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Analysis of *Enterococcus faecalis* Isolates from Intercontinental Sources by Multilocus Enzyme Electrophoresis and Pulsed-Field Gel Electrophoresis

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Pulsed-field gel electrophoresis (PFGE) and multilocus enzyme electrophoresis (MLEE) were compared in this study of 65 *Enterococcus faecalis* isolates recovered over a 20-year period from diverse geographic sources. Clonal relationships recognized by PFGE were also recognized by MLEE; however, MLEE recognized a greater number of isolates as belonging to clonal groups than did PFGE. Both techniques were reproducible and discriminatory, but MLEE more readily recognized relationships among large numbers of isolates. MLEE confirmed the previously reported clonal spread of β-lactamase-producing *E. faecalis* to six hospitals in five states. MLEE provided a useful population framework of the *E. faecalis* isolates in this sample, while PFGE was able to differentiate among isolates within some MLEE clonal groups.

Enterococci are frequently recognized nosocomial pathogens; they are isolated from 12% of all such infections (17). Compounding the problem of their high incidence, enterococci are resistant or relatively resistant to commonly used antibiotics (10). Enterococcal infections were originally thought to arise from a patient's own gut flora; however, recent studies have clearly demonstrated both intra- and interhospital spread of these organisms (13, 16). Enterococcal infections can be difficult to eradicate; indeed, for some infections caused by multiresistant isolates, there is no therapy with established efficacy (2). In such instances, controlling the spread of these organisms becomes of paramount importance. Molecular typing tools aid the identification of clonal outbreaks and can establish a clonal population framework useful for further study of the organism. A population structure of enterococci has not been previously reported.

Successful epidemiologic investigations are aided by typing techniques that demonstrate good discriminatory reliability. Pulsed-field gel electrophoresis (PFGE) has shown success in typing enterococci and has facilitated the study of local outbreaks and dissemination of antibiotic resistance among enterococci (13). Our concerns about the subjective nature of determining clonal relationships among isolates by using PFGE, and the difficulties associated with comparing large numbers of isolates, stimulated our interest in comparing results obtained from PFGE with those generated by multilocus enzyme electrophoresis (MLEE). MLEE offers an advantage over other techniques in that its output is a list of allele profiles collectively known as an electrophoretic type (ET). These ETs can be readily organized in a database, facilitating the recognition of unexpected relationships among isolates. Furthermore, the relatively objective nature of interpretation allows investigators to compare findings without necessarily exchanging isolates. Finally, MLEE has been established to have utility as a bacterial population genetics tool by its characterization of the genetic structures of *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, and many other species (1, 3, 14).

In this study of *Enterococcus faecalis* isolates cultured from intercontinental sources, we compared the results of MLEE with those obtained by PFGE. We confirmed, by MLEE, our previous findings of a widespread, interstate β-lactamase-producing (Bla^+) *E. faecalis* clone (13). In addition, we explored the relative strengths and limitations of these typing techniques when applied to *E. faecalis.*

MATERIALS AND METHODS

Bacterial isolates. The 65 strains used in this study were all *E. faecalis* isolates chosen to represent geographic and temporal diversity and, in instances in which PFGE had previously been performed, to represent strains with a diversity of PFGE patterns (Table 1). The isolates had been collected over a 20-year period from various locations in the United States, Thailand, Lebanon, Chile, and Argentina (11–13). Most of these isolates were from urine, wounds, and blood. Some were from patients with infectious endocarditis, notably, the Mayo Clinic isolates and those labelled END from Boston, Mass. (4). Isolates were not chosen for any particular antibiotic susceptibility pattern except to represent the Bla^+ isolates previously reported as representative of clonal spread to six hospitals in five states (13). Other Bla^+ isolates, clonally distinct from the aforementioned Bla^+ organisms, were also included. They were from different regions, including West Haven, Conn.; Beirut, Lebanon; and Buenos Aires, Argentina (11, 13, 15). Bla^- isolates from the same hospitals were included when available. Several fecal isolates from normal volunteers were included (4). Two laboratory isolates, JH2-2 and OG1-RF (ATCC 47077), were included (6, 9). Isolates were identified to species level by routine biochemical tests (5).

