JOURNAL OF CLINICAL MICROBIOLOGY, June 1993, p. 1570–1574
0095-1137/93/061570-05$02.00/0

Vol. 31, No. 6

Comparison of Ribotyping and Pulsed-Field Gel Electrophoresis for Subspecies Differentiation of Strains of *Enterococcus faecalis*

MANUEL E. GORDILLO,[1]† KAVINDRA V. SINGH,[1] AND BARBARA E. MURRAY[1,2]*

Department of Internal Medicine[1] and Department of Microbiology and Molecular Genetics,[2] Center for Infectious Diseases, The University of Texas Medical School at Houston, Houston, Texas 77030

Received 21 December 1992/Accepted 12 March 1993

Hybridization of *Eco*RI- and *Hin*dIII-digested chromosomal DNAs from 41 isolates of *Enterococcus faecalis* with probes for rRNA genes was performed (ribotyping). The ability of ribotyping to distinguish strains at the subspecies level was compared with results previously determined by pulsed-field gel electrophoresis (PFGE). With *Eco*RI, seven ribopatterns (usually differing by only one band) were found, while PFGE had previously shown 25 clearly different patterns plus six related variants. Digestion with *Hin*dIII generated a few additional patterns but still failed to differentiate some strains that had very different PFGE patterns. Ribotyping with *Bsc*I has also been reported to be inadequate for subspecies strain differentiation (L. M. Hall, B. Duke, M. Guiney, and R. Williams, J. Clin. Microbiol. 30:915–919, 1992). Although ribotyping with other restriction endonucleases may perform better in distinguishing different strains, at present PFGE appears to be superior for strain differentiation.

Enterococci are important causes of clinical infections such as urinary tract infections, endocarditis, and bacteremia (9). The national Nosocomial Infections Surveillance System ranked the enterococcus as the second most common agent of nosocomial infections for the years 1986 to 1989 (18). This trend is likely to continue or worsen, in part because of the increasing resistance of this organism in the hospital setting, such as high-level gentamicin resistance (6), penicillin resistance resulting from β-lactamase production (10), and glycopeptide resistance (1). For years it was thought that enterococcal infections were acquired from the patient's own flora, but classical and molecular epidemiologic techniques have now confirmed that this organism can be spread in the hospital setting (5, 11–13, 20). Until recently, epidemiologic evaluations of enterococcal infections were hampered by the lack of a sufficiently discriminatory typing system. Antibiograms and biotypes show little variation within an enterococcal species, and serotyping, bacteriophage typing, and enterococcinotyping have never become widely used because of the tedious nature of the tests and the need for special reagents (4). Total plasmid content has been used, but in our hands, it has yielded less consistent and reproducible results than those that we have obtained with members of the family *Enterobacteriaceae*. We have recently used pulsed-field gel electrophoresis (PFGE) in the epidemiologic assessment of *Enterococcus faecalis*, and this technique appears promising for such studies (11–14). Ribotyping is another technique that has recently been applied to microorganisms for epidemiologic purposes. Ribotyping uses less expensive equipment and might be more appropriate for the routine microbiology laboratory. In the present study, we compared the results obtained after hybridization of an rRNA probe derived from *Escherichia coli* to a Southern blot of digested enterococcal DNA (ribotyping) with results previously obtained by PFGE (12, 13).

* Corresponding author.
† Present address: Manasota Infectious Disease Consultants, Sarasota, FL 34239.

MATERIALS AND METHODS

Bacterial isolates. The *E. faecalis* isolates used in the present study have been described previously (12, 13) and are listed in Table 1, along with their origins, original PFGE restriction endonuclease digestion patterns, and ribotypes, as determined and then arbitrarily named here. In addition, we studied three reference strains, also listed in Table 1.

