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Comparison of the β-Lactamase Gene Cluster in Clonally Distinct Strains of *Enterococcus faecalis*

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Ten β-lactamase-producing *Enterococcus faecalis* isolates were examined for the presence of the staphylococcal β-lactamase repressor and antirepressor genes. Four isolates, previously shown to be unrelated to each other by pulsed-field gel electrophoresis analysis, were positive for both genes by PCR, although β-lactamase production was not induced with methicillin. Six isolates, previously shown to be clonally related, were negative for both genes by PCR. The *blaZ* sequences of eight β-lactamase-producing *E. faecalis* isolates were determined. Seven isolates from five distinct clones had sequences identical to that previously reported for *E. faecalis* HH22, regardless of whether the repressor or antirepressor was demonstrated by PCR. However, *blaZ* from one isolate differed from those of the other enterococci by 11 nucleotides; this isolate is part of the large clone, as defined by pulsed-field gel electrophoresis and multilocus enzyme analysis, that includes HH22. These findings suggest either that enterococci have acquired the *bla* gene cluster from more than one source or that the gene cluster has undergone considerable change since acquisition by this clone.

The first reported β-lactamase-producing (Bla^+) enterococcal isolate, HH22, was recognized in Houston, Tex., in the early 1980s (8). Since that time, other Bla^+ enterococci, nearly all of which have been *Enterococcus faecalis*, have been reported. The nucleotide sequences of the β-lactamase (*blaZ*) genes from *E. faecalis* HH22 and CH19 have been determined and are identical to the sequences of the staphylococcal type A *blaZ* genes of pC1 and pS1 and very similar to those of other staphylococcal β-lactamase genes (11, 23). Although the structural β-lactamase genes of enterococci and staphylococci are highly related, phenotypic differences in the production of the enzyme have been observed. In enterococci the β-lactamase is produced constitutively and in relatively small amounts, whereas in most *Staphylococcus aureus* isolates, β-lactamase production is inducible, under the control of a repressor (*blaI*) and antirepressor (*blaR1*), and produced in large amounts after induction. In a recent study, *E. faecalis* HH22 was found to contain only 51% of *blaR1* and no hybridization was seen with a DNA probe for *blaI* (24). In the case of HH22, constitutive production is apparently due to the absence of functioning regulatory genes.

By using pulsed-field gel electrophoresis (PFGE) of *Sma*I-digested genomic DNA, Bla^+ *E. faecalis* isolates have been characterized into several distinct clones. Our laboratory has provided evidence of the interstate spread of one clone, named the mid-Atlantic clone (PFGE pattern 5, which includes HH22), to seven hospitals in the United States and characterized clonally distinct isolates from other U.S. locations, Beirut, Lebanon, and Argentina (9) (unpublished observations). These findings have recently been corroborated by multilocus enzyme electrophoresis (20). Although it is generally accepted that enterococci likely acquired β-lactamase from staphylococci and that there are clonally distinct strains of enterococci displaying this trait, the genetic diversity and nature of transfer events of this trait remain unknown.

The purposes of this study were to compare the findings for HH22 with those for other Bla^+ enterococci and to determine whether there is evidence of β-lactamase gene cluster diversity in enterococci. To do this, we sequenced *blaZ* from eight representatives of six PFGE clonal groups and analyzed Bla^+ enterococcal strains for the presence of *blaI* and *blaR1*. Assays to determine whether enzyme production was inducible or constitutive were also performed.

MATERIALS AND METHODS

Bacterial isolates. The Bla^+ *E. faecalis* isolates used in this study and their PFGE patterns (3, 7, 9, 10, 21) are listed in Table 1. The nucleotide sequence of HH22 was previously reported (GenBank and EMBL DNA accession no. M60253) (23). OG1RF (ATCC 47077) is a plasmidless, Bla^- strain used as a control in the enzyme assay and as a negative control in PCR amplifications. All isolates were single colony purified, and β-lactamase production was reconfirmed by nitrocefin assay. The isolate named Toronto (kindly provided by Joseph Yao) was shown to be unrelated to other Bla^+ *E. faecalis* isolates by PFGE, performed as reported previously (9). Fla18 was reconfirmed as being identical to that isolate reported previously (9) by PFGE (this isolate was referred to as Florida-1 in a previous publication [9]).

Primer selection. The PCR primer pairs for the repressor and antirepressor were chosen according to the DNA sequence of Tn*552* published by Rowland and Dyke (15) and are listed in Table 2. The structural gene primers were chosen on the basis of the published HH22 sequence (23) and are also listed in Table 2. Primers were obtained from Genosys Biotechnologies, The Woodlands, Tex.