PFGE of genomic DNA. Genomic DNA was prepared in agarose plugs as previously described (12). For restriction endonuclease digestion of DNA, small slices of the agarose plugs were placed into a mixture of 200 μl of distilled water, 25 μl of reaction buffer, and 16 U of *Sma*I (New England BioLabs, Beverly, Mass.) and incubated at 25°C for 6 h or overnight. After digestion, the plugs were washed for 1 h at 37°C. The slices were placed in wells of a 1.2% SeaPlaque GTG agarose gel (FMC) made with 0.5× TBE (10× TBE is 0.89 M Tris, 0.89 M boric acid, and 0.025 M EDTA), and the wells were sealed with the same agarose. Gels were electrophoresed by using clamped homogeneous electric fields (CHEF-DRII; Bio-Rad) (12), stained with ethidium bromide, and photographed with a UV light source.

Interpretations of PFGE patterns were made by comparing isolates on the same agarose gel. The total numbers of visible bands were counted for each isolate, and patterns were compared visually. Once isolates were recognized as having identical patterns, a representative isolate of the group was used to compare its pattern with those of similar isolates. When isolates run on different gels demonstrated apparent similarities, a subsequent gel was run so that isolates could be compared in close proximity. Isolates were considered to be members of the same clone when they shared most bands and thus had similar, but not necessarily identical, patterns. As clonal groups became larger, isolates were

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TABLE 1. *E. faecalis* isolates used in this study

Isolate	Origin (reference)	PFGE pattern	MLEE ET
E-3	Richmond, Va.	1	25
E-12	Richmond, Va.	2	24
E-24	Richmond, Va.	3	5
E-36	Richmond, Va.	4	4
E-47[a]	Richmond, Va. (13)	5	21
E-48[a]	Richmond, Va. (13)	5	20
E-78	Richmond, Va.	6	14
E-228[a]	Richmond, Va.	5	20
E-278[a]	Richmond, Va.	5	20
E-340[a]	Richmond, Va.	5	20
E-366[a]	Richmond, Va.	5	20
FLA-1[a]	Florida (13)	5	20
FLA-2[a]	Florida (13)	5	20
DEL[a]	Delaware (13)	5	20
CH-570[a]	Pittsburgh, Pa. (13)	5	20
HH-22[a]	Houston, Tex. (13)	5	20
MCP-270	Philadelphia, Pa.	20	12
MCP-273	Philadelphia, Pa.	5	20
MCP-276	Philadelphia, Pa.	5	20
BEIRUT[a]	Beirut, Lebanon (13)	7	7
WH-245[a]	West Haven, Conn. (15)	8	5
WH-257[a]	West Haven, Conn. (15)	8	5
WHVA-249	West Haven, Conn.	9	8
WHVA-603	West Haven, Conn.	10	2
WHVA-3356	West Haven, Conn.	11	1
WHVA-3358	West Haven, Conn.	18	12
WHVA-4604	West Haven, Conn.	13	3
HG-1081	Buenos Aires, Argentina	17	13
HG-1086	Buenos Aires, Argentina	15	10
HG-1090	Buenos Aires, Argentina	12	12
HG-1096	Buenos Aires, Argentina	12	12
HG-1097	Buenos Aires, Argentina	12	12
HG-1100	Buenos Aires, Argentina	14	22
HG-1113	Buenos Aires, Argentina	16	6
HG-1116	Buenos Aires, Argentina	12	12
HG-1184	Buenos Aires, Argentina	12	12
HG-6280[a]	Buenos Aires, Argentina (11)	19	16
HG-9829[a]	Buenos Aires, Argentina (11)	19	16
HG-10528[a]	Buenos Aires, Argentina (11)	19	16
BE-17	Bangkok, Thailand (12)	B-6	18
BE-18	Bangkok, Thailand (12)	B-9	18
BE-86	Bangkok, Thailand (12)	B-1	15
BE-88	Bangkok, Thailand (12)	B-3	18
MC 02152	Mayo Clinic (4)	MC-1	18
MC 08036	Mayo Clinic (4)	MC-2	18
MC 22208	Mayo Clinic (4)	MC-3	18
MC 22499	Mayo Clinic (4)	MC-4	18
MC 25561	Mayo Clinic (4)	MC-5	18
END 6	Boston, Mass. (4)	E-1	9
END 11	Boston, Mass. (4)	E-1	9
END 16	Boston, Mass. (4)	E-2	18
END 27	Boston, Mass. (4)	E-3	18
SE-3	Houston, Tex. (4)	S-1	11
SE-7	Houston, Tex. (4)	S-1	11
SE-18a	Houston, Tex. (4)	S-2	15
SE-20	Houston, Tex. (4)	S-3	23
SE-21	Houston, Tex. (4)	S-4	18
OG1-RF	Laboratory strain (6, 9)	VIII	25
JH2-2	Laboratory strain (9)	VII	17
Phaneuf	Boston, Mass. (4)	E-4	26
PA[a]	Philadelphia, Pa. (13)	5	17
CE-13	Santiago, Chile (12)	C-1	17
CE-36	Santiago, Chile (12)	C-2	15
Sm22	Santiago, Chile (12)	C-4	18
K4	Santiago, Chile (12)	C-1	17