Ribotyping and PFGE. Plugs containing bacterial chromosomal DNA were processed and digested with *Sma*I by previously described methods (12). For riboprobing, the DNA in the plugs was digested with *Eco*RI or *Hin*dIII (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) under the manufacturer's suggested conditions, washed with TE (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA) at 37°C for at least 1 h, and then electrophoresed in a 0.7% agarose gel for 12 h at 40 V. The gels were denatured, neutralized, and transferred to Hybond-N filters (Amersham, Arlington Heights, Ill.) by following the methods described by Sambrook et al. (17). DNA containing 16S and 23S rRNA genes from *E. coli* (Boehringer Mannheim Biochemicals) was end labelled with [^{32}P]ATP using the 5′ DNA terminus labelling kit (Boehringer Mannheim Biochemicals) with T4 polynucleotide kinase (Bethesda Research Laboratories, Bethesda, Md.) by the manufacturer's instructions. After 60 min of incubation at 37°C, the reaction was terminated by the addition of 0.2% sodium dodecyl sulfate (SDS)–20 mM EDTA. The product was then passed over a Bio Spin 30 chromatography column (Bio-Rad Laboratories, Richmond, Calif.) according to the instructions in the insert. The Hybond-N filters were prehybridized for at least 3 h at 65°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× FPG (1× FPG is 0.02% Ficoll 400, 0.02% polyvinylpyrrolidone 350, and 0.02% glycine)–0.5% SDS–100 μg of calf thymus DNA per ml. The 16S plus 23S rRNA probe was then added (10^6 counts per cm^2 of filter), and the hybridization mixture was incubated at 65°C overnight. Filters were washed three times in 2× SSC–0.1% SDS at 65°C for 15 min (one time in 0.1× SSC–0.1% SDS for 15 min, once at 65°C, and then once at room temperature), air dried,

TABLE 1. Details of *E. faecalis* isolates used in the present study

Isolate	Origin[a]	PFGE pattern	*Eco*RI ribotype
BE18	Bangkok, Thailand	B-9[b]	A
BE78	Bangkok, Thailand	B-2	D
BE81	Bangkok, Thailand	B-4	E
BE82	Bangkok, Thailand	B-1	A
BE83	Bangkok, Thailand	B-1	A
BE86	Bangkok, Thailand	B-1	A
BE88	Bangkok, Thailand	B-3	B
BE114	Bangkok, Thailand	B-1	A
BE117	Bangkok, Thailand	B-10	A
BE120	Bangkok, Thailand	B-8	A
BE125	Bangkok, Thailand	B-1	A
CE13	Santiago, Chile	C-1	A
CE30	Santiago, Chile	C-1	A
CE36	Santiago, Chile	C-2	B
K1	Santiago, Chile	C-3	A
K4	Santiago, Chile	C-1b	A
S22	Santiago, Chile	C-4	A
CE39	Santiago, Chile	C-1a	B
HH31	Houston, Tex.	H-3	A
HH52	Houston, Tex.	H-4	A
HH54	Houston, Tex.	H-1	B
HH98	Houston, Tex.	H-2	C
HH123	Houston, Tex.	H-5	A
HH181	Houston, Tex.	H-1	B
HH22	Houston, Tex.	I[c]	A
PA	Philadelphia, Pa.	I	A
CH570	Pittsburgh, Pa.	I	A
WH245	West Haven, Conn.	II	G
Delaware	Wilmington, Del.	I	A
Florida-1	Jacksonville, Fla.	I	A
Florida-2	Jacksonville, Fla.	I	A
Virginia-1	Richmond, Va.	I	A
Virginia-2	Richmond, Va.	I	A
CH25	Boston, Mass.	III	G
Beirut	Beirut, Lebanon	IV	G
Argentina-1 (HG6280)	Buenos Aires, Argentina	V	G
Argentina-2 (HG6049)	Buenos Aires, Argentina	V	G
Argentina-3 (HG4354)	Buenos Aires, Argentina	V	G
ATCC 29212	American Type Culture Collection, Rockville, Md.	VI (this study)	A
JH2-2	Commonly used laboratory strain[d]	VII (this study)	F
OG1RF	Commonly used laboratory strain[d]	VIII[e]	D