PCR and DNA sequencing. Bacterial genomic DNA was obtained by using an Instagene DNA matrix (Bio-Rad, Hercules, Calif.), and PCRs were carried out according to the instructions of a GeneAmp DNA amplification kit, GeneAmp PCR carryover prevention kit (Perkin-Elmer Cetus, Norwalk, Conn.), and/or PCR optimizer kit (InVitrogen, San Diego, Calif.). All PCRs were carried out with a Perkin-Elmer Cetus DNA Thermal Cycler. Prior to sequencing, amplified DNA was purified by using the Wizard PCR DNA cleanup system according to the package insert (Promega Corporation, Madison, Wis.). The nucleotide sequences were determined by the dideoxy-chain termination method (16) utilizing an Applied Biosystems model 373A DNA automatic sequencer and a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.). Data were processed with a sequencing analysis program from Applied Biosystems.

DNA sequence analysis. Nucleotide sequences were analyzed by using MacVector version 3.5 (International Biotechnologies, Inc., New Haven, Conn.),

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TABLE 1. Isolates of *E. faecalis* used in this study

Strain	Origin	PFGE pattern[a] (reference[s])
OG1RF	Laboratory strain	VIII (3, 20)
HH22[b,c]	Houston, Tex.	5 (9, 20)
WH257[c,d]	West Haven, Conn.	8 (10, 20)
HG6280[d]	Buenos Aires, Argentina	19 (7, 20)
HG9829[c,d]	Buenos Aires, Argentina	19 (7, 20)
HG10528[d]	Buenos Aires, Argentina	19 (7, 20)
CH25[c,d]	Boston, Mass.	21 (9)
Beirut[c,d]	Beirut, Lebanon	7 (9, 20)
Fla18[c,d]	Florida	5 (9, 20)
Del[c]	Delaware	5 (9, 20)
CH570[c]	Pittsburgh, Pa.	5 (9, 20)
PA[c]	Philadelphia, Pa.	5 (9, 20)
E-47[c]	Richmond, Va.	5 (9, 20)
Toronto[d]	Toronto, Canada	22 (this study)

[a] Patterns 8 and VIII are different patterns and are the designations used in the respective studies.
[b] *blaZ* sequence previously reported (23).
[c] β-Lactamase-producing isolate used for PCR analysis of *blaI* and *blaR1*.
[d] β-Lactamase-producing isolate used for sequencing of *blaZ*.

and sequences were compared through the National Center for Biotechnology Information by using the BLAST network service.

Enzyme assay. β-Lactamase activities were determined for selected isolates (see Table 3). Isolates were grown overnight with or without 8 μg of methicillin per ml for induction, as reported previously (5). Suspensions of induced and uninduced organisms were made in 3-ml aliquots of 0.1 M sodium phosphate buffer (pH 6.0) and adjusted to an optical density of 0.8 at 600 nm. Adjusted enterococcal cultures (150 μl) were added to 2.5 ml of a 10^{-4} M solution of nitrocefin (BD Microbiology Systems, Cockeysville, Md.). Spectrophotometric readings (Gilson recording spectrophotometer; Gilson Medical Electronics, Woburn, Mass.) at 482 nm were recorded every 10 min for 1 h at 25°C and reported in nanomoles of nitrocefin hydrolyzed per minute per milliliter of standardized culture. Each reported value is the average of two determinations.

RESULTS

Comparison of *blaZ* sequences. Parts of the *blaZ* sequences from eight Bla$^+$ *E. faecalis* isolates (Table 1) were determined by sequencing the amplified genomic DNA in both directions. By utilizing combinations of the seven structural gene primers reported in Table 2, approximately 810 nucleotides of sequence were determined for each gene. These sequences were then compared with the available sequences of staphylococcal β-lactamase genes (2, 15) and with that of HH22. Four of eight *E. faecalis* isolates (Beirut, CH25, WH257, and Toronto) contained a *blaZ* sequence which was 100% identical, over more than 800 nucleotides, to that previously reported for HH22 (data not shown). Three other isolates, HG9829, HG6280, and HG10528, were partially sequenced (approximately 650 to 720 bp), and the sequenced regions were also identical. These eight isolates and HH22 represent six distinct clones, as characterized by PFGE (Table 1). As previously reported by Zscheck and Murray, the *blaZ* sequence of strain HH22 is identical to those of the type A β-lactamase genes of staphylococcal plasmids pC1 (accession no. M25252) and pS1 (accession no. M25253) and differs by two nucleotides within the coding region from the published sequence of a constitutively produced variant of the staphylococcal type A β-lactamase from pI258 (accession no. J04551) (22, 23). It is also identical to that found on the staphylococcal transposable element Tn*552* (accession no. X52734) (15). Other staphylococcal β-lactamase gene sequences in the GenBank database had greater differences from that of HH22.

