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Comparison of Genomic DNAs of Different Enterococcal Isolates Using Restriction Endonucleases with Infrequent Recognition Sites

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Epidemiologic evaluation of enterococci has been limited by the lack of a simple and effective method for comparing strains. In this study, we have compared chromosomal restriction endonuclease digestion patterns of 27 isolates of *Enterococcus faecalis* from three different locations by using pulsed-field electrophoresis of large chromosomal fragments (14 to 1,000 kilobases). All but two isolates generated a clear, evaluable pattern with a single lysis and digestion, and the remaining two were visualized when a larger quantity of bacteria was used. All isolates from different locations generated different restriction patterns, as did most isolates within a single location; there was also evidence of spread of strains between individuals in each location. The ease with which this analysis can be performed, together with the clarity and polymorphism seen in the patterns, suggests that this technique will be very useful for epidemiological evaluations of nosocomial enterococcal infections.

Enterococci are important causes of clinical infections, including endocarditis, urinary tract infections, and superinfections in persons who are receiving or have recently received antibiotics (17). Although enterococci are normal inhabitants of the gastrointestinal tract and may migrate from this area to cause infections, these organisms can also be spread nosocomially (14, 35). However, epidemiologic studies of enterococci have been limited by the lack of a convenient and accessible method for comparing strains. For example, biotypes and antibiograms often show little variation within an enterococcal species and are generally not sufficiently discriminatory to be helpful. Bacteriophage typing and enterococcinotyping have been used with some success but require access to special reagents and performance of a large number of tests (11). Total plasmid content has been used to compare some strains of enterococci (14, 35); however, we have experienced much more difficulty and inconsistency in the generation of clearly visible plasmid patterns with enterococci than with other organisms such as *Escherichia coli* and *Shigella* sp. (personal observations). This study was initiated (i) to determine whether enterococci can be easily and reliably lysed by using a protocol designed for analysis of chromosomal DNA and (ii) to determine the extent of restriction pattern polymorphism when chromosomal DNA is analyzed with a low-frequency-cleavage restriction endonuclease and pulsed-field electrophoresis. The overall goal was to assess the potential utility of this methodology for epidemiologic analyses of clinical enterococcal isolates.

MATERIALS AND METHODS

Bacterial strains. Isolates of *Enterococcus faecalis* had been collected in 1980 and 1981 from the United States, Thailand, and Chile during studies of high-level resistance (HLR) to aminoglycosides (MICs > 2,000 μg/ml) (16, 19); for each location, all enterococci were isolated from patients in a single hospital over a 1- to 2-month period. For this study, 27 isolates were selected and retested for HLR to streptomycin, kanamycin, and gentamicin. In an effort to avoid studying a single strain per location, isolates with different patterns of HLR to aminoglycosides were chosen; all were from different patients except for BE 82 and BE 83, which had different patterns of HLR to aminoglycosides. The resistance patterns and isolates studied are shown in Table 1. *E. coli* MG1655 was used as a control for molecular size determination (2).

Restriction fragment analysis. Genomic DNA from the enterococcal isolates and from *E. coli* MG1655 was prepared by a modification of the method described by Smith and Cantor (28). Enterococci were grown overnight in 5 ml of brain heart infusion broth at 37°C. The cells were harvested and suspended in an equal volume of PIV buffer (1 M NaCl, 10 mM Tris hydrochloride [pH 7.6]). A portion (2.5 ml) of this suspension was mixed with 2.5 ml of 1.6% low-melting-temperature agarose (InCertAgarose; FMC Corp., Marine Colloids Div., Rockland, Maine) in water at 40 to 50°C and then pipetted into a plug mold (Bio-Rad Laboratories, Richmond, Calif.) and allowed to solidify. For lysis, one to four plugs were placed in 10 ml of fresh lysis solution (6 mM Tris hydrochloride [pH 7.6], 1 M NaCl, 100 mM EDTA [pH 7.5], 0.5% Brij 58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, 20 μg of RNase [DNase free] per ml, 1 μg of lysozyme per ml). Following incubation overnight at 37°C with gentle shaking, this solution was replaced with 10 ml of ESP (0.5 M EDTA [pH 9 to 9.5], 1% sodium lauroyl sarcosine, 50 μg of proteinase K per ml) and then incubated overnight at 50°C with gentle shaking. The plugs were washed three times for 30 min each with 15 ml of TE (10 mM Tris hydrochloride [pH 7.5], 0.1 mM EDTA) and then stored at 4°C.