[a] Origins were described previously (12, 13).
[b] PFGE pattern designations previously used for the first 24 isolates (12).
[c] Pattern designations I to VIII were assigned in the present study to new or previously published but unnamed PFGE restriction endonuclease digestion patterns.
[d] See reference 3.
[e] See reference 8.

and subsequently exposed on X-ray film for 98 h at −70°C. Every effort was made to reproduce the lane order in the figures reported in the two previous studies (12, 13), where these isolates were typed by PFGE. Autoradiograms showing potential partial digestion of DNA were also repeated at least once for verification.

RESULTS

Forty-one isolates of *E. faecalis* were studied. Using *Eco*RI, we found seven different ribotypes among these isolates; when these isolates were previously studied by PFGE, they were found to belong to 25 distinct patterns with six variants (differences of one to two bands) (12, 13). By ribotyping, each isolate yielded between 7 and 10 bands per strain, whereas PFGE yielded 15 to 20 bands per strain. Because relatively few bands were obtained by ribotyping, we arbitrarily chose to call a pattern different even when there was a difference of only one band between isolates. In previous enterococcal PFGE typing studies, patterns with differences of one to several bands have been variously called "same," "similar," or "variant" (12, 13) and interpreted as indicating that the isolates represent the same strain or that they were derived from a common ancestral strain. Figures 1 to 3 show some of the autoradiographs obtained with these isolates. Interpretations were based on the results of these and other autoradiographs (data not shown).

Figure 1A shows *Eco*RI ribotype pattern A which was found in the four isolates from Chile (K1 [only faintly seen in Fig. 1A], K4, S22, CE13) and three isolates from Houston (HH31, HH52, HH123); JH2-2, also shown in Fig. 1A, differed from the other isolates mentioned above in only one *Eco*RI band. When these strains were examined by PFGE,

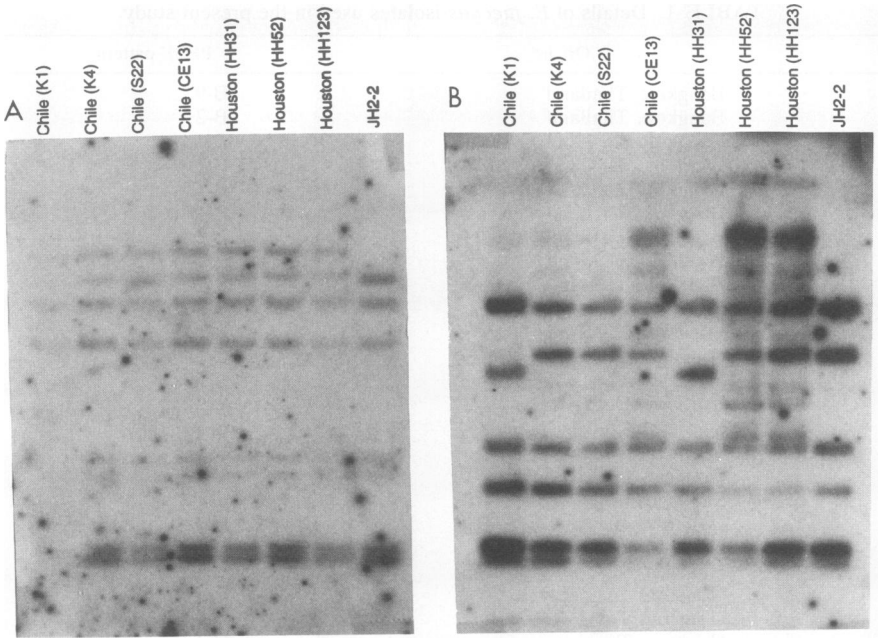

FIG. 1. Autoradiograms of enterococcal chromosomal DNAs after riboprobing. (A) Digestion with *Eco*RI. The size range of the hybridizing bands was from about 2 to 23 kb. (B) Digestion with *Hin*dIII.

