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Chromosomal DNA Restriction Endonuclease Digestion Patterns of β-Lactamase-Producing *Enterococcus faecalis* Isolates Collected from a Single Hospital over a 7-Year Period

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Twenty-three β-lactamase (β-lac)-producing, highly gentamicin-resistant *Enterococcus faecalis* isolates collected over a 7-year period from the same hospital were examined by pulsed-field gel electrophoresis of *Sma*I-digested genomic DNA. The β-lac$^+$ isolates appeared to form a single clonal group, which had been previously designated the mid-Atlantic pattern. Eleven variations of the mid-Atlantic clone, differing by one to six bands, were identified; some of the changes were likely due to plasmid bands. However, a number of isolates had indistinguishable patterns, including some recovered over a 4-year period. There was a surprising lack of movement of the β-lac determinant to other strains, although this trait was transferable in vitro by conjugation. We conclude that a single clone (the mid-Atlantic clone) of β-lac$^+$ *E. faecalis* has remained endemic in this hospital for at least 7 years. The reason(s) for the apparent lack of spread to other strains of *E. faecalis* is unknown.

Enterococci are increasingly implicated as important causes of clinical infections, including endocarditis, septicemia, urinary tract infections, and wound infections. Various typing methods have been used in an attempt to study the epidemiology of enterococcal infections, such as biotyping, serotyping, bacteriophage typing, bacteriocin typing (6), and plasmid analysis (7, 17). None of these methods has proven to be sufficiently discriminatory, reproducible, or convenient, nor are any of these methods widely accepted for comparing enterococcal strains. The drawbacks of these methods have led to the development of molecular 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-resistant enterococci to hospitals in different states (1, 11).

In this study, we compared the restriction endonuclease digestion patterns of 23 β-lactamase-producing (β-lac$^+$) *Enterococcus faecalis* isolates, collected over a 7-year period from the same hospital after a large outbreak of infections caused by β-lac$^+$, highly gentamicin-resistant *E. faecalis* (16). The aim was to determine the degree of change in PFGE chromosomal digestion patterns with time. Our hypothesis was that there would be evidence of spread into new strains since the isolates had been recovered over an extended period and since some of those isolates were previously shown to have β-lac and gentamicin resistance encoded on a transferable 55- to 65-MDa plasmid (8). We also examined the influence of plasmid bands on PFGE patterns to gain insight into whether plasmids may be responsible for any of the variations in PFGE patterns of isolates that appear to be clonally related.

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MATERIALS AND METHODS

Bacterial isolates. The 23 isolates used in this study were all β-lac$^+$ *E. faecalis* isolates chosen to represent different patients and temporal diversity. Isolates had been collected over a 7-year period (July 1988 to March 1995) from prospective screening of all enterococcal isolates by using nitrocefin disks (Cefinase; BBL Microbiology Systems, Cockeysville, Md.) following an outbreak of β-lac$^+$, highly gentamicin-resistant *E. faecalis* at a tertiary-care teaching hospital in Virginia in 1988 (16). Most of the isolates were from urine, wounds, and sputum. The *E. faecalis* isolates studied and date of isolation are listed in Table 1. Five non-β-lac-producing (β-lac$^-$) *E. faecalis* isolates and one *Enterococcus faecium* isolate (E2) from the same hospital were also included. Five laboratory isolates, JH2-7 (an enterococcal recipient strain), XH22 (the transconjugant obtained from filter mating of JH2-7 with HH22, the β-lac$^+$ isolate recovered in Texas in 1981) (9), OG1RF (an enterococcal recipient strain resistant to rifampin and fusidic acid) (4), and XE421 (a transconjugant obtained from a filter mating of OG1RF with E421) (Table 1) were also studied.

