SF3502) transferred gentamicin resistance by filter mating at high frequency (1.4×10^{-6} to 5×10^{-2}) and one isolate (SF350) transferred at low frequency (1×10^{-9}). Gentamicin resistance plasmids (pYN111, pYN112, and pYN113) had different molecular sizes ranging from 53 to 56 kb and different restriction enzyme (*Hin*dIII and *Eco*RI) patterns (Fig. 2A). Common fragments were observed. Hybridization of the gentamicin resistance determinant occurred on different plasmid fragments of four isolates (SF339, SF370, SF381, and SF3502) (Fig. 2B) and on the chromosomes of two isolates (SF350 and SF4855). Data are not shown for SF4855.

TABLE 1. Properties of gentamicin resistance plasmids lacking high-level streptomycin resistance from clinical isolates of *E. faecalis*

E. faecalis strain	Resistance plasmid designation	Plasmid properties[a]	Molecular size (kb)	Transfer frequency
SF370	pYN111	Tra^+, GM, EM, TC, Hly^-	54	1.4×10^{-6}
SF381	pYN112	Tra^+, GM, EM, TC, Hly^-	53	4.4×10^{-4}
SF3502	pYN113	Tra^+, GM, TC, EM	56	5×10^{-2}

[a] Abbreviations: EM, resistance to macrolide, lincosamide, and streptogramin B-type antibiotics; GM, resistance to gentamicin; Hly^-, no production of beta-hemolysin or bacteriocin; TC, resistance to tetracycline; Tra^+, conjugative.

The treatment of enterococcal infections has recently been complicated by the emergence of penicillin-, glycopeptide-, and aminoglycoside-resistant strains (2–4, 6, 7, 13–22, 24–32,

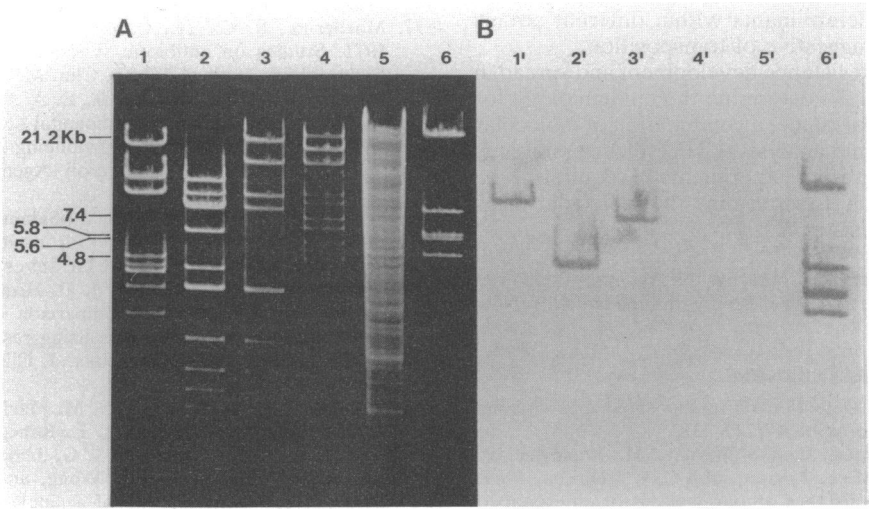

FIG. 2. (A) Agarose gel electrophoresis of restriction endonuclease digestion with *Eco*RI. Lanes: 1, pYN111; 2, pYN112; 3, pYN113; 4, SF350 purified plasmid DNA; 5, SF350 chromosome; 6, bacteriophage lambda. (B) Hybridization of a probe specific for the gentamicin resistance determinant (bifunctional 6′-aminoglycoside acetyltransferase 2″-phosphotransferase enzyme) and DNA specific for bacteriophage lambda to the gel in panel A.