[a] β-Lactamase-producing isolate.

compared with several members of the group, and a common, or modal, pattern was identified. In a similar manner, isolates were considered to be members of a large clonal group when they shared most bands and had a pattern similar to the modal pattern.

Preparation of enzyme extracts for MLEE. To obtain sufficient concentrations of enzymes in lysate preparations, approximately 10^{11} cells of each isolate were obtained by growth in 200 ml of brain heart infusion broth supplemented with 0.5% glycine overnight with agitation at 37°C. Cells were harvested by centrifugation, resuspended in 1.5 ml of TE (50 mM Tris, 5 mM EDTA pH 7.5), and kept on ice. Five hundred microliters of lysozyme (25 mg/ml) was added to the suspensions, which were then incubated at 37°C for 45 min. The cells were lysed by sonication with a model 200 Branson Cell Disrupter for 30 s in an ice bath. After lysis and centrifugation at 20,000 × *g* for 20 min, the lysate supernatants were stored at −70°C. Starch gels were prepared and loaded as previously described and then electrophoresed at a constant voltage in a cooled environment (4°C) (18). Following electrophoresis, the gel was cut into three horizontal slices 1 to 2 mm thick and incubated individually in various enzyme staining solutions at 37°C. The enzyme staining solutions contained an enzyme-specific substrate, necessary coenzymes and cofactors, buffer, salts, and a dye. The staining solutions were as previously reported (18). Gels were incubated until bands appeared (the time varied with the activity of each enzyme), rinsed with water, and fixed with a 1:5:5 mixture of acetic acid, methanol, and water.

The following 15 metabolic enzymes were chosen for their visual clarities after staining and for degrees of polymorphism adequate to resolve relationships among the isolates studied: leucine aminopeptidase, phosphoglucose isomerase, carbamylate kinase, adenylate kinase, nucleoside phosphorylase, phosphoglucomutase, 6-phosphogluconate dehydrogenase, glutamate dehydrogenase, esterase, lactate dehydrogenase, glucose 6-phosphate dehydrogenase, leucylglycylglycine peptidase-1, leucylglycylglycine peptidase-2, phenylalanylleucine peptidase-1, and phenylalanylleucine peptidase-2. Comparisons of the mobilities of enzymes from different isolates were made visually against one another on the same gel; for each enzyme, distinctive electromorphs were given numbers in order of decreasing anodal migration. The absence of enzyme activity was scored as a null allele. Isolates were then chosen as standards in terms of their electrophoretic mobilities and used for relative comparison on subsequent gels. Each isolate was characterized by its combination of electromorphs over the number of enzymes assayed, yielding a unique ET. Analyses of the data, including the calculations of genetic distance and genetic diversity, were performed as previously reported (14, 18).