Antimicrobial susceptibilities. The presence of high-level resistance to aminoglycosides was detected by streaking growth from overnight cultures onto brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) containing 2,000 μg of streptomycin (Sigma Chemical Co., St. Louis, Mo.) per ml or 500 μg of gentamicin (Schering-Plough Research Institute, Kenilworth, N.J.) per ml and incubating at 37°C for 24 to 48 h. Susceptibilities to ciprofloxacin (Miles Pharmaceuticals, West Haven, Conn.), ampicillin and sulbactam (Pfizer, Inc., Groton, Conn.), rifampin (Merrell Dow, Cincinnati, Ohio), chloramphenicol (Sigma Chemical Co.), erythromycin (Abbott Laboratories, North Chicago, Ill.), trimethoprim (Sigma Chemical Co.), tetracycline (United States Biochemical Corp., Cleveland, Ohio), and vancomycin (Eli Lilly and Co., Indianapolis, Ind.) were determined by the agar dilution method with breakpoints set by the National Committee for Clinical Laboratory Standards (12). All isolates were screened for β-lac production by using nitrocefin disks.

Resistance transfers. For filter matings, samples (50 μl) of stationary-phase cultures of HH22, E421, and E586 in BHI broth were added to separate tubes containing 4.5 ml of BHI broth and 0.5 ml of stationary-phase OG1RF cells. The bacterial mixture was collected on filters (0.45-μm pore size), placed on BHI agar, and incubated overnight at 37°C; the resulting growth was suspended in 1 ml of sterile 0.9% NaCl, and 0.1 ml was plated onto BHI agar containing 500 μg of gentamicin per ml or 25 μg of fusidic acid per ml and onto agar containing both.

PFGE of genomic DNA. Genomic DNA was prepared in agarose plugs, digested with the enzyme *Sma*I (Promega Corp., Madison, Wis.) or *Sfi*I (Promega Corp.), and electrophoresed by using clamped homogeneous electric fields with a CHEF-DRII (Bio-Rad Laboratories, Richmond, Calif.) as previously described (10). Interpretations of PFGE patterns were made by comparing isolates on the same agarose gel. 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 other isolates. Isolates were considered to be closely or possibly

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

Strain	Variant	Date of isolation	Source	Phenotype[a]
E47	MA-1	September 1988	Urine	β-lac$^+$ Gm^r Em^r Cm^r Tc^r
E48	MA-2	September 1988	Urine	β-lac$^+$ Gm^r Em^r Cm^r Tc^r Sm^r
E87	MA-1	July 1989	Sputum	β-lac$^+$ Gm^r Em^r Cm^r Tc^r
E95	MA-3	August 1989	Wound	β-lac$^+$ Gm^r Em^r Cm^r Tc^r
E103A	MA-3	September 1989	Blood	β-lac$^+$ Gm^r Em^r Cm^r Tc^r Sm^r
E123	MA-1	October 1989	Wound	β-lac$^+$ Gm^r Em^r Cm^r Tc^r
E228	MA-4	February 1990	Urine	β-lac$^+$ Gm^r Em^r Cm^r Tc^r
E278	MA-4	June 1990	Wound	β-lac$^+$ Gm^r Em^r Cm^r Tc^r
E340	MA-5	December 1990	Urine	β-lac$^+$ Gm^r Em^r Cm^r Tc^r
E366	MA-6	March 1991	Sputum	β-lac$^+$ Gm^r Em^r Cm^r Tc^r Sm^r
E400	MA-6	August 1991	Urine	β-lac$^+$ Gm^r Em^r Cm^r Tc^r Sm^r
E405	MA-7	October 1991	Sputum	β-lac$^+$ Gm^r Em^r Cm^r Tc^r
E409	MA-8	January 1992	Wound	β-lac$^+$ Gm^r Em^r Cm^r Tc^r
E420	MA-6	March 1992	Urine	β-lac$^+$ Gm^r Em^r Cm^r Tc^r Sm^r
E421	MA-9	March 1992	Urine	β-lac$^+$ Gm^r Em^r Cm^r Tc^r Sm^r
E429	MA-10	June 1992	Urine	β-lac$^+$ Gm^r Tc^r
E434	MA-11	August 1992	Wound	β-lac$^+$ Gm^r Em^r Cm^r Tc^r Sm^r
E439	MA-1	October 1992	Wound	β-lac$^+$ Gm^r Em^r Cm^r Tc^r
E553[b]	MA-10	September 1993	Wound	β-lac$^+$ Gm^r Tc^r
E581	MA-4	December 1993	Sputum	β-lac$^+$ Gm^r Em^r Cm^r Tc^r Sm^r
E586	MA-4	December 1993	Wound	β-lac$^+$ Gm^r Em^r Cm^r Tc^r Sm^r
E661	MA-6	October 1994	Wound	β-lac$^+$ Gm^r Em^r Cm^r Tc^r Sm^r
E666	MA-6	March 1995	Wound	β-lac$^+$ Gm^r Em^r Cm^r Tc^r Sm^r
E2	ND[c]	October 1988	Urine	β-lac$^-$ Gm^s
E3	ND	August 1988	Urine	β-lac$^-$ Gm^r
E12	ND	August 1988	Urine	β-lac$^-$ Gm^s
E24	ND	August 1988	Urine	β-lac$^-$ Gm^r
E36	ND	September 1988	Urine	β-lac$^-$ Gm^r
E78	ND	October 1988	Urine	β-lac$^-$ Gm^s