there were marked restriction fragment length polymorphisms among all these strains except strains K4 and CE13 (Fig. 1A), which had similar PFGE patterns (see Fig. 4 in the previous report [12]). Ribotyping of these same isolates after digestion with *Hin*dIII (Fig. 1B) generated three subtypes within the A ribopattern. Two isolates from Chile (K4, S22) and isolate JH2-2 had the same *Hin*dIII ribopattern, although JH2-2 differed from the isolates from Chile by one band in the *Eco*RI ribopattern. One isolate from Chile (K1) and one isolate from Houston (HH31) had identical patterns which differed from those of the isolates described above by a single *Hin*dIII band. Two other isolates from Houston (HH52, and HH123) shared a third *Hin*dIII pattern, despite marked differences in their PFGE patterns (12).

Figure 2 shows two *Eco*RI rRNA patterns among 10 isolates, while PFGE of these isolates previously showed four very different digestion patterns (patterns I, II, III, and V). Isolates Virginia-1, Virginia-2, Delaware, Pittsburgh, and Houston (HH22) (marked with asterisks) had a common ribotype (the widespread pattern A), which was in agreement with previously reported PFGE results (13). A different, shared ribopattern (pattern G) was found among the Argentina-1, Argentina-2, West Haven, and Boston isolates, which have been shown to be clearly different (except for the two isolates from Argentina) by PFGE (13). Isolate Argentina-3 probably has the same ribopattern, since the extra bands (smudge at the top band and then the very faint band five bands below) were not seen consistently and may represent partially digested DNA.

Figure 3A shows the patterns of seven isolates with the ubiquitous *Eco*RI A pattern. Riboprobing after digestion of these same isolates with *Hin*dIII (Fig. 3B) shows that only isolates BE117 and ATCC 29212 had different rRNA patterns (each differing from the others by a single band). PFGE (Fig. 3C) revealed four distinctly different patterns (C-1, B-10, I, and VI) among these seven isolates; also shown in Fig. 3C are the PFGE patterns of isolates JH2-2 (pattern VII) and OG1 (pattern VIII).

DISCUSSION

Ribotyping is an increasingly used typing system that is being applied to an ever increasing number of organisms. It

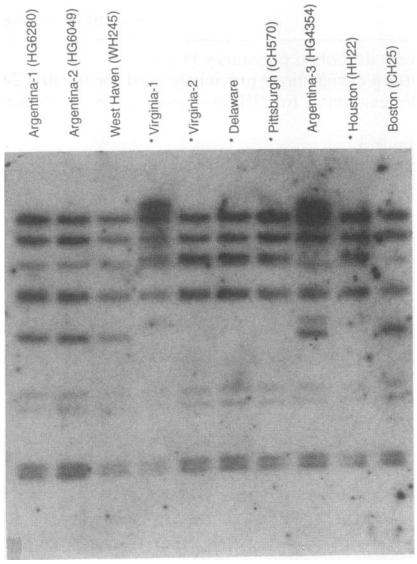

FIG. 2. Autoradiogram after riboprobing of enterococcal chromosomal DNAs from β-lactamase-positive enterococci digested with *Eco*RI. Those isolates represented in lanes marked with an asterisk were previously interpreted by PFGE to be clonally derived (13).