RESULTS

The isolates studied and their respective PFGE patterns and MLEE ETs are listed in Table 1.

PFGE. With *Sma*I, PFGE yielded between 13 and 20 visible bands for each isolate. Among the 65 isolates studied, PFGE recognized 42 clonal patterns, including 7 clones that contained multiple isolates (PFGE patterns 5, 8, 12, 19, E-1, S-1, and C-1). There were a total of 30 isolates found among the seven clonal groups containing multiple isolates. Although most of the clones contained isolates that demonstrated patterns identical or almost identical (one to three band differences) to others of the same clone, the larger clonal groups contained isolates with greater differences. Depending on which two isolates from a clonal group were compared, differences in up to five or six bands could be demonstrated; however, fewer differences (one to three bands) existed between these isolates when they were compared with an isolate demonstrating the common or modal pattern. When clonal isolates differed by five or six bands, the differences were generally in fragments of 50 to 100 kb.

MLEE. In this collection of 65 *E. faecalis* isolates, 13 of 15 loci examined by MLEE were polymorphic for alleles encoding electrophoretically distinct metabolic enzymes, and 2 loci were monomorphic (Table 2). MLEE defined 26 ETs (Table 1), including 9 ETs containing more than one isolate (ET-5, -9, -11, -12, -15, -16, -17, -18, and -20) (Table 3). There were 48 isolates found among the nine ETs containing multiple isolates.

Comparison of PFGE and MLEE. Comparison of the groups containing multiple isolates revealed identical clonal relationships for 28 isolates recognized by both MLEE and PFGE. The techniques differed in classifying 21 isolates representing six clonal groups. Table 3 shows the MLEE ETs with

TABLE 2. Allele frequencies and genetic diversities at 15 enzyme loci in 26 ETs of *E. faecalis*

Enzyme locus[a]	Frequency of allele:					Genetic diversity (*h*)
	1	2	3	4	5	
LAP	0.038	0.769	0.154	0.038		0.397
PGI	1.000					0.000
CAK	0.885	0.115				0.212
ADK	1.000					0.000
NSP	0.923	0.038	0.038			0.151
PGM	0.038	0.154	0.808			0.335
6PG	0.077	0.923				0.148
GLU	0.077	0.385	0.423	0.077	0.038	0.686
EST	0.038	0.115	0.731	0.115		0.455
LDH	0.038	0.846	0.115			0.280
G6P	0.808	0.192				0.323
LGGF	0.962	0.038				0.077
LGGS	0.962	0.038				0.077
PLEF	0.038	0.846	0.115			0.280
PLES	0.308	0.615	0.038	0.038		0.545

[a] LAP, leucine aminopeptidase; PGI, phosphoglucose isomerase; CAK, carbamylate kinase; ADK, adenylate kinase; NSP, nucleoside phosphorylase; PGM, phosphoglucomutase; 6PG, 6-phosphogluconate dehydrogenase; GLU, glutamate dehydrogenase; EST, esterase; LDH, lactate dehydrogenase; G6P, glucose 6-phosphate dehydrogenase; LGGF, leucylglycylglycine peptidase-1; LGGS, leucylglycylglycine peptidase-2; PLEF, phenylalanylleucine peptidase-1; PLES, phenylalanylleucine peptidase-2.

multiple isolates; 20 of these isolates were not recognized as belonging to the corresponding PFGE clonal group. From Table 3, one can appreciate that MLEE recognized clonal relationships that were not demonstrated by PFGE (ET-15 and -18) and that PFGE clonal groups were usually a subset of MLEE clones (ET-5, -12, and -17).

The MLEE clonal group ET-20 represents a large collection of isolates with PFGE pattern 5. Most of the isolates in ET-20 are highly gentamicin resistant and Bla^+; MCP-273 and MCP-276 are highly gentamicin resistant, Bla^- isolates collected from a hospital where a Bla^+ isolate of this clonal strain was also found. The isolate E-47, also PFGE pattern 5, was not included in ET-20 by MLEE. However, E-47's ET, ET-21, differs at only one locus from ET-20, demonstrating its close relationship to the other isolates in that ET (Fig. 1). MLEE, by recognizing the clonal relationship of these isolates, confirmed our previous findings by PFGE of clonal dissemination of this strain to hospitals in five states (13).