[a] r, resistant, s, susceptible, Cm, chloramphenicol, Em, erythromycin, Gm, gentamicin, Sm, streptomycin, Tc, tetracycline. All isolates were susceptible to ampicillin-sulbactam, ciprofloxacin, rifampin, and vancomycin.
[b] Resistant to trimethoprim.
[c] ND, not determined.

related if they differed by changes consistent with one or two genetic events (one to six band differences) (14).

Plasmid analysis and hybridization. Plasmid DNA was prepared by a modification of the procedure described by Currier and Nester (3), purified by cesium chloride gradient centrifugation, then solidified in agarose plugs, digested with *Sma*I or *Sfi*I, and processed as described for genomic DNA. DNA was transferred to filters as previously described (11) and hybridized with a probe specific for the enterococcal *aac(6′)-aph(2″)* gene (5).

RESULTS

Antimicrobial susceptibilities and transfer of resistance. Table 1 shows the antibiograms of the isolates. All the β-lac$^+$ isolates had high-level resistance to gentamicin and were resistant to tetracycline. Most of them were also resistant to erythromycin and chloramphenicol, and a few were resistant to streptomycin. HH22, E421, and E586 all readily transferred Gm^r to OG1RF on filters, although frequencies of transfer (expressed as transconjugants per donor) were ~10-fold higher when HH22 was the donor (6×10^{-5}). The transconjugants were also β-lac$^+$, and the other resistances that transferred in the case of XE421 and XE586 were to erythromycin, chloramphenicol, and streptomycin.

PFGE. When *Sma*I was used, PFGE yielded 16 to 19 visible bands for each of the 23 β-lac$^+$ isolates. The β-lac$^-$ *E. faecalis* isolates were markedly different from the β-lac$^+$ isolates, with more than 10 band differences (Fig. 1). However, the β-lac$^+$ isolates appeared to be similar to strains E47 and E48, isolated at this hospital in 1988, which we have previously classified as belonging to a large clone referred to as the mid-Atlantic (MA) clone (11). Eleven different restriction digestion variations of this pattern were observed among the isolates. Table 2 and Fig. 2 show the β-lac$^+$ isolates classified into variants of the MA clone (MA-1 to MA-11); isolates belonging to a particular variant, for instance, MA-4 (isolates E228, E278, E581, and E586), had identical restriction patterns. Most of the variant groups contained isolates that demonstrated patterns almost identical (one to three band differences) to those of other groups; e.g., isolated E48 from MA-2 and E95 from MA-3 showed three band differences. However, differences in up to six bands could be seen in some cases, for example, E421 from MA-9 and E553 from MA-10 (Fig. 2); however, fewer differences were seen between these isolates and others of the clone. Fragments in the 50- to 100-kb range seemed to show the most variability when isolates E421 and E553 were compared with the other clonal variants (Fig. 3). There was no obvious tem-