34–37). Since up to 49% of strains exhibiting high-level resistance to gentamicin lack high-level streptomycin resistance in some institutions, screening for these resistances is indicated for the management of infections for which an aminoglycoside is required. The difficulty in obtaining streptomycin in the United States further complicates therapy. To date, almost all β-lactamase-producing enterococcal strains show high-level aminoglycoside resistance (18–21, 27, 28, 30). Rare exceptions include one previously described strain lacking high-level gentamicin resistance (27) and an isolate reported in the present study which is β-lactamase producing and lacks high-level resistance to streptomycin. Prior studies have shown in vitro synergy of penicillin and streptomycin against strains lacking high-level resistance to streptomycin (22). This study confirms those earlier observations and in addition shows synergy of ampicillin-sulbactam plus streptomycin for the β-lactamase-producing strain examined. Since all isolates evaluated in the present study showed in vitro synergy of bactericidal killing with the combination of penicillin or ampicillin-sulbactam plus streptomycin, these agents can be considered for therapy of serious infections.

On the bases of CHEF analysis of chromosomal DNA and plasmid analysis, all clinical isolates in the present study were different strains. In addition, distinct conjugative plasmids and a nonconjugative plasmid were responsible for gentamicin resistance in different clinical isolates. Therefore, the spread of this resistance trait in examined isolates was not due to the spread of a single strain or a single plasmid. There was similarity of high-level gentamicin resistance genes as determined by DNA-DNA hybridization studies using a probe specific for the bifunctional 6′-aminoglycoside acetyltransferase 2″-phosphotransferase enzyme in *E. faecalis*. Earlier studies have shown similarity of gentamicin resistance determinants in different clinical enterococcal isolates and between enterococci and staphylococci (12, 28, 29, 31). Transposons mediating gentamicin, erythromycin, and tetracycline resistances in enterococci have been recently identified (3, 4, 12). In this study, the finding of the gentamicin resistance determinant on both the plasmid and chromosome and on the chromosome in the absence of coresident plasmids is suggestive of transposition. The apparent transfer of the gentamicin resistance determinant from chromosome and the homology among gentamicin resistance determinants within different strains and plasmids are also suggestive of transposition.

The epidemiology of resistance development and spread in enterococci is complex. To determine the epidemiology for high-level gentamicin resistance, a combination of molecular methods including plasmid analysis, CHEF electrophoresis, and DNA-DNA hybridization experiments is required. Further study will be needed to determine the molecular basis for the spread of this resistance.

This study was supported in part by the William Beaumont Hospital Research Institute and Pfizer Roerig Pharmaceuticals, New York, N.Y.