Five other ETs (ET-5, -12, -15, -17, and -18) contained isolates which had not been recognized as being related by PFGE. Three of these, ET-5, -12, and -17, contained a subset of isolates recognized by PFGE as related (Table 3), as well as isolates that had not been included in the same PFGE clonal group, namely, E-24 (ET-5), WHVA-3358 (ET-12), MCP-270

TABLE 3. ETs with multiple isolates

ET	Isolates
5	E-24,[a] WH-257, WH-245
9	END 6, END 11
11	SE-3, SE-7
12	MCP-270,[a] WHVA-3358,[a] HG-1090, HG-1096, HG-1097, HG-1116, HG-1184
15	SE-18a,[a] BE-86,[a] CE-36[a]
16	HG-6280, HG-9829, HG-10528
17	K4, CE-13, JH2-2,[a] PA[a]
18	SE-21,[a] MC 22499,[a] MC 22208,[a] MC 25561, MC 02152, MC 08036,[a] BE-17,[a] BE-18,[a] BE-88,[a] END 16,[a] END 27,[a] Sm22[a]
20	E-48, E-228, E-278, E-340, E-366, FLA-1, FLA-2, DEL, CH-570, HH-22, MCP-273, MCP-276

[a] Not recognized as clonally related by PFGE.

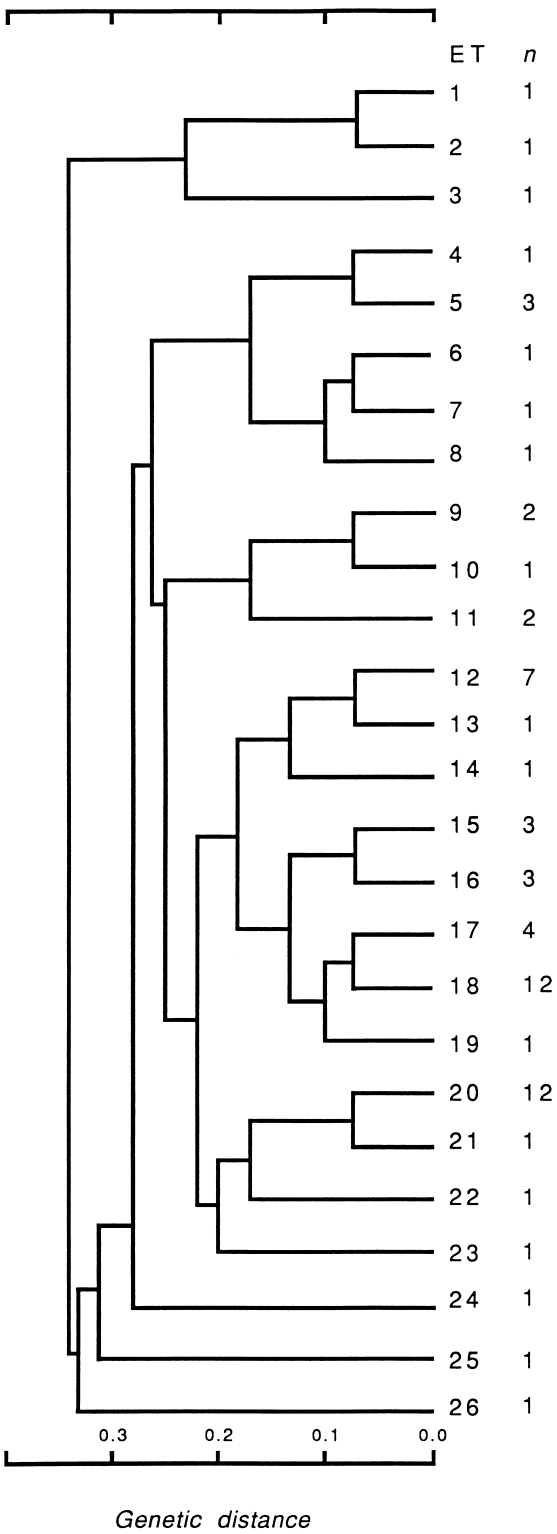

FIG. 1. Dendrogram showing estimates of genetic relationships among 26 ETs of 65 *E. faecalis* isolates based on allelic profiles at 15 enzyme loci.