*Sma*I was chosen for digestion of enterococci because it has a G+C-rich recognition sequence, while *E. faecalis* has a G+C content of ~40% (26). Digestion with *Sma*I was performed by placing a small slice (~1 mm thick) of an agarose plug in a microcentrifuge tube with 200 μl of distilled water followed by 25 μl of reaction buffer and 2 μl of *Sma*I (Bethesda Research Laboratories, Inc., Gaithersburg, Md.); this was then incubated for 6 h at 25°C. The slices were washed with TE (1 ml) for 1 h at 37°C. They were then

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TABLE 1. Resistance patterns and isolates used in this study

Isolate and source[a]	High-level aminoglycoside resistance[b]			Chromosome pattern
	Km	Gm	Sm	
Thailand				
BE 11	−	−	−	B-5
BE 17	−	−	−	B-6
BE 18	+	−	+	B-9
BE 59	+	+	+	B-7
BE 78	+	−	+	B-2
BE 81	−	−	−	B-4
BE 82	−	−	+	B-1
BE 83	+	+	+	B-1
BE 86	+	+	+	B-1
BE 88	+	+	+	B-3
BE 114	−	−	+	B-1
BE 117	+	−	+	B-10
BE 120	−	−	+	B-8
BE 125	−	−	+	B-1
Chile				
CE 13	+	+	+	C-1
CE 30	+	+	+	C-1
CE 36	+	+	+	C-2
CE 39	+	+	+	C-1a[c]
CE K1	+	+	−	C-3
CE K4	+	+	+	C-1b[c]
CE S22	+	+	+	C-4
Houston, Tex.				
HH 31	+	−	+	H-3
HH 52	+	−	+	H-4
HH 54	+	+	+	H-1
HH 98	+	+	+	H-2
HH 123	+	−	+	H-5
HH 181	+	+	+	H-1

[a] All isolates were from different patients, except for BE 82 and BE 83.
[b] Gm, Gentamicin resistance; Km, kanamycin resistance; Sm, streptomycin resistance.
[c] Patterns C-1a and C-1b appear to be variants of pattern C-1.

melted at 55 to 65°C and loaded into the wells of 1.2% agarose gels (SeaPlaque GTG agarose; FMC Corp.) in 0.5× TBE buffer. *E. coli* MG1655 digested with *Not*I or lambda concatamers (FMC Corp.) or both was used as a size standard. The gels were processed by using the contour-clamped homogeneous electric fields device (CHEF-DRII) from Bio-Rad; the pulse time was increased from 5 to 35 s over 30 h at 200 V. Gels were then subjected to staining with ethidium bromide followed by 1 to 10 h of destaining in distilled water (longer destaining gives sharper definition of bands) and photographed with UV illumination.

RESULTS

By using our standard protocol for *E. coli*, 25 of the 27 *E. faecalis* isolates showed an evaluable restriction endonuclease digestion pattern with the first lysis and digestion procedure. Two strains (Fig. 1, lane d; Fig. 2, lane c) had insufficient DNA; a repeat digestion with a larger slice generated an evaluable pattern for these two strains.

The restriction endonuclease digestion patterns of 26 of the 27 *E. faecalis* isolates studied are shown in Fig. 1 through 4. On these and other gels obtained by running different combinations of strains together, 19 different patterns and 2 variants were seen. These were arbitrarily designated patterns B-1 to B-10 for strains from Bangkok, Thailand; C-1 to C-4 for strains from Chile; and H-1 to H-5 for strains from Houston, Tex. (Table 1). Fragment sizes ranged from <30 to ~400 kilobases; some of these fragments might represent plasmid DNA. No isolate from one location had a restriction endonuclease digestion pattern that was identical to or closely resembled that of an isolate from another location. Within each geographic location, there was also considerable restriction fragment length polymorphism among the various isolates. However, some isolates from a given location had the same restriction pattern and presumably represent a single strain which was spread between patients. Isolates that have identical restriction endonuclease digestion patterns were found in Chile (Fig. 1, lanes a and b), in Houston (Fig. 1, lanes e and g), and in Thailand (Fig. 3, lanes