REFERENCES

1. **Bennett, P. M.** 1984. Detection of transposable elements on plasmids. Methods Microbiol. **17**:227–231.
2. **Bush, L. M., J. Calmon, C. L. Cherney, M. Wendeler, P. Pitsakis, J. Poupard, M. E. Levison, and C. C. Johnson.** 1989. High-level penicillin resistance among isolates of enterococci: implications for treatment of enterococcal infections. Ann. Intern. Med. **110**:515–520.
3. **Clewell, D. B.** 1981. Plasmids, drug resistance, and gene transfer in the genus *Streptococcus*. Microbiol. Rev. **45**:409–436.
4. **Clewell, D. B.** 1990. Movable genetic elements and antibiotic resistance in enterococci. Eur. J. Clin. Microbiol. **9**:90–102.
5. **Clewell, D. B., Y. Yagi, G. M. Dunny, and S. K. Schultz.** 1974. Characterization of three plasmid deoxyribonucleic acid molecules in a strain of *Streptococcus faecalis*: identification of a plasmid determining erythromycin resistance. J. Bacteriol. **117**: 283–289.
6. **Courvalin, P., C. Carlier, and E. Collatz.** 1980. Plasmid-mediated resistance to aminocyclitol antibiotics in group D streptococci. J. Bacteriol. **143**:541–551.
7. **Eliopoulos, G. M., C. Wennersten, S. Zighelboim-Daum, E. Reiszner, D. Goldmann, and R. C. Moellering, Jr.** 1988. High-level resistance to gentamicin in clinical isolates of *Streptococcus* (*Enterococcus*) *faecium*. Antimicrob. Agents Chemother. **32**:1528–1532.
8. **Facklam, R. R., and M. D. Collins.** 1989. Identification of *Enterococcus* species isolated from human infections by a conventional test scheme. J. Clin. Microbiol. **27**:731–734.
9. **Falcow, S.** 1975. Infectious multiple drug resistance. Pion, London.
10. **Ferretti, J. J., K. S. Gilmore, and P. Courvalin.** 1986. Nucleotide sequence analysis of the gene specifying the bifunctional 6′-aminoglycoside acetyltransferase 2″-aminoglycoside phosphotransferase enzyme in *Streptococcus faecalis* and identification and cloning of gene regions specifying the two activities. J. Bacteriol. **167**:631–638.
11. **Forbes, B. A., and D. R. Schaberg.** 1983. Transfer of resistance plasmids from *Staphylococcus epidermidis* to *Staphylococcus aureus*: evidence for conjugative exchange of resistance. J. Bacteriol. **153**:627–634.
12. **Hodel-Christian, S. L., and B. E. Murray.** 1991. Characterization of the gentamicin resistance transposon Tn*5281* from *Enterococcus faecalis* and comparison to staphylococcal transposons Tn*4001* and Tn*4031*. Antimicrob. Agents Chemother. **35**:1147–1152.
13. **Horodniceanu, T., L. Bougueleret, N. El-Solh, G. Bieth, and F. Delbos.** 1979. High-level, plasmid-borne resistance to gentamicin in *Streptococcus faecalis* subsp. *zymogenes*. Antimicrob. Agents Chemother. **16**:686–689.
14. **Kaplan, A. H., P. H. Gilligan, and R. R. Facklam.** 1988. Recovery of resistant enterococci during vancomycin prophylaxis. J. Clin. Microbiol. **26**:1216–1218.
15. **Leclercq, R., E. Derlot, J. Duval, and P. Courvalin.** 1988. Plasmid-mediated resistance to vancomycin and teicoplanin in *Enterococcus faecium*. N. Engl. J. Med. **319**:157–161.
16. **Mederski-Samoraj, B. D., and B. E. Murray.** 1983. High-level resistance to gentamicin in clinical isolates of enterococci. J. Infect. Dis. **147**:751–757.
17. **Moellering, R. C., Jr., C. Wennersten, and A. N. Weinberg.** 1971. Studies on antibiotic synergism against enterococci. I. Bacteriologic studies. J. Lab. Clin. Med. **77**:821–828.
18. **Murray, B. E., H. A. Lopardo, E. A. Rubeglio, M. Frosolono, and K. V. Singh.** 1992. Intrahospital spread of a single gentamicin-resistant, β-lactamase-producing strain of *Enterococcus faecalis* in Argentina. Antimicrob. Agents Chemother. **36**:230–232.
19. **Murray, B. E., and B. Mederski-Samoraj.** 1983. Transferable β-lactamase: a new mechanism for in-vitro penicillin resistance in *Streptococcus faecalis*. J. Clin. Invest. **72**:1168–1171.
20. **Murray, B. E., K. V. Singh, J. D. Heath, B. R. Sharma, and G. M. Weinstock.** 1990. Comparison of genomic DNAs of different enterococcal isolates using restriction endonucleases with infrequent recognition sites. J. Clin. Microbiol. **28**:2059–2063.
21. **Murray, B. E., K. V. Singh, S. M. Markowitz, H. A. Lopardo, J. E. Patterson, M. J. Zervos, E. Rubeglio, G. M. Eliopoulos, L. B. Rice, F. W. Goldstein, S. G. Jenkins, G. M. Caputo, R. Nasmas, L. S. Moore, E. S. Wong, and G. Weinstock.** 1991. Evidence for clonal spread of a single strain of β-lactamase-producing *Enterococcus* (*Streptococcus*) *faecalis* to six hospitals in five states. J. Infect. Dis. **163**:780–785.
22. **Nachamkin, I., P. Axelrod, G. H. Talbot, S. H. Fischer, C. B. Wennersten, R. C. Moellering, Jr., and R. R. MacGregor.** 1988.