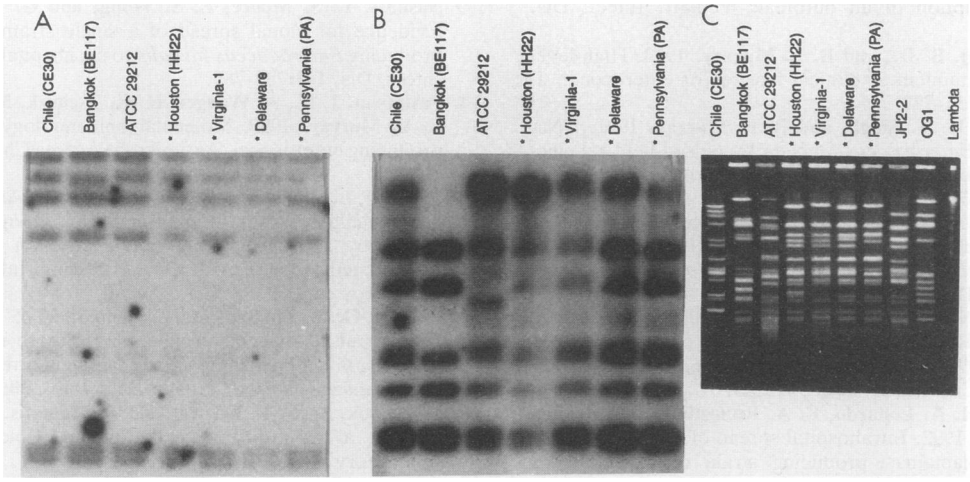

FIG. 3. Autoradiograms of enterococcal chromosomal DNAs after riboprobing. Those lanes marked with an asterisk represent β-lactamase-positive and clonally related isolates. (A) Digestion with *Eco*RI. (B) Digestion with *Hin*dIII. (C) PFGE of isolates.

has the advantage over other genotyping systems such as PFGE in that it can be accomplished at a relatively minor expense and does not require specialized equipment (19). The need for radioactive material has been circumvented by the use of nonradioactive probes such as digoxigenin (2) and biotinylated probes (15). PFGE has now been used by several different groups, including our own, to study enterococci and has shown a great deal of diversity among patterns of epidemiologically unrelated strains (11–14). On the basis of the data presented here, ribotyping with *Eco*RI was inferior to PFGE for subspecies differentiation of strains of *E. faecalis* since the ribopatterns did not show sufficient polymorphisms to distinguish between epidemiologically unrelated isolates that were previously found to have clearly different PFGE restriction endonuclease digestion patterns. In no instance was rRNA probing able to further resolve isolates that were identical by PFGE. This persisted even when attempts were made to demonstrate polymorphism by riboprobing after digesting chromosomal DNA with a different restriction endonuclease. Although a second enzyme did generate a few additional subtypes, many isolates with different PFGE patterns still had the same ribopatterns. Although our method for DNA extraction differs from the one routinely used for riboprobing, we have shown it to be effective and to yield evaluable patterns. We did have some problems with partial digestions which may or may not have been attributable to the DNA extraction method that we used.

Our results with ribotyping are perhaps not surprising if one considers the nature of the test. PFGE detects the distribution of restriction sites throughout the chromosome, and ribotyping is based on the distribution of restriction sites within or surrounding regions of the chromosome (rRNA operons) that are known to be highly conserved among all bacteria; thus, it is possible that restriction sites may be conserved as well. PFGE has also been found to be more effective than ribotyping in differentiating methicillin-resistant *Staphylococcus aureus* strains (16). Recently, after digestion with *Bsc*I, Hall et al. (2) ribotyped 12 *E. faecalis*, 5 *E. faecium*, and several other enterococci isolated on more than one occasion from the same individual. They found highly related patterns within *E. faecalis* and within *E. faecium* and were unable to differentiate strains they found to be different by conventional gel electrophoresis of *Sst*I-digested DNA. They were also unable to demonstrate polymorphism in the ribotype of isolates from one of two individuals who harbored two enterococcal isolates, although these two isolates had different DNA fingerprints by conventional gel electrophoresis. In that report (2), DNA restriction fragment analysis by conventional electrophoresis was found to be effective in typing enterococcal isolates. However, their results showed only 8 to 13 bands in the 1.6- to 8-kb size range, which (considering that the enterococcal genome has an estimated size of 2,000 to 2,800 kb [7]) leaves a large part of the genome unresolved. In addition, typing based on the use of small fragments without a probe raises the possibility that what is being assessed may be plasmid DNA or insertions or deletions in plasmid or chromosomal DNA.