TABLE 2. PCR primers used in this study

Primer	Sequence	Nucleotide positions[a]
blaI F	5′-ATGTCTCGCAATTCTTCAA-3′	3190–3208
blaI R	5′-CTATGGCTGAATGGGAT-3′	3520–3503
blaR1 F	5′-GGTATCTAACTCTTCTTGC-3′	3612–3630
blaR1 R	5′-CATCTGATAAATGTGTAGC-3′	5177–5159
blaZ 1F	5′-TACAACTGTAATATCGGAGGG-3′	5372–5392
blaZ 1R	5′-CATTACACTCTTGGCGGTTTC-3′	6212–6192
blaZ 2F	5′-GAGGCTTCAATGACATATAGTG-3′	5741–5762
blaZ 2R	5′-TCTATCTCATATCTAACTGG-3′	5855–5836
blaZ 3F	5′-CACCTGCTGCTTTCGGTAAGAC-3′	5916–5937
blaZ 3R	5′-AGGAGAATAAGCAACTATATCATC-3′	5669–5646
blaZ 4F	5′-GCTCATATTGGTGTTTATG-3′	5504–5522

[a] Based on the sequence of Tn*552* (GenBank and EMBL accession no. X52734) published by Rowland and Dyke (15).

Among the isolates sequenced in this study, only Fla18 demonstrated a sequence different from that of the aforementioned enterococcal isolates; this sequence was generated a number of different times from different PCR amplification preparations and with different primers, including from a cloned fragment with T7 and *blaZ* primers. The PFGE pattern of Fla18 was also reverified from a single colony. By using the BLAST network service to compare this sequence with other sequences in the GenBank database, two staphylococcal *blaZ* sequences demonstrated more similarities to the Fla18 sequence than did that of HH22 (Fig. 1). The sequences of other staphylococcal β-lactamases in the GenBank database either demonstrated more differences or had only partial sequences available. The *blaZ* sequence of Fla18 differed by 11 nucleotides from that reported for HH22. There were only seven nucleotide differences between this sequence and the published sequences of the type A β-lactamase gene of the staphylococcal plasmid pI1071 (accession no. M25254) and of the type D β-lactamase of the staphylococcal plasmid pUB101 (also referred to as pUB10; accession no. M25257) (2); however, these seven differences were not in the same positions. Figure 1 depicts 720 nucleotides of these sequences; nucleotides 721 to 800 were identical. As shown in Fig. 1, *blaZ* of Fla18 demonstrated unique nucleotides at positions 396, 434, and 486, two of which are silent.

The translation of the Fla18 *blaZ* nucleotide sequence showed seven amino acid changes compared with that from HH22 (Fig. 1). Five of the amino acid changes in Fla18 (occurring at Ambler codon positions L9, 93, 121, 226, and 229) were also present in the deduced sequences from both pI1071 and pUB101, and one of the Fla18 changes (at position L22) was also found in the deduced sequence of pI1071. The nucleotide 434 change unique to Fla18 resulted in a glutamine-to-glycine amino acid substitution (Ambler amino acid 154).

Regulatory genes and enzyme expression. DNA fragments corresponding to both *blaI* (0.4 kb) and *blaR1* (1.6 kb) were amplified from four Bla$^+$ enterococci, namely, Beirut, CH25, HG9829, and WH257, all unrelated by PFGE. The *blaR1* product of CH25 was consistently larger than those of the other isolates (2.9 kb). No amplification was observed for six isolates from the mid-Atlantic clone, including Fla18. In the case of *blaI*, these results were confirmed by the lack of hybridization with a previously described DNA probe for *blaI* (18).

Table 3 shows the amounts of β-lactamase produced under induced and uninduced conditions. HH22, an isolate previously shown to lack an intact *blaI* and *blaR1* (24), had an induction ratio of 1.1, indicating constitutive enzyme production. Strains Beirut, CH25, HG9829, and WH257, which were positive by PCR for the regulatory genes, also had induction ratios of around 1.0.