Multiply high-level-aminoglycoside-resistant enterococci isolated from patients in a university hospital. J. Clin. Microbiol. **26:**1287–1291.

23. **National Committee for Clinical Laboratory Standards.** 1990. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 2nd ed. Approved standard document M7-A2. National Committee for Clinical Laboratory Standards, Villanova, Pa.
24. **Noskin, G. A., M. Till, B. K. Patterson, J. T. Clarke, and J. R. Warren.** 1991. High-level gentamicin resistance in *Enterococcus faecalis* bacteremia. J. Infect. Dis. **164:**1212–1215.
25. **Oster, S. E., V. A. Chirurgi, A. A. Goldberg, S. Aiken, and R. E. McCabe.** 1990. Ampicillin-resistant enterococcal species in an acute-care hospital. Antimicrob. Agents Chemother. **34:**1821–1823.
26. **Patterson, J. E., B. L. Masecar, C. A. Kauffman, D. R. Schaberg, W. J. Hierholzer, Jr., and M. J. Zervos.** 1988. Gentamicin resistance plasmids of enterococci from diverse geographic areas are heterogeneous. J. Infect. Dis. **158:**212–216.
27. **Patterson, J. E., B. L. Masecar, and M. J. Zervos.** 1988. Characterization and comparison of two penicillinase-producing strains of *Streptococcus* (*Enterococcus*) *faecalis*. Antimicrob. Agents Chemother. **32:**122–124.
28. **Patterson, J. E., A. Wanger, K. K. Zscheck, M. J. Zervos, and B. E. Murray.** 1990. Molecular epidemiology of β-lactamase-producing enterococci. Antimicrob. Agents Chemother. **34:**302–305.
29. **Patterson, J. E., and M. J. Zervos.** 1990. High-level gentamicin resistance in enterococcus: microbiology, genetic basis and epidemiology. Rev. Infect. Dis. **12:**644–652.
30. **Rhinehart, E., N. E. Smith, C. Wennersten, E. Gross, G. Eliopoulos, and R. Moellering.** 1990. Rapid dissemination of gentamicin-resistant, β-lactamase-producing, aminoglycoside-resistant *Enterococcus faecalis* among patients and staff on an infant/toddler surgical ward. N. Engl. J. Med. **323:**1814–1818.
31. **Schaberg, D. R., and M. J. Zervos.** 1986. Plasmid analysis in the study of the epidemiology of nosocomial gram-positive cocci. Rev. Infect. Dis. **8:**705–712.
32. **Shlaes, D. M., A. Bouvet, C. Devine, J. H. Shlaes, S. Al-Obeid, and R. Williamson.** 1989. Inducible, transferable resistance to vancomycin in *Enterococcus faecalis* A256. Antimicrob. Agents Chemother. **33:**198–203.
33. **Smith, C. L., and C. R. Cantor.** 1987. Purification, specific fragmentation, and separation of large DNA molecules. Methods Enzymol. **155:**449–467.
34. **Uttley, A. H. C., C. H. Collins, J. Naidoo, and R. C. George.** 1988. Vancomycin-resistant enterococci. Lancet **i:**57–58. (Letter.)
35. **Wurtz, R., D. Sahm, and J. Flaherty.** 1991. Gentamicin-resistant, streptomycin-susceptible *Enterococcus* (*Streptococcus*) *faecalis* bacteremia. J. Infect. Dis. **163:**1393–1394.
36. **Zervos, M. J., C. A. Kauffman, P. M. Therasse, A. G. Bergman, T. S. Mikesell, and D. R. Schaberg.** 1987. Nosocomial infection by gentamicin-resistant *Streptococcus faecalis*: an epidemiologic study. Ann. Intern. Med. **106:**687–691.
37. **Zervos, M. J., T. S. Mikesell, and D. R. Schaberg.** 1986. Heterogeneity of plasmids determining high-level resistance to gentamicin in clinical isolates of *Streptococcus faecalis*. Antimicrob. Agents Chemother. **30:**78–81.