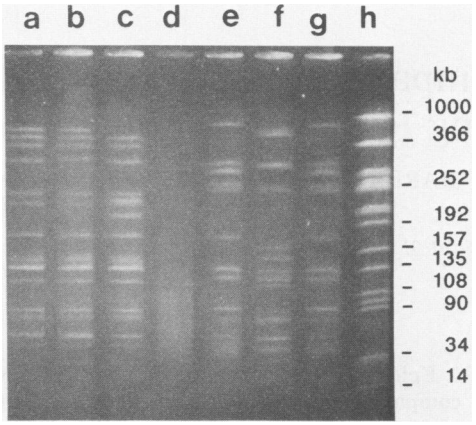

FIG. 1. Chromosomal digestion patterns of enterococci from Chile and Houston, Tex. All digestions were performed with *Sma*I. Among isolates from Chile, pattern C-1 is seen in isolates CE 13 (lane a) and CE 30 (lane b); pattern C-1a (isolate CE 39 in lane c) appears to differ by two to three fragments. Strain CE 36 (lane d) is not well seen. Among isolates from Houston, pattern H-1 is seen in isolates HH 54 (lane e) and HH 181 (lane g); lane f contains HH 98, pattern H-2. Lane h contains *Not*I-digested chromosomal DNA from *E. coli* MG1655 which was used as a molecular size standard. kb, Kilobases.

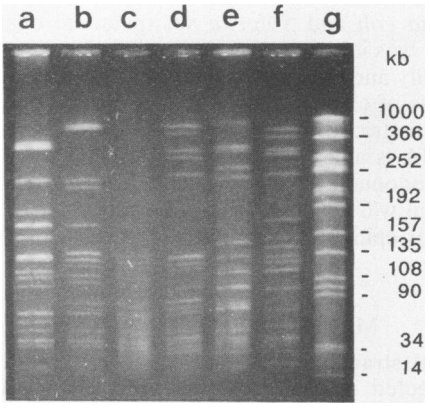

FIG. 2. Chromosomal digestion patterns of enterococci from Bangkok, Thailand, and Chile generated by *Sma*I. Lane a contains isolate BE 120 (pattern B-8). Lane b shows BE 125 (pattern B-1); lane c (BE 114) also shows pattern B-1 but had insufficient DNA on this gel. Lane d contains BE 18 (pattern B-9), and lane e contains BE 117 (pattern B-10). Lane f contains CE K1 from Chile (pattern C-3). Lane g contains *E. coli* MG1655 chromosomal DNA digested with *Not*I. kb, Kilobases.

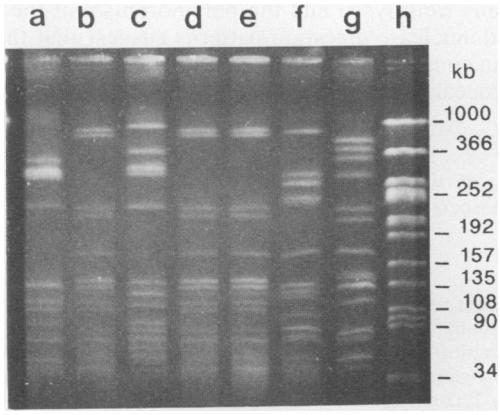

FIG. 3. Chromosomal digestion patterns of enterococci from Bangkok, Thailand, and Chile generated by *Sma*I. Among Bangkok isolates, lane a shows pattern B-4 (isolate BE 81); lanes b (isolate BE 82), d (BE 83), and e (BE 86) show pattern B-1; lane c (isolate BE 78) shows pattern B-2; and lane f (isolate BE 88) shows pattern B-3. Lane g contains CE 13 (pattern C-1) from Chile. Lane h contains *E. coli* MG1655 digested with *Not*I. kb, Kilobases.

b, d, and e). Two isolates from Chile (Fig. 1, lane c; Fig. 4, lane b) appeared to have a variation of the pattern shown in Fig. 1 (lanes a and b) and Fig. 4 (lane d).