In summary, ribotyping with *Eco*RI and *Hin*dIII (this study) or *Bsc*I (2) does not appear to be a suitable method for differentiating enterococcal strains for epidemiologic purposes; whether other enzymes will generate greater diversity remains to be seen. At present, PFGE appears to be more discriminatory and has been used more often than other methods for subspecies differentiation of these organisms.

ACKNOWLEDGMENT

This work was supported in part by grant AI27249 from the USPHS.

REFERENCES

1. **Derlot, E., and P. Courvalin.** 1991. Mechanisms and implications of glycopeptide resistance in enterococci. Am. J. Med. **91**(Suppl. 3B):82S–85S.
2. **Hall, L. M., B. Duke, M. Guiney, and R. Williams.** 1992. Typing of *Enterococcus* species by DNA restriction fragment analysis. J. Clin. Microbiol. **30**:915–919.
3. **Hodel, S. L., and B. E. Murray.** Mobilization of the gentamicin resistance gene in *Enterococcus faecalis*. Antimicrob. Agents Chemother. **34**:1278–1280.
4. **Kuhnen, E., F. Richter, K. Richter, and L. Andries.** 1988. Establishment of a typing system for group D streptococci. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **267**:322–330.
5. **Luginbuhl, L. M., H. A. Rotbart, R. R. Facklam, M. H. Roe, and J. A. Elliot.** 1987. Neonatal enterococcal sepsis: case-control

study and description of an outbreak. Pediatr. Infect. Dis. **6**:1022–1030.

6. **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.
7. **Miranda, A. G., K. V. Singh, and B. E. Murray.** 1991. DNA fingerprinting of *Enterococcus faecium* by pulsed-field gel electrophoresis may be a useful epidemiologic tool. J. Clin. Microbiol. **29**:2752–2757.
8. **Miranda, A. G., K. V. Singh, and B. E. Murray.** 1992. Determination of the chromosomal size of three different strains of *Enterococcus faecalis* and one strain of *Enterococcus faecium*. DNA Cell Biol. **11**:332–335.
9. **Murray, B. E.** 1990. The life and times of the enterococcus. Clin. Microbiol. Rev. **3**:46–65.
10. **Murray, B. E.** 1992. β-Lactamase-producing enterococci. Antimicrob. Agents Chemother. **36**:2355–2359.
11. **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.
12. **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.
13. **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. Nasnass, L. S. Moore, E. S. Wong, and G. Weinstock.** 1991. Evidence for clonal spread of a single strain of β-lactamase-producing *Enterococcus faecalis* to six hospitals in five states. J. Infect. Dis. **163**:780–785.
14. **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.
15. **Preheim, L., D. Pitcher, R. Owen, and B. Cookson.** 1991. Typing of methicillin resistant and susceptible *Staphylococcus aureus* strains by ribosomal RNA gene restriction patterns using a biotinylated probe. Eur. J. Clin. Microbiol. Infect. Dis. **10**:428–436.
16. **Prevost, G., B. Jaulhac, and Y. Piemont.** 1992. DNA fingerprinting by pulsed field electrophoresis is more effective than ribotyping in distinguishing among methicillin resistant *Staphylococcus aureus* isolates. J. Clin. Microbiol. **30**:967–973.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Schaberg, D. R., D. H. Culver, and R. P. Gaynes.** 1991. Major trends in the microbial etiology of nosocomial infection. Am. J. Med. **91**(Suppl. 3B):72S–75S.
19. **Stull, T. L., J. J. LiPuma, and T. D. Eldind.** 1988. A broad spectrum probe for molecular epidemiology of bacteria: Ribosomal RNA. J. Infect. Dis. **157**:280–286.
20. **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.