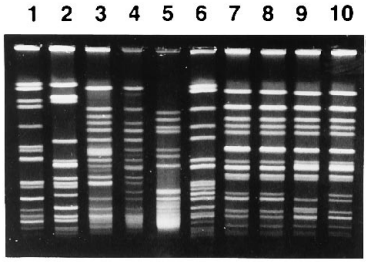

FIG. 1. PFGE patterns of *Sma*I-digested genomic DNA from 10 enterococcal isolates from a single hospital. Lanes 1 to 4 and 6, β-lac$^-$ *E. faecalis* isolates with different restriction patterns; lane 5, an *E. faecium* isolate; and lanes 7 to 10, β-lac$^+$ *E. faecalis* isolates E228 (MA-4), E278 (MA-4), E340 (MA-5), and E366 (MA-6) with related patterns.

TABLE 2. PFGE pattern variants among β-lac$^+$ *E. faecalis* isolates

PFGE variant	Isolate(s)
MA-1	E47, E87, E123, E439
MA-2	E48
MA-3	E95, E103A
MA-4	E228, E278, E581, E586
MA-5	E340
MA-6	E366, E400, E420, E661, E666
MA-7	E405
MA-8	E409
MA-9	E421
MA-10	E429, E553
MA-11	E434

poral relatedness in the restriction endonuclease digestion patterns of isolates showing the 11 variant patterns; for instance, isolates E47, E87, E123, and E439 belonging to the variant group MA-1 had been collected over a 4-year period.

Plasmid analysis. With conventional electrophoresis of cesium chloride-purified plasmid DNA, *Sma*I did not digest the plasmid from XH22, and *Sfi*I digested this plasmid once (data not shown). PFGE patterns of genomic DNA from isolates XH22 and JH2-7 differed by only one band, equivalent to a linear fragment of ~58 kb (in the case of *Sfi*I) and ~75 kb (in the case of *Sma*I) (Fig. 4A). As shown in Fig. 4B, under different electrophoresis conditions, the new band in *Sfi*I-digested XH22 that was not seen in JH2-7 is again visible. The slight difference in migration of the band from the digested preparation in lanes 4 and 5 results from addition of the plasmid DNA to agarose to form a plug, in contrast with direct addition to the well.

To determine if any of the bands causing E421 to differ from other isolates might be a plasmid band, *Sma*I-digested purified plasmid DNA from this strain was run in a lane next to *Sma*I-digested genomic DNA (Fig. 4C). The migration of two of the bands in the genomic preparation matched the migration of the two plasmid bands. In the genomic DNA, these two bands appeared more dense than bands above or below them, which usually suggests a possible doublet but, in this case, may be due to greater copy numbers. Hybridization with the radiolabelled gentamicin resistance gene probe showed that the only bands in Fig. 4C that hybridized were the larger band (~85 kb) in lane 2 and a band with the same migration in the digested genomic DNA in lane 1 (data not shown).

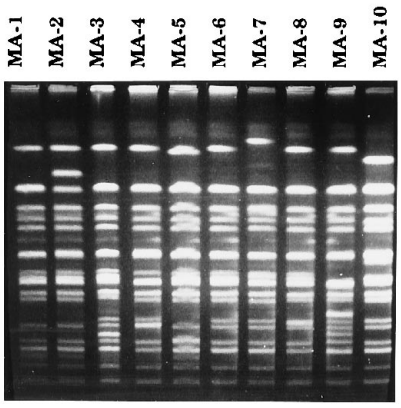

FIG. 2. PFGE *Sma*I digestion patterns of representatives of the variants of the MA clone. The pattern for variant MA-11 is not shown.