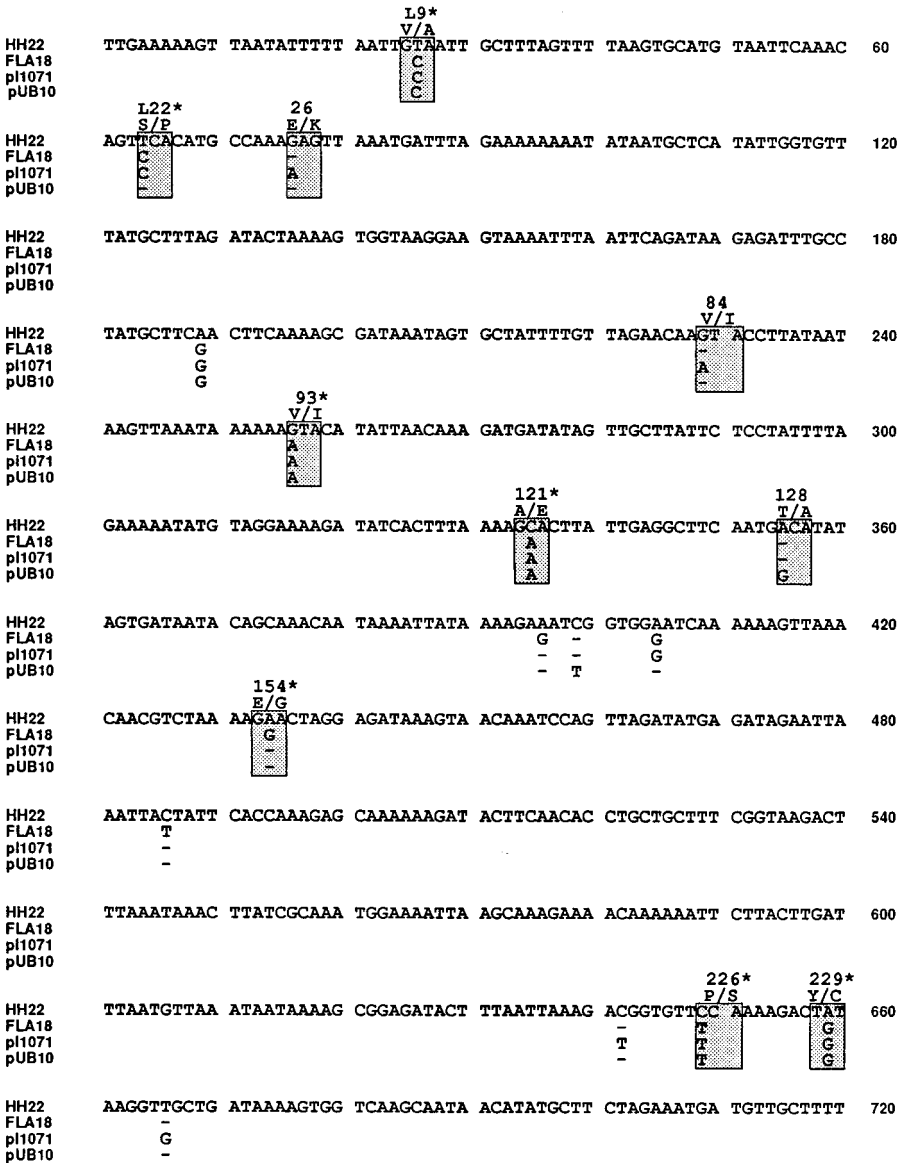

FIG. 1. Comparison of nucleotide sequences of amplified *blaZ* from enterococci and staphylococci. The HH22 *blaZ* nucleotide sequence was identical to that obtained from strains Beirut, CH25, WH257, HG9829, HG6280, HG10528, and Toronto (data not shown). Differences in nucleotide sequences are reported; −, no change from the *blaZ* sequence of HH22. Shaded boxes indicate codons in which nucleotide substitutions resulted in amino acid changes; silent nucleotide substitutions are not shaded. Amino acid substitutions are listed above boxes with amino acid position numbers (Ambler et al. numbering scheme [1]); ∗, position differs between HH22 and Fla18 deduced sequences. pUB10 is also known as pUB101.

DISCUSSION

Interest in β-lactamase began soon after the introduction of penicillin in the 1940s. Subsequently, staphylococcal β-lactamases were divided into four variants (A, B, C, and D) by serologic and, later, kinetic techniques (6, 25). Type A is the most common, whereas type B appears to be limited to isolates typeable by group II bacteriophages (13). Type C β-lactamases have been reported for up to 20% of methicillin-resistant *S. aureus* isolates, and type D β-lactamases appear to be limited to less than 2% of surveyed staphylococci (4, 13, 14, 23).