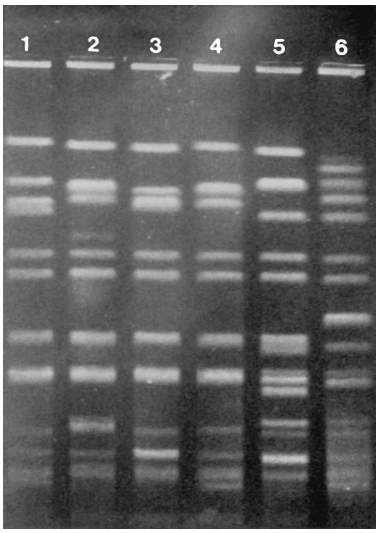

FIG. 2. *Sma*I genomic digestion patterns of isolates in ET-12. Lanes 1 to 4, isolates from Argentina (HG-1090, HG-1096, HG-1097, and HG-1116, respectively); lane 5, WHVA-3358; lane 6, MCP-270.

(ET-12), PA (ET-17), and JH2-2 (ET-17). Further PFGE analysis was performed on these isolates by placing them next to other representatives from their respective ETs. Figure 2 shows isolates WHVA-3358 and MCP-270 run on a gel with other representatives of ET-12. Lanes 1 to 4 show isolates from Argentina determined to be clonally related by both techniques, lane 5 contains WHVA-3358, and lane 6 contains MCP-270. Although the patterns for the isolates shown in lanes 1 to 4 were not identical, they demonstrate extensive similarities. The genomic digestion pattern of WHVA-3358 (lane 5) did not demonstrate sufficient similarities to the other isolates in this clonal group (lanes 1 to 4) for us to recognize a clonal relationship, even knowing the MLEE results. However, it is of interest that this isolate shared many bands with those in that clonal group. There were few similarities between the pattern of MCP-270 and the others on the gel. Similarly, ET-5 contained three isolates; PFGE recognized the Bla^+ isolates WH-245 and WH-257 as clonally related (PFGE pattern 8), whereas the third isolate, E-24 (Bla^-), showed no similarities to the other isolates of this ET. Finally, ET-17 contained four isolates, two of which were very similar by PFGE (Table 1, pattern C-1). The Bla^+ isolate PA, which was collected at the same hospital as the Bla^- isolates MCP-273 and MCP-276 and which also showed PFGE pattern 5, as well as the laboratory isolate JH2-2, which showed PFGE pattern VII, were included in this ET by MLEE.

MLEE recognized two ETs containing multiple isolates, all of which had different PFGE patterns. ET-15, found on three continents, contained two clinical isolates and a stool isolate from a healthy volunteer. ET-18 contained 12 isolates, 7 of which were from patients with infectious endocarditis. Figure 3 shows PFGE patterns for eight of the isolates in ET-18. In this example, one can see some similarities in the patterns, mainly in the lower half of the gel; however, the patterns differed considerably in the larger fragments, and none of these relationships were considered clonal by PFGE, except possibly MC 02152 and MC 25561.

DISCUSSION

A number of typing techniques have been applied to the epidemiologic investigation of enterococci, but few have performed well enough to gain wide acceptance. Simple-to-perform techniques such as antibiograms and biotypes generally show insufficient variation among enterococci, whereas serotyping and bacteriophage typing, although reportedly effective, are of limited use because they are tedious and require special reagents (7, 8). Plasmid profiling of undigested *E. faecalis* plasmids has worked for some investigators, but in our hands it was inconsistent and generated results that were less reproducible than those we obtained with gram-negative organisms (19, 20). We also found that ribotyping by using *Eco*RI- or *Hin*dIII-digested enterococcal genomic DNA with 16S plus 23S rRNA probes was less discriminatory than PFGE (6).