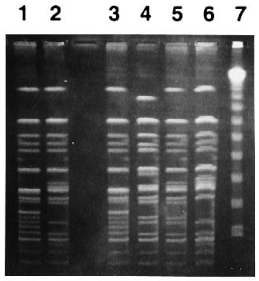

FIG. 3. Illustration of the differences between *Sma*I-digested genomic DNA from isolates from certain variant groups. Lanes 1 and 3, E421 of MA-9; lane 2, E47 of MA-1; lane 4, E553 of MA-10; lane 5, E400 of MA-6; lane 6, E434 of MA-11; and lane 7, lambda concatemers. The sizes of the smallest four bands in lane 7, from the bottom up, are 48.5, 97, 145.5, and 194 kb (see also Fig. 4).

DISCUSSION

β-Lactamase production was first described for enterococci in 1983 (9), and such organisms have now been reported from at least eight states and three continents, both as individual isolates and as clusters in hospitalized patients. The first outbreak caused by β-lactamase-producing enterococci was described in an infant-toddler surgical unit (13). Despite the documented colonization of 68 children, no infections were observed. The isolates we examined were collected during a 7-year period (1988 to 1995) after the first large nosocomial outbreak of infections caused by β-lactamase-producing, highly gentamicin-resistant *E. faecalis* (16). This provided us with a unique opportunity to characterize the changes in PFGE chromosomal digestion patterns that occurred in nature over a prolonged period.

As shown here and previously (11), most *E. faecalis* strains have 15 to 20 *Sma*I chromosomal digestion fragments. None of the β-lac$^+$ isolates examined in this study had patterns that resembled those of the β-lac$^-$ *E. faecalis* strain from the same hospital. Moreover, the β-lac$^+$ isolates seemed to form a single clonal group, either being indistinguishable from other isolates in this clone or differing by a few of their fragments, which would categorize them as closely related or possibly related according to the criteria referred to in reference 14. Variations of two to three bands have been observed in strains of some species when they have been cultured repeatedly over time or

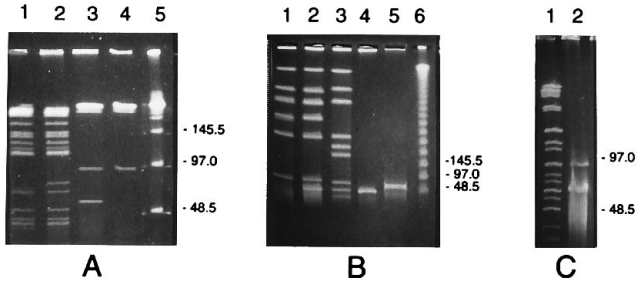

FIG. 4. Illustration of plasmid bands on PFGE pattern. (A) Lanes 1 and 2, genomic DNA from JH2-7 and XH22 digested with *Sma*I; lanes 3 and 4, genomic DNA from XH22 and JH2-7 digested with *Sfi*I; and lane 5, lambda concatamers (sizes are in kilobases). (B) Different electrophoresis parameters were used. Lanes 1 and 2, genomic DNA from JH2-7 and XH22 digested with *Sfi*I; lane 3, genomic DNA from HH22 digested with *Sfi*I; lane 4, plasmid DNA from XH22 digested with *Sfi*I in agarose; lane 5, plasmid DNA from XH22 digested with *Sfi*I added in buffer to the well (note the slight difference in migration between the two bands); and lane 6, lambda concatemers (sizes are in kilobases). (C) Lane 1, *Sma*I-digested genomic DNA from isolate E421; lane 2, *Sma*I-digested plasmid DNA purified from this isolate.

isolated multiple times from the same patient (14), while variations of four to six bands have been observed among isolates collected over longer periods (>6 months) or taken from large numbers of patients involved in extended outbreaks (14).