Since isolates with identical restriction patterns are interpreted as representing the same strain or a recent derivative, such isolates usually have identical resistance patterns; this also means that strains with different resistance patterns usually have different restriction endonuclease digestion patterns. This was observed with all enterococci in this study except those showing pattern B-1 from Thailand. Within this pattern, isolates BE 83 and BE 86 had HLR to streptomycin and gentamicin, while isolates BE 82 (isolated from the same patient as BE 83), BE 114, and BE 125 had HLR to streptomycin but not gentamicin. This pattern was further pursued by analyzing the plasmids of these isolates and by hybridizing chromosomal and plasmid DNA to a gene probe for gentamicin resistance; this probe contains the bifunctional 6′-aminoglycoside acetyltransferase–2″-aminoglycoside phosphotransferase and was prepared as previously described (33). Plasmid DNA from BE 83 (resistant to high levels of gentamicin) had several restriction fragments not present in plasmid DNA from BE 125 (lacking resistance to high levels of gentamicin), one of which hybridized to the gentamicin resistance gene probe (data not shown). There was no hybridization to plasmid DNA from BE 125 or to chromosomal DNA from either strain.

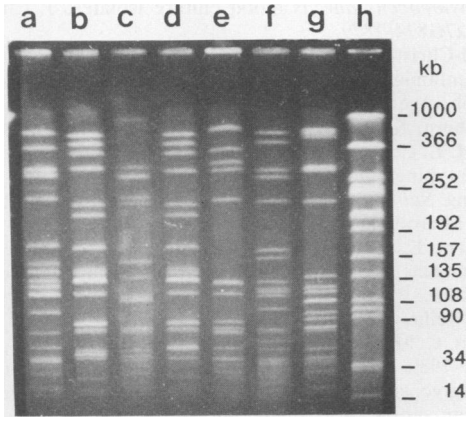

FIG. 4. Chromosomal digestion patterns of enterococci from Chile and Houston, Tex., generated by *Sma*I. Lane a shows pattern C-3 (isolate CE K1), lane b shows pattern C-1b (isolate CE K4) which differs from pattern C-1 (lane d, isolate CE 13) by one to two bands, and lane c shows pattern C-4 (isolate CE S22). Among Houston strains, lane e shows pattern H-3 (isolate HH 31), lane f shows pattern H-4 (isolate HH 52), and lane g shows pattern H-5 (isolate HH 123). Lane h contains chromosomal DNA from *E. coli* MG1655 digested with *Not*I. kb, Kilobases.

DISCUSSION

It is often important to be able to compare different isolates of a particular species. This may be helpful in epidemiological studies, in which the demonstration that different persons are infected with a single strain would suggest that an outbreak has occurred, while the presence of different strains would point away from an outbreak. Comparing isolates may also have clinical relevance for individual patients; for example, the demonstration that the same strain is present in a posttherapy urine culture has clinical and therapeutic implications different from those of the demonstration of different strains. In the past, bacteria have been compared by using phenotypic properties, including those relating to biochemical reactions, antibiotic resistance, phage typing, bacteriocin typing, and serotyping. Each of these techniques has some difficulties. Biotyping can be useful when multiple tests are done in a standardized manner, such as with multilocus enzyme analysis (6, 27), but routinely available kits, such as API, are not usually sufficient, especially for organisms which grow poorly or are largely nonfermentative. Other problems include the following: some techniques may be useful for only a limited number of species, some require individualized reagents for each species or genus, some require a large number of individual assays, and some may not be applicable to all members of a species (5, 8, 10, 12, 32).