In our experience, PFGE has been consistently proven to have utility as a typing tool for enterococci; however, it does have limitations. Without the aid of sophisticated digital analysis, isolates must be compared visually on the same gel. Furthermore, as the number of isolates in an analysis increases, the limitations of human recognition may result in overlooked clonal relationships. In addition, uncertainty remains in determining how much similarity isolates should demonstrate in order to be called clonal. MLEE has not been previously applied to enterococci, but in general it is an efficient method and can more easily identify relationships among large groups of isolates than can PFGE.

In this study of 65 *E. faecalis* isolates, there was general agreement between the two techniques for most of the isolates. MLEE confirmed our previous findings of a clonal relationship among Bla^+ isolates from six hospitals in five states and supported the inclusion of several additional Bla^+ isolates, as well as two Bla^- isolates, MCP-273 and MCP-276, recovered from one of these hospitals (13). MLEE also confirmed that Bla^+ clones from Buenos Aires, Argentina (ET-16), and West Haven, Conn. (ET-5), as well as the single Bla^+ isolate from Beirut, Lebanon (ET-7), were different from this group and from each other (13).

Although in many instances, clones defined by PFGE were a subset of an MLEE ET, two clonal groups, ET-15 and -18, demonstrated few apparent PFGE pattern similarities among the isolates, and in ET-5, -12, and -17, MLEE recognized a broader clonal relationship than PFGE by including additional isolates with different PFGE patterns within a group of isolates sharing a common PFGE pattern. These data suggest that MLEE may recognize more ancestral relationships than those

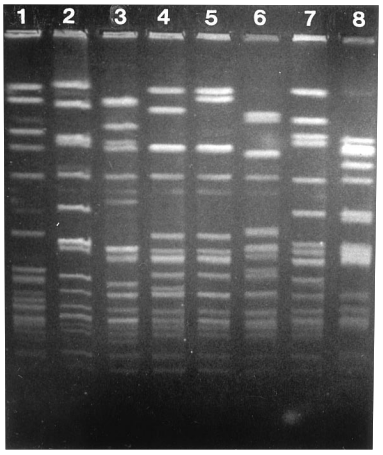

FIG. 3. *Sma*I genomic digestion patterns of isolates in ET-18. Lane 1, END 16; lane 2, END 27; lane 3, MC 08036; lane 4, MC 02152; lane 5, MC 25561; lane 6, MC 22499; lane 7, MC 22208; lane 8, SE-21.

recognized by PFGE and that PFGE may be able to discriminate among isolates in some MLEE clones. In another case two isolates from a PFGE clone were not part of the corresponding MLEE clone. These isolates, PA and E-47, are Bla^+ isolates which shared PFGE pattern 5 with other members of ET-20. In the case of isolate E-47 (ET-21), a close relationship with ET-20 was demonstrated by MLEE in that they differed at only one locus (Fig. 1). Finally, while MLEE generally recognized a broader (but consistent) clonal relationship among the sampled isolates than did PFGE, in the case of isolate PA (PFGE pattern 5), MLEE included it in a clone, ET-17, distinctly different from those of other Bla^+ isolates demonstrating pattern 5.

Several of the isolates in this study were obtained from patients with endocarditis. Whereas both techniques found END 6 and END 11 to be clonal, MLEE demonstrated an unexpected clonal structure among other endocarditis isolates in ET-18. This recognized relationship may be useful in studying the pathophysiology of enterococcal endocarditis.

In conclusion, this study illustrates some of the relative strengths of PFGE and MLEE in the epidemiologic investigation of *E. faecalis*. Both techniques demonstrated the ability to differentiate isolates at the subspecies level. MLEE appears to recognize broader clonal groups than PFGE. Hence, PFGE may be more useful in the study of the nosocomial spread of infection when isolates are shown or suspected to have had a recent spread and a more narrow genetic diversity, whereas MLEE can identify unexpected relationships among isolates that may later be shown to have common properties.

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