The common restriction pattern depicted here has been observed before and designated the MA pattern (11). We identified 11 variations of the MA clone in this study. The differences in the isolates with the common MA restriction endonuclease digestion pattern occurred in fragments of various sizes; for example, in the case of MA-7, the largest fragment (~437 kb) was larger than the largest fragment of other variant groups, while for MA-5 and MA-10, this fragment was slightly smaller (<437 kb). Below 194 kb, the patterns of the variant groups MA-9, MA-10, and MA-11 differed the most. A subset of the isolates studied—e.g., isolates E47, E48, E228, E340, and E366, which showed one to three band differences—have also been classified as closely related by multilocus enzyme electrophoresis (15); this finding further supports our interpretation that these isolates are derived from the same clone or have the same genetic lineage but have undergone some changes (genetic events), which account for the observed variations. However, there was no obvious temporal pattern to these changes that would allow one to track these variations sequentially. Some isolates which were indistinguishable, e.g., E47, E87, E123, and E439 (MA-1), were recovered 4 years apart (Table 1). This indicates that chromosomal digestion patterns can be stable in nature over a number of years. On the other hand, the variants MA-1 to MA-6, which differed by one to three bands, might not be classified as the outbreak strain by the criteria described in reference 14 (and, indeed, were not representative of recent direct spread between patients), but they would be categorized as probably related (14).

As was previously described with β-lac$^+$ *E. faecalis* from this hospital (8), the Gm^r, β-lac-encoding plasmid was easily transferred in conjugation experiments by using E421 and E586 as donors. Thus, theoretically, during a prolonged outbreak, spread of the resistance phenotype would be expected to occur by the conjugal transfer of plasmid DNA from one enterococcal isolate to another. Although a β-lac$^+$ *E. faecium* isolate has been reported from this institution (2), the *E. faecalis* isolates obtained at different times during (16) and after (in this study) the outbreak were all found to be related, indicating that the continuous and frequent isolation of β-lac$^+$ *E. faecalis* in this setting was due to intrahospital spread of one strain that contained the resistance plasmid rather than to intraspecies transmission of this plasmid to other strains (16). This suggests that it may be easier to spread this enterococcal strain from patient to patient than to spread this particular plasmid from strain to strain.

β-lac$^+$ isolates were also characterized by their antibiograms. A few differences were noted in the susceptibilities of isolates belonging to the same MA variant. For example, E228 and E278 (MA-4) were susceptible to streptomycin, whereas E581 and E586, in the same variant group, were resistant. Similarly, isolate E429 (MA-10) was susceptible to trimethoprim, while E553, also in this group, was resistant. Otherwise, most of the isolates belonging to the same variant had similar antibiograms, indicating that there was a fair correlation between antibiograms and PFGE patterns.

Parallel analysis of *Sma*I- and *Sfi*I-digested genomic DNA (Fig. 4A) from JH2-7 and from XH22 (a transconjugant derived from HH22 which was previously shown to belong to the MA pattern [11, 15]) revealed that a single new band was present in the transconjugant and that the new band migrated to different positions following exposure to different enzymes. This illustrates that digested (*Sfi*I) and undigested (*Sma*I) plasmid bands migrate differently and also indicates that some of the smaller fragments in the restriction pattern of the MA clone may be derived from plasmids. Similarly, two of the bands in the PFGE pattern of E421 had the same migration as the bands generated by *Sma*I digestion of plasmid DNA from this strain. The larger of the two plasmid bands (Fig. 4C, lane 2) and a band of the same size in the genomic digests (Fig. 4C, lane 1) were the only bands that hybridized to an intragenic probe from the *aac(6′)-aph(2″)* gene, which encodes high-level gentamicin resistance, further supporting the plasmid origin of some of the fragments seen in PFGE of digested genomic DNA.

In conclusion, our data showed that there was little variation in the PFGE patterns of the β-lac$^+$, gentamicin-resistant *E. faecalis* isolates collected over a 7-year period, although some isolates differed more from the original isolates (E47 and E48) than did others. We also demonstrated that plasmid bands, both digested and undigested, can influence PFGE patterns. There was a surprising lack of movement of the plasmid encoding β-lac and gentamicin resistance to other *E. faecalis* strains despite the demonstration that the plasmid was transferable. The persistence of a single endemic clone for at least 7 years emphasizes the continuing need to screen for resistance traits and to institute measures to reduce their spread between patients.

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