The isolation of β-lactamase-producing enterococci remains a relatively rare event, although a number of clonally distinct strains exist. In this study, we have examined the molecular epidemiology of β-lactamase genes and shown that isolates from six distinct clones have identical *blaZ* nucleotide se-

TABLE 3. β-Lactamase activities of enterococcal strains

Strain	β-Lactamase activity[a]		Induction ratio[b]
	Uninduced	Induced	
OG1RF	0.0	0.0	NA[c]
HH22	3.5	3.9	1.1
WH257	3.6	4.6	1.2
HG9829	3.7	3.6	0.9
CH25	2.5	1.7	0.7
Beirut	1.4	1.9	1.3

[a] Expressed in nanomoles of nitrocefin hydrolyzed per minute per milliliter of culture.
[b] Induction ratio = induced activity/uninduced activity.
[c] NA, no β-lactamase activity.

quences. Only within a geographically diverse large clone, the mid-Atlantic clone, did we find a different nucleotide sequence. This sequence, from Fla18, appeared to be somewhat more similar to the *blaZ* sequences derived from the staphylococcal plasmids pI1071 and pUB101 than to the *blaZ* sequence of HH22. Of interest, these kinetically different staphylococcal β-lactamases, pI1071 (type A) and pUB101 (type D), have nucleotide sequences that are more similar to each other than the pI1071 sequence is to other type A sequences (2). Kernodle and Voladri have also found a β-lactamase in an *S. aureus* isolate, DK 6014, whose *blaZ* sequence is more similar to those of pI1071 and pUB101 than to the Tn*552 blaZ* sequence, which is identical to that in HH22 (4a, 21). This *blaZ* nucleotide sequence possesses the same novel nucleotide 434 substitution that results in the Glu-to-Gly change found in Fla18, and although it does not share Fla18's two other silent, unique nucleotide substitutions, the translated sequences differ by only one amino acid (4a).

Consistent with differences between the *blaZ* sequences of HH22 and Fla18 is the report by Smith and Murray in which they demonstrated two distinct restriction endonuclease digestion patterns on autoradiograms of *Eco*RV-digested plasmid DNAs from these strains after hybridization to an 840-bp staphylococcal *blaZ* probe (17). They found that HH22 exhibited the same pattern seen with strains Beirut and HG9829, which have identical *blaZ* sequences, whereas Fla18 exhibited a different pattern, which was similar to those seen with two other isolates of the mid-Atlantic clone (E-47 and PA) whose *blaZ* sequences were not determined (17).

It was surprising for us to find diversity in the *blaZ* sequence within the mid-Atlantic clone, particularly in view of the identities of sequences from strains other than this clone. One possibility is that the mid-Atlantic clone has received in transfer different *blaZ* sequences, but analysis of the presence of *bla* regulatory genes would argue against this.

In this study, we could not amplify a *blaR1* fragment from HH22 or five other isolates of the mid-Atlantic clone by PCR using primers for *blaR1* outside the truncated sequence previously found in HH22 (24). However, there was evidence of these regulatory genes in four clonally distinct isolates analyzed in this study (Beirut, CH25, HG9829, and WH257). It is interesting that in CH25, the amplified *blaR1* product was consistently larger than expected, 2.9 versus 1.6 kb. In CH19, isolated during the same outbreak as was CH25, Rice and Marshall found an IS*256*-like element inserted within *blaR1*; the same arrangement probably exists in CH25 as well, which would explain the size difference of the PCR product (12).

Among the isolates studied, the induction ratios demonstrated low-level constitutive production of enterococcal β-lactamases, even in isolates containing DNA sequences that are amplified by primers for both *blaI* and *blaR1* (Table 3). It could be that the regulatory genes do not function in the enterococcal background or, alternatively, that *blaI* and *blaR1* contain mutations that result in the loss of function and, therefore, in constitutive production. An analogous case was described by Suzuki et al. when they investigated the regulatory genes (*mecI* and *mecR1*) of methicillin resistance in *S. aureus*, which are homologs of *blaI* and *blaR1* (4, 19). Many of the strains they tested had regulatory genes but produced MecA constitutively because of point mutations in *mecI* (19).

In conclusion, in this study we found that the presence of β-lactamase regulatory gene sequences corresponded to previously determined clonal lineages, that is, isolates of the mid-Atlantic clone did not have evidence of an intact *blaR1* or *blaI*, while isolates other than this clone did; however, β-lactamase expression was constitutive in all instances. On the other hand, the *blaZ* sequences of all isolates were identical regardless of strain background, except for that of Fla18, which belongs to the same clonal group as does HH22. These apparent discrepancies have not been explained and could be secondary to the acquisition of *blaZ* by this clone from different sources or to significant sequence divergence after acquisition.

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