Genetic techniques have also been used to compare strains. One type of analysis compares the total plasmid content of isolates (5, 10, 12, 15, 23, 32, 34; B. E. Murray and S. L. Hodel-Christian, *in* V. Lorian, ed., *Antibiotics in Laboratory Medicine*, 3rd ed., in press); this technique is most useful when there is a reliable and easy lysis method for the organism being investigated and when plasmids, preferably two or more, are present in most of the isolates examined. Comparisons of chromosomal digestion patterns, using the same electrophoretic conditions as those used for plasmid analyses, have also been performed in epidemiologic studies; however, results typically show a large number of fragments that are close together and may be difficult to analyze (21, 22, 31). This technique has been more successful when combined with hybridization to a gene probe to select a small number of fragments that generate more readily visible, and thus more easily compared, patterns (13, 29).

Recently, pulsed-field electrophoresis has been used to visualize very large fragments of DNA (1, 7, 21); such fragments can be generated from bacterial genomic DNA by restriction endonucleases that have few recognition sites. The choice of the restriction endonuclease depends on the G+C content of the organism being studied as well as the recognition sequence of the enzyme. *Not*I, for example, has an 8-base-pair recognition sequence and cleaves *E. coli* chromosomal DNA an average of 25 times. Enzymes such as *Sma*I, whose recognition sequence is CCCGGG, cleave *E.*

coli chromosomal DNA many times but cleave organisms with a lower G+C content, such as staphylococci, streptococci, and enterococci, less frequently. *Sma*I has been recently used for digestion in an epidemiologic investigation of staphylococci (7).

In this study, we have established that enterococci can be easily lysed and prepared for restriction endonuclease digestion of genomic DNA; indeed, the procedure used is the one we had been using for *E. coli* and was applied to enterococci without modification. This is in contrast to most plasmid lysis protocols for enterococci which differ from the procedures for *E. coli* by requiring growth in glycine or L-threonine, additional lysozyme, and/or mutanolysin to achieve adequate lysis. This study also showed that there is considerable restriction fragment length polymorphism among enterococcal chromosomes, even among contemporary isolates from a single geographic location. The presence of polymorphism is important, since it allows the interpretation that isolates which have the same genomic restriction pattern likely represent a single strain. Thus, we interpret isolates BE 82, BE 83, BE 86, BE 114, and BE 125 as representing a single strain, even though some lacked HLR to gentamicin. Since it has been well documented that bacteria can lose or acquire resistance in vivo in a single patient, this finding is not really surprising (3, 19, 24, 25). In general, the lack of variation in the chromosomal digestion pattern B-1, even though the resistance pattern differs, could be due to the gain or loss of a transposon which did not have a *Sma*I site and which was not large enough to make a discernible difference in fragment size or to a plasmid which might not be seen at the bottom of the gel or which had the same size as another fragment. Mutational gain or loss of resistance could also change a resistance phenotype without changing the restriction pattern. In this study, gentamicin resistance was explained by the presence of new plasmid DNA which hybridized to a gentamicin resistance gene probe. Since the gentamicin resistance gene appears to be on a transposon in enterococci as well as staphylococci (9), this finding is not surprising.

With this or any other technique, the presence of what appears to be the same strain does not prove a direct epidemiologic relationship. This has been illustrated in epidemiologic studies of group B streptococci, in which the same *Eco*RI chromosomal patterns were found in epidemiologically unrelated individuals, and with plasmid pattern analyses of *Shigella sonnei*, which showed a single pattern in several locations, including one location in which the single pattern occurred for five consecutive years (4, 23). Another situation that can make interpretation of chromosomal patterns difficult is that in which isolates differ by only a few fragments. Such differences could arise within a single individual from inversions, deletions, or other rearrangements of the chromosome or from the acquisition or loss of a prophage, transposon, insertion sequence, or plasmid. On the other hand, such differences could indicate that the isolates are more distantly related. Although it is not known how to interpret small variations, an evaluation of the variation present in a number of other isolates in the same locale may be useful. The possibility of in vivo changes in phenotype or genotype is not limited to this technique and represents potential limitations of any typing scheme.

In summary, we have shown that the comparison of chromosomal restriction endonuclease digestion patterns by using enzymes that generate large DNA fragments, a technique recently applied to other organisms, can also be applied to *E. faecalis*. The ease and reliability with which organisms were lysed and the polymorphism of the restriction endonuclease digestion patterns suggest that this technique, in comparison with other techniques used to compare enterococcal isolates, represents a significant